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Genetic Diversity in Antimicrobial Resistance Determinants Among Pathogenic *Pseudomonas aeruginosa* in India

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Abstract

The drastic rise in antibiotic resistance has become a global challenge, including India, due to high morbidity. The delayed identification and lack of treatment are the major causes of death. However, there is a shortage of precise information on the specific resistance pattern and sequence types of *Pseudomonas aeruginosa* from India that can help in diagnostics and therapy. A total of 16 clinical isolates were collected from the western region of India, along with 181 *P. aeruginosa* genomes of India from public database were retrieved and thoroughly analysed for antibiotics resistance determinants for associated sequence types and O-serotypes using different bioinformatics tools. Of all collected isolates ($n=16$), 9 were extensively drug-resistant (XDR), 6 were multidrug-resistant (MDR), and only 1 isolate was susceptible to selected antibiotics. ST357 ($n=23$; 11.6%) was the most frequent, followed by ST308, and ST1203. In serotyping, O11 ($n=85$; 43%) was most prevalent. A novel ST4937 was reported and submitted to PubMLST. $bla_{\text{NDM-1}}$ carbapenemase was found in ($n=45$; 22.8%) isolates, whereas class D $bla_{\text{OXA-488}}$ was present in ($n=38$; 19.2%) isolates, further, several variants were found for class C bla_{PDC} genes, where $bla_{\text{PDC-3}}$ and $bla_{\text{PDC-19a}}$ were found to be predominant. We discovered that the amounts of carbapenemases and extended spectrum beta-lactamases (ESBL) genes were lower in India. This can be a relief sometimes, but a rise in high-risk clones could lead to longer hospital stays and more deaths. Therefore, ongoing surveillance of these strains is essential for effective infection management and containment of their spread.

Introduction

Pseudomonas aeruginosa, a gram-negative opportunistic pathogen, is known to cause life-threatening infections, particularly in patients with weakened immune systems [1]. *Pseudomonas aeruginosa* infections are mostly associated with urinary tract infections, ventilator-associated pneumoniae (VAP) and endotracheal aspirate [2]. The bacterium has a genome size measuring from 5.5 to 7 million base-pairs (Mbp). This characteristic grants it an exceptional ability to adapt and flourish in hostile conditions [3].

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Carbapenem-resistant *P. aeruginosa* is identified as high-priority pathogen by the World Health Organization (WHO) and it was also listed in the Indian Priority Pathogen List (IPPL) as one of six bacterial species that need the development of new medicines for the treatment of infections [4]. Acute infections caused by antibiotic-resistant *P. aeruginosa* lead to thousands of fatalities globally each year [5, 6]. Infectious diseases resulting from *P. aeruginosa* are typically challenging to manage and enduring due to the notably high incidence of multidrug-resistant (MDR) or extensively drug-resistant (XDR) strains [7, 8]. *Pseudomonas aeruginosa* exhibits inherent resistance to numerous antimicrobials, including β-lactams [9]. A primary mechanism of medication resistance is the synthesis of β-lactamase, which is an enzyme that hydrolyzes β-lactam antibiotics, resulting in their deactivation [10]. The increasing resistance of *P. aeruginosa* to many antibiotics, due to inappropriate antibiotic use, has led to the rise in drug resistance and cross-resistance across antibiotics, culminating in the emergence of MDR strains of *P. aeruginosa*. Traditionally, carbapenem resistance in *P. aeruginosa* is facilitated by nonenzymatic processes, including the upregulation of the efflux pumps and

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modifications to porins [11]. Nevertheless, the prevalence of carbapenemase-producing *P. aeruginosa* strains has been rising globally [11]. This resistance mechanism significantly affects treatment, as certain carbapenemases can inactivate not only carbapenems but also antipseudomonal agents like cefepime and ceftazidime, along with newer therapeutic options such as ceftolozane–tazobactam, ceftazidime–avibactam, and imipenem–relebactam [11].

Furthermore, molecular typing techniques such as multilocus sequence typing (MLST) have revealed that the population structure of *P. aeruginosa* is primarily influenced by particular lineages, often termed high-risk clones, which are broadly distributed across numerous hospitals globally and are typically multidrug or extensively drug-resistant [12]. MLST is more suitable for the comparative investigation of strain types, irrespective of geography or source. Furthermore, in a comparative analysis of genetic approaches for typing *P. aeruginosa*, MLST had the highest predictive value (100%) in identifying strains as unique [13]. These high-risk clones are characterized by their dominance, virulence, and heightened drug resistance, including the sequence types (STs) ST111, ST175, ST233, ST244, ST298, ST308, ST357, and ST654 [14]. Further, ST277 has been intermittently documented in Asian, North American, and European nations, but it is notably frequent in Brazil [15, 16].

The O-polysaccharide (OPS), a highly diverse segment of lipopolysaccharide (LPS), is crucial for virulence and determines serogroup identification [17]. The International Antigenic Scheme (IATS) classified *P. aeruginosa* into 20 distinct serotypes (O1 to O20) based on the configuration of their O-polysaccharide [18]. Research indicates that isolates of serotypes O1, O6, O11, and O12 comprise over 65% of *P. aeruginosa* illnesses [19, 20], while serotypes O4 and O12 are more commonly linked to resistance against several antibiotic classes [12, 21].

Genomic characterization of Gram-negative pathogens by whole-genome sequencing (WGS) is essential for comprehending the sequence variability of resistance genes. While several studies have reported the prevalence of molecular antimicrobial resistance profiles in India for *K. pneumoniae* [22, 23], *Vibrio cholerae* [23], and a few other enteric pathogens [24], however, the data are lacking for *P. aeruginosa*. We aimed to examine resistance allele heterogeneity in genomes of *P. aeruginosa* across several regions of India.The present study was conducted to compile the antibiotic resistance genes circulating in genomes of *P. aeruginosa* from India. We collected ($n=16$) *P. aeruginosa* isolates from different parts of western India and retrieved ($n=181$) genomes from Bacterial and Viral Bioinformatics Resource Centre (BV-BRC) database. Using MLST profiles and Resistance Genes Identifier (RGI) tool we identified dominance of sequence types, serotypes, antimicrobial resistance gene's different geographic locations in India. We also correlated the presence of antibiotic resistance genes and their association with sequence types and serotypes.

Materials and Methods

Collection of Clinical Isolates

A total of ($n=16$) clinical samples were collected from Dr. Toprani's Lab ($n=4$), Vadodara, Gujarat, and Mahatma Gandhi Medical College and Hospital ($n=12$), Jaipur, Rajasthan, during the time period from December 2021 to February 2022. Samples were collected from urine ($n=6$), ear swab ($n=3$), endotracheal (ET) secretions ($n=2$), pus swab ($n=2$), blood ($n=1$), sputum ($n=1$), and ($n=1$) from an unknown site. Isolates were named as TL7, TL8, TL9, TL12, 140, U, J3, J11, J13, J20, J22, J23, J24, J25, J27, and J29 as shown in Table 1.

Antibiotic Susceptibility Testing (AST), DNA Extraction and Whole Genome Sequencing

Antibiotic Susceptibility Testing (AST) was performed for 13 antibiotics from 6 drug classes: Aminoglycosides (Amikacin and Gentamicin), Monobactam (Aztreonam), 3rd Generation Cephalosporins (Ceftazidime, Cefoperazone and Cefepime), Fluoroquinolones (Ciprofloxacin and Levofloxacin), Penicillins (Piperacillin and Ticarcillin) and Carbapenems (Doripenem, Imipenem and Meropenem) by disk diffusion method to check sensitivity towards different antibiotics as per CLSI guidelines [25]. Minimum inhibitory concentration (MIC) was performed using broth microdilution method for 2 antibiotics Glycylcycline (Tigecycline) and Polymyxin (Colistin) and results were interpreted as per CLSI guidelines.

We used the MagGenome Express DNA extraction Kit (MagGenome, India) to carry out genomic DNA extraction according to the manufacturer's protocol. Agarose Gel electrophoresis was performed to check the quality of extracted DNA. DNA QC was performed by nanodrop followed by quantification with a Qubit fluorometer. Library preparation was done with the TruSeq sample preparation kit. Library QC was done with Agilent Tape Station, and sequencing was done on Illumina (Illumina, USA) with 2X150 base pair PE chemistry; 0.5–1 GB data were generated. Sequences were submitted to NCBI under BioProject number PRJNA1163665, and accession numbers allotted to each sequence are mentioned in Table 1.

Whole Genome Data Recovery and Analysis

Raw data were analysed using FastQC (Galaxy Version 0.74 + galaxy1) followed by filtering and trimming of

Table 1 List of collected isolates, site of isolation and year of isolation along with their resistance category and genome accession numbers

Sr. No.	Isolate name	Year of isolation	Site of isolation	State	Resistance category	Genome accession number
1	TL7	2021	Urine	Gujarat	XDR	JBHOAA000000000
2	TL8	2021	Ear swab	Gujarat	XDR	JBHOAB000000000
3	TL9	2021	Urine	Gujarat	XDR	JBHOAC000000000
4	TL12	2021	Urine	Gujarat	XDR	JBHOAD000000000
5	140	2022	Blood	Rajasthan	XDR	JBHNZP000000000
6	U	2022	Urine	Rajasthan	XDR	JBHOAE000000000
7	J3	2022	Ear swab	Rajasthan	MDR	JBHNZQ000000000
8	J11	2022	Ear swab	Rajasthan	MDR	JBHNZR000000000
9	J13	2022	ET secretions	Rajasthan	XDR	JBHNZS000000000
10	J20	2022	Pus swab	Rajasthan	MDR	JBHNZT000000000
11	J22	2022	ET secretions	Rajasthan	XDR	JBHNZU000000000
12	J23	2022	Urine	Rajasthan	Susceptible	JBHNZV000000000
13	J24	2022	Urine	Rajasthan	MDR	JBHNZW000000000
14	J25	2022	Pus swab	Rajasthan	XDR	JBHNZX000000000
15	J27	2022	Sputum	Rajasthan	MDR	JBHNZY000000000
16	J29	2022	Unknown	Rajasthan	MDR	JBHNZZ000000000

adaptors using Trimmomatic (Galaxy Version 0.39 + galaxy2), and filtered reads were assembled using SPAdes (Galaxy Version 3.15.5 + galaxy2). Quality assessment of assembled sequences was performed using QUAST (Galaxy Version 5.3.0 + galaxy0). Annotation of assembled genomes was done using Prokka (Galaxy Version 1.14.6 + galaxy1). Additional *P. aeruginosa* ($n = 181$) genomes from 1995 to 2022, that were submitted by various research groups across India were retrieved from BV-BRC database (https://www.bv-brc.org/) using following filters (Public: True; Genome Status: WGS; Genome Quality: Good; Isolation Country: India; Host Common Name: Human) [26]. Information such as collection year, geographic location, and site of isolation was collected from the database. Further, to study genomics of AMR in *P. aeruginosa*, we included 16 isolates along with retrieved 181 genomes from India. Whereas PAO1 was taken as reference genome.

MLST (Software version-2.0.9) (https://cge.food.dtu.dk/services/MLST/) was performed to check the sequence type of the clinical isolates. 7 housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA, trpE*) and their allelic combinations were used for detection of sequence types. Efflux genes and antibiotic resistance genes were identified using Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/).

Pseudomonas aeruginosa serotyper (PAst 1.0) (https://cge.food.dtu.dk/services/PAst/) program was used for in-silico serotyping of *P. aeruginosa* isolates. PAst programme runs on next generation sequencing data and is based on BLAST analysis of O-specific antigen (OSA) gene cluster.

Phylogeny Tree Preparation and Annotation

Phylogeny tree was prepared using REALPHY (https://realphy.unibas.ch/realphy/) and RECOPHY (https://recophy.unibas.ch/recophy/) tools. Interactive Tree of Life (iTOL) (https://itol.embl.de/) online tool was used for phylogeny tree display and data representation. Various annotations were added to the phylogeny tree using the iTOL tool.

Results

The present study was conducted to compile the presence of AMR genes in isolates collected from western region of India. A total ($n = 16$) isolates were collected and processed for antibiotic susceptibility testing followed by whole genome sequencing. Further, previously reported genomes of clinical origin ($n = 181$) from India were compared with isolates collected in the present study. Year-wise collection of isolates and their source are shown in Fig. 1. Figure 1 illustrates that in 2020, the predominant isolates were from the respiratory tract ($n = 55$), followed by urine ($n = 42$), eye ($n = 36$), pus ($n = 11$), blood ($n = 9$), ET ($n = 8$), tissue ($n = 4$), unknown ($n = 3$), with two each from sputum, ear and bile, and one each from CSF and knee joint. Notably, the total number of isolates for each year was below 15, except for 2020. In the state wise comparison of isolates depicted in Fig. 3a, the highest number of genomic data submissions originated from Tamil Nadu ($n = 77$; 38.8%) followed by Chhattisgarh ($n = 38$; 19.1%), an unknown site ($n = 26$; 13.1%), Telangana ($n = 15$; 7.5%), Andhra Pradesh ($n = 14$; 7%) and Rajasthan ($n = 12$; 6%). Each of Maharashtra,

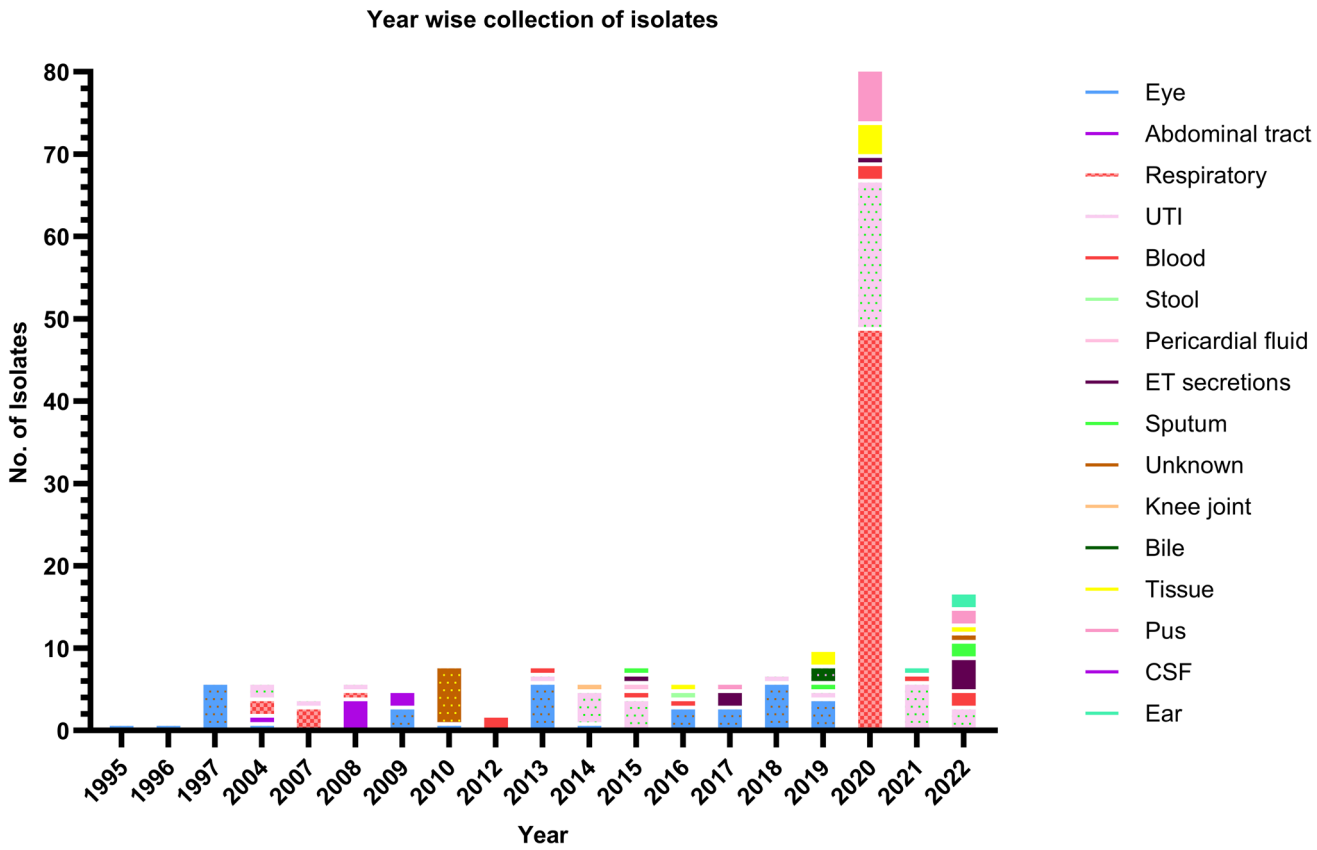


Fig. 1 Year wise site of isolation of clinical isolates. Bars in the graph represents year wise submission of genomes to BV-BRC database. Color and pattern in the bar indicate origin site of the isolate. GraphPad Prism 10.0.1 software was used to create this graph (Color figure online)

Gujarat, Karnataka and West Bengal contributed less than 5% of the total isolates submitted.

Antibiotic Susceptibility Testing

Based on AST, isolates were categorized as susceptible, MDR or XDR. Out of the 16 isolates, 1 isolate (J23) was found to be susceptible, 6 isolates (J3, J11, J20, J24, J27, and J29) were found to be MDR and 9 isolates (TL7, TL8, TL9, TL12, U, J13, J22, J25, and 140) were found to be XDR. For aminoglycoside antibiotics (gentamicin and amikacin), isolates U, J11 and J23 were found susceptible to gentamicin and amikacin, whereas isolate J27 was found susceptible to gentamicin and intermediate to amikacin. Isolates TL7, TL8, TL9, TL12, J3, J13, J20, J22, J24, J25, J29, and 140 were resistant to both aminoglycoside antibiotics. All isolates showed resistance towards aztreonam (monobactam) antibiotic. For penicillin antibiotics (penicillin and ticarcillin), isolates J3 and J23 were susceptible to penicillin and ticarcillin, whereas, J11, J23, J24, and J25 were only susceptible to penicillin and resistant to ticarcillin. Isolates J20 and J27 were intermediate to penicillin and resistant to ticarcillin. Isolates TL7, TL8, TL9, TL12, U, J13, J22, J29, and 140 were resistant towards both penicillin antibiotics. For fluoroquinolone antibiotics (ciprofloxacin and levofloxacin), only J23 was susceptible to both antibiotics, and J29 was sensitive towards ciprofloxacin whereas TL7, TL8, TL9, TL12, U, J3, J11, J13, J20, J22, J24, J25, and 140 were resistant towards both antibiotics. For carbapenem antibiotics (doripenem, imipenem and meropenem), J3, J11, and J23 were sensitive towards all 3 antibiotics and J29 was sensitive towards meropenem and intermediate to doripenem and imipenem, whereas, TL7, TL8, TL9, TL12, U, J13, J20, J22, J24, J25, J27, and 140 were resistant towards all three carbapenem antibiotics. All isolates were found sensitive towards polymyxin (colistin) and glycylcycline (tigecycline) antibiotics. AST data were also useful in corelating the presence of resistance gene with its resistance profile. AST and MIC data for all clinical isolates are shown in Supplementary Material section.

Whole Genome Sequence-Based MLST and Serotyping

Multilocus sequence typing was performed for all clinical isolates. A total of 61 different sequence types were found in

P. aeruginosa clinical isolates in India. As shown in Fig. 2a, high-risk clones were found to be circulating in highest numbers in India which included ($n=23$; 11.6%) isolates belonged to ST357 sequence type, which is a high-risk clone, was the most frequent across genomes, followed by ST308 ($n=15$; 7.6%), ST1203 ($n=14$; 7%), ST1047 ($n=13$; 6.5%), ST244 (n = 10; 5%) and ST654 (n = 10; 5%). Whereas, non-high-risk clones included ST316 ($n=12$; 6%), ST664 ($n=8$; 4%), ST235 ($n=7$; 3.5%), ST823 ($n=6$; 3%), ST303 ($n=5$; 2.5%) and ($n=8$, 4%) isolates with unknown sequence types.

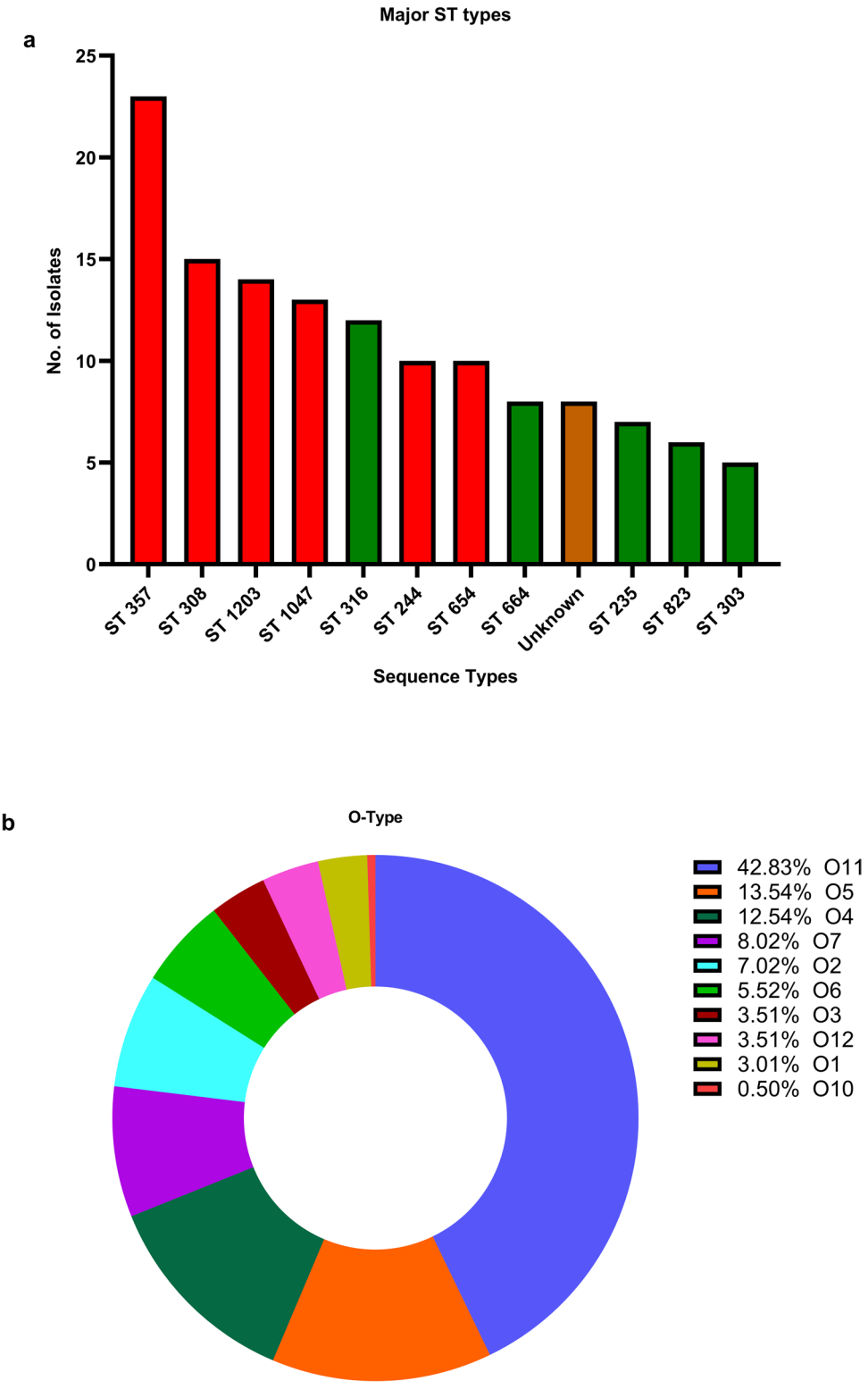
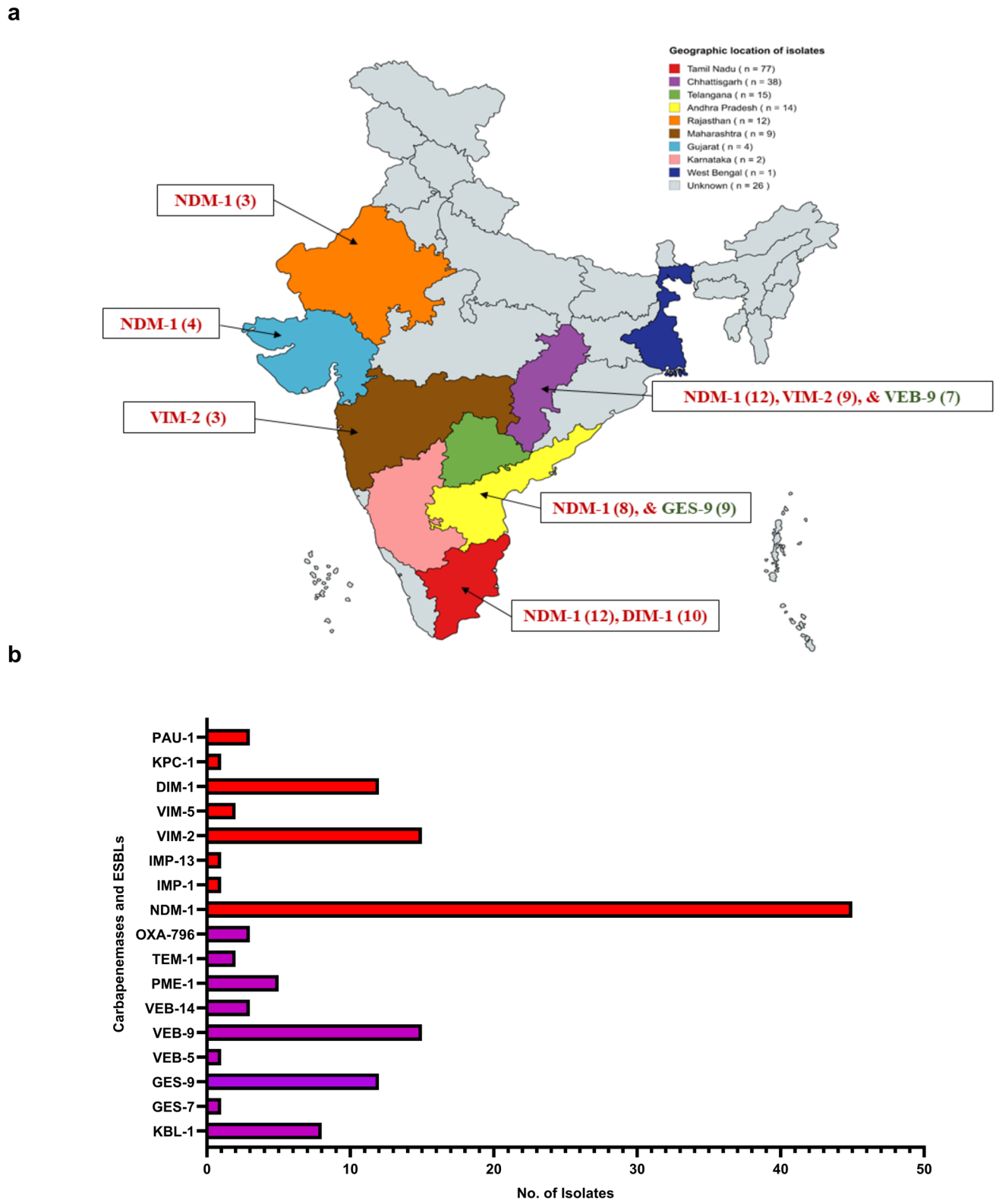


Fig. 2 Major sequence types and serotypes found in clinical *P. aeruginosa*. *a* Bars in the graph indicate the number of isolates associated with specific sequence type. Bars with red color indicate the given ST as a high-risk clone, bars in green color indicate the given ST to be a non-high-risk clone and brown color bar indicate unknown STs. **b** Parts of the circle indicate the association of serotype with percentage of *P. aeruginosa* isolates. O11 serotype was found to be predominant among *P. aeruginosa* in India. GraphPad Prism 10.0.1 software was used to create this graph (Color figure online)



Isolate J11 which was collected from Mahatma Gandhi Hospital, Jaipur was found to have a novel allelic combination of housekeeping genes (*acsA*-140; *aroE*-203; *guaA*-64; *mutL*-26; *nuoD*-28; *ppsA*-24; *trpE*-32), was submitted to Bacterial Isolate Genome Sequence Database (BigSdb) and ST4937 was assigned to this novel allelic combination. As shown in Fig. 2b, Serotyping analysis results showed that, serotype O11 ($n = 85$; 42.7%) was the most circulating, followed by

◂**Fig. 3** Carbapenemases and ESBLs found in *P. aeruginosa* in India and distribution of carbapenemases, ESBL and other beta-lactamases in India. **a** NDM-1 carbapenemase was found to be most prevalent among all the carbapenemases in India. Gujarat, Rajasthan, Andhra Pradesh, Tamil Nadu and Chhattisgarh showed presence of NDM-1 in 3 or more than 3 clinical isolates. Tamil Nadu showed presence of highest number of isolates possessing DIM-1 whereas, Chhattisgarh showed highest number of isolates with VIM-2. ESBLs such as GES-9 and VEB-9 were found to be predominant in Andhra Pradesh and Chhattisgarh. **b** Bars in the graph represent the number of isolates possessing specific gene. Bars in red color represent the carbapenemase genes found in *P. aeruginosa* whereas, bars in purple color represents ESBLs. GraphPad Prism 10.0.1 software was used to create this graph (Color figure online)

O5 ($n=27$; 13.6%), O4 ($n=25$; 12.6%), O7 ($n=16$; 8%), O2 ($n=14$; 7%), O6 ($n=11$; 5.5%), O3 & O7 each ($n=7$; 3.5%), O1 ($n=6$; 3%), and O10 ($n=1$; 0.5%).

Presence of Carbapenemases, Extended Spectrum Beta-lactamases (ESBL), and Other Beta-lactamases

$bla_{\text{NDM-1}}$ carbapenemase was first found in UTI isolates in year 2014. $bla_{\text{NDM-1}}$ was found to be present in ($n=45$, 22.8%) isolates associated with ST357 ($n=12$), ST1203 ($n=9$), ST654 ($n=8$), ST244 ($n=4$), ST308 ($n=4$), ST1047 ($n=3$), ST773 ($n=2$) and ST1601 and unknown ST ($n=1$) each and was associated with UTI, respiratory, blood, ET, sputum and tissue infections. Surprisingly, 1 isolate each from ear and stool were found to harbour $bla_{\text{NDM-1}}$. Fortunately, no isolates from eye infections showed presence of $bla_{\text{NDM-1}}$. ($n=12$; 50%) isolates from UTI were found to possess $bla_{\text{NDM-1}}$. $bla_{\text{VIM-2}}$ carbapenemase was associated with (n = 15) isolates belonging to ST823, ST132, ST233, ST773, ST357 and ST1047 and no isolate was found to be co-harbouring $bla_{\text{NDM-1}}$ and $bla_{\text{VIM-2}}$. ($n=12$, 6%) isolates were found to be harbouring $bla_{\text{DIM-1}}$ carbapenemase and were associated with ST1203, ST 1047, and ST 1601 were found with infections from respiratory, pus and stool. 11 out of 12 isolates were found to co-harbour $bla_{\text{NDM-1}}$ and $bla_{\text{DIM-1}}$. Geographic distribution and epidemiology of carbapenemases in India are shown in Fig. 3a.

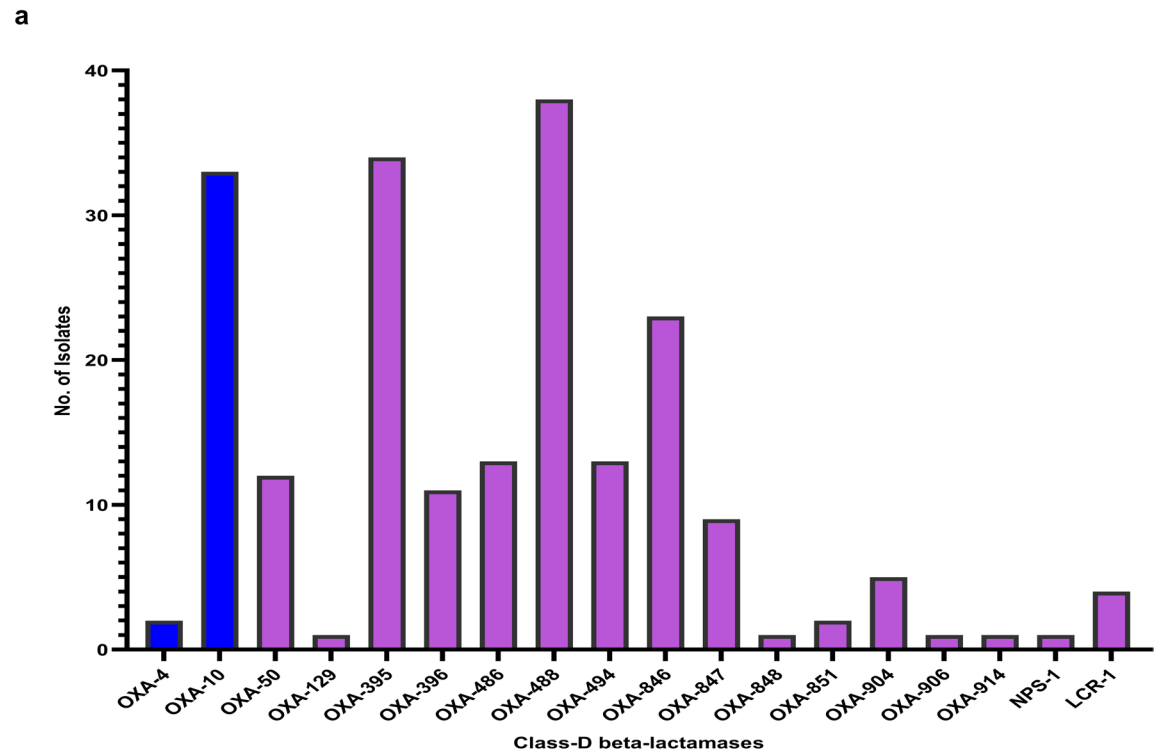
$bla_{\text{VEB-9}}$ ESBL gene was found to be present in ($n=15$, 7.5%) isolates belonging to ST357, ST111, and ST235 from ET, pus, UTI, and tissue infections. $bla_{\text{GES-9}}$ ESBL was found to be present in ($n=12$) isolates belonging to ST313, ST1203, and ST654 from UTI, pericardial fluid, and pus. ($n=8$, 4%) isolates were found to be harbouring the $bla_{\text{KBL-1}}$ gene and were associated with ST654 from UTI infections, and all isolates showed copresence of $bla_{\text{NDM-1}}$ with $bla_{\text{KBL-1}}$. $bla_{\text{PME-1}}$ ESBL gene was found to be present in ($n=5$, 2.5%) isolates belonging to ST235, ST244, ST260, ST1047, and ST308 from eye, blood, UTI, pus, and ear origin. Carbapenemases and ESBLs found in Indian isolates of *P. aeruginosa* is shown in Fig. 3b.

$bla_{\text{OXA-10}}$ (narrow spectrum) was found to be present in combinations with $bla_{\text{OXA-846}}$, $bla_{\text{OXA-129}}$, $bla_{\text{OXA-847}}$, and $bla_{\text{OXA-488}}$ and was associated with ST357, ST244, ST534, ST308, ST1047 from UTI, ET, ear, eye, sputum, and pus. $bla_{\text{OXA-396}}$ was found to be present only in isolates associated with UTI with ST654. No other OXA were found to be in combination with $bla_{\text{OXA-396}}$. $bla_{\text{OXA-395}}$ was associated with ST773, ST1203, and 1 unknown ST from blood, UTI, sputum, respiratory, and stool. $bla_{\text{OXA-395}}$ was not found in combination with other OXAs. $bla_{\text{OXA-488}}$ was associated with ST308, ST534, and ST1047 from isolation sites such as UTI, tissue, ET, sputum, and pus. $bla_{\text{OXA-488}}$ was found to be in combination with $bla_{\text{OXA-10}}$. $bla_{\text{OXA-846}}$ was associated with ST357 and was also found to be in combination with $bla_{\text{OXA-10}}$ and was associated with UTI, tissue, and ET infections. From our collected isolates, susceptible isolate J23 and MDR isolates J3, J11, J24, and J29 showed absence of bla_{OXA}. Variants of bla_{OXA} found in *P. aeruginosa* are shown in Fig. 4a.

In a genomic analysis, $n=22$ isolates (11%) were identified as possessing $bla_{\text{PAC-1}}$ extended spectrum AmpC (ESAC) gene. $bla_{\text{PDC-3}}$ was identified as being linked to ST654 from UTIs. In $bla_{\text{PDC-19a}}$, two combinations were identified: O5 serotype with ST1203, and O11 serotype with ST308. The isolation sites included stool, respiratory, sputum, UTI, and ET samples. $bla_{\text{PDC-11}}$ was linked to O11, and ST357 covered eye, urinary tract, endotracheal, and tissue infections. $bla_{\text{PDC-1}}$ was correlated with O12 and ST244, which were associated with UTI and ear infections. $bla_{\text{PDC-12}}$ was linked to O7 and ST1047 from pus, UTIs, and tissue infections. $bla_{\text{PDC-16}}$ was identified alongside O11, ST773, and unidentified sequence types from urine and sputum infections. $bla_{\text{PDC-35}}$ was linked to O11, ST534 from ET infections. $bla_{\text{PDC-39}}$ was linked to the O11 serotype, ST1601, originating from respiratory infection sites. Variants of bla_{PDC} identified in *P. aeruginosa* in India, along with their correlation to clinical isolates, are depicted in Fig. 4b.

Presence of Other Antibiotic Resistance Genes

aac(3)Ic, an aminoglycoside acetyltransferase, was found to be present in ($n=2$) isolates where both isolates belonged to ST235. *aac(3)Id*, another aminoglycoside acetyltransferase was found to be associated with ($n=3$) isolates of ST316, and ST1284 from eye infections. *aac(6')Ib* was associated with ($n=2$) isolates associated with ST1203. *aadA* aminoglycoside nucleotidyltransferase was found to be present in ($n=2$) isolates with ST316. *aadA2* encoded by integrons was found in ($n=3$) isolates of ST244, ST233, and ST132. *aadA5*, another integron-encoded aminoglycoside transferase was found in only ($n=1$) isolate of ST1203. *dfrA5*, and *dfrA15* which are integron-encoded dihydrofolate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No. of Isolates
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10
20
30
40
OXA-4
OXA-10
OXA-50
OXA-129
OXA-395
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OXA-486
OXA-488
OXA-494
OXA-846
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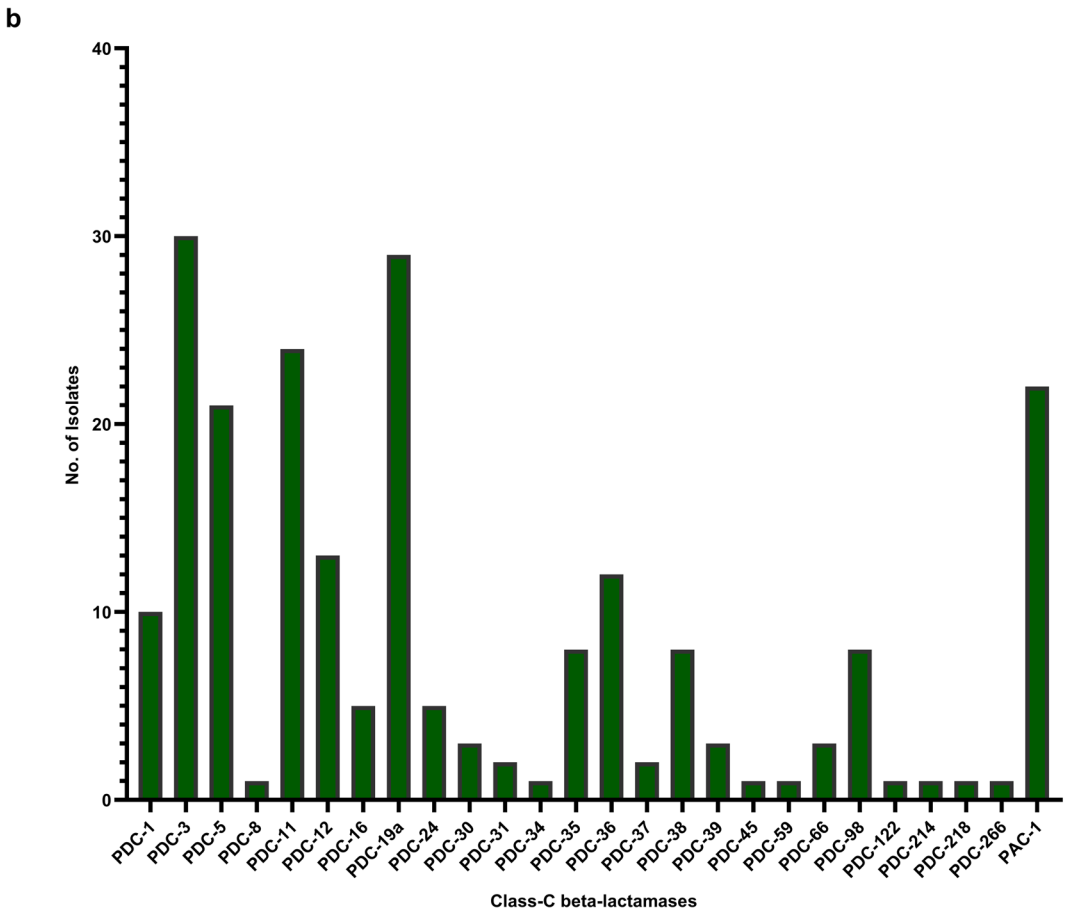
LCR-1
Class-D beta-lactamases


b
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10
20
30
40
PDC-1
PDC-3
PDC-5
PDC-8
PDC-11
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PDC-16
PDC-19a
PDC-24
PDC-30
PDC-31
PDC-34
PDC-35
PDC-36
PDC-37
PDC-38
PDC-39
PDC-45
PDC-59
PDC-66
PDC-98
PDC-122
PDC-214
PDC-218
PDC-266
PAC-1
Class-C beta-lactamases

◂**Fig. 4** Class-C and Class-D beta-lactamases found in *P. aeruginosa*. **a** Bars in the graph marks the number of isolates found to harbour specific variant of Class-D beta-lactamase. Bars with blue color represents narrow spectrum beta-lactamases whereas, bars in purple represents Oxacillinases. GraphPad Prism 10.0.1 software was used to create this graph. **b** Bars in the graph indicated the number of isolates found to harbour specific variant of Class-C beta-lactamase. PDC-19a, PDC-3 and PDC-11 were found to be present in highest number of isolates. GraphPad Prism 10.0.1 software was used to create this graph (Color figure online)

reductase were found in only ($n=1$) isolate each from ST1203, and ST1284. *dfrB5*, a dihydrofolate reductase and trimethoprim resistance gene was found in ($n=9$) isolates of ST308, ST354, and ST823.

mphA which is known to phosphorylate macrolides was found in ($n=10$) of ST1203 and all were associated with respiratory infection isolates. *mphE*, a macrolide phosphotransferase and resistance gene were found to be present in ($n=15$) isolates of respiratory origin isolates belonging to ST1203, ST357, ST308, ST1047, and unknown ST. *msrE* which is associated with plasmid DNA was found in ($n=18$) isolates of respiratory, abdominal, stool, eye, UTI, pus, ear, and ET origin. *msrE* was associated with ST308, ST244, ST1047, ST1203, ST357, and an unknown ST. *Mrx* which is part of macrolide inactivation gene cluster was found in ($n=14$) isolates from respiratory, ear, UTI, and eye origin and were found to be associated with ST244, ST235, ST1203, and ST308. Presence of all antibiotic resistance genes found in *P. aeruginosa* in India is shown as iTOL image in Fig. 5.

Discussion

The present study was carried out to understand the prevalence and distribution of antibiotic resistance genes in clinical *P. aeruginosa* in India. Sixteen isolates of *P. aeruginosa* were gathered from the western part of India. In addition, we retrieved 181 whole genome data of *P. aeruginosa* isolates submitted to the BV-BRC database and conducted a bioinformatics study to analyse the resistance pattern. International high-risk clones ST357, ST308, ST1203, ST654 and ST235 which are prevalent in Asian countries, were found to be associated with highest number of isolates ($n=23$), ($n=15$), ($n=14$), ($n=10$), and ($n=8$) respectively [12, 27–29]. In Spain [12], France, Belgium, and Switzerland [30] O6 and O1 serotypes were found to be most frequent among *P. aeruginosa*, whereas our study showed that in India, O11 serotype is found to be dominant and was associated with multiple drug resistance. It is reported that O11 serotype is associated with high mortality and increased antibiotic resistance [31]. However, in another report, isolates of serotype O11 exhibited the highest sensitivity to nearly all tested antimicrobials in comparison to isolates of other frequently encountered serotypes [32]. No high-risk clones were found to be associated with O1, O2, O3, O7, and O10 serotypes.

$bla_{\text{NDM-1}}$ was first reported in *Klebsiella pneumoniae* from a Swedish patient in the year 2008 [33] however, in our analysis, the first isolate harbouring $bla_{\text{NDM-1}}$ was discovered in the year 2014. Since then, there are regular encounters of $bla_{\text{NDM-1}}$ in India. We observed that in the year 2021 and 2022, out of total 25 isolates (16 collected by us and 9 from genome data submitted to BV-BRC), ($n=15$, 60%) isolates were found to be harbouring $bla_{\text{NDM-1}}$. We found that no isolates originating from eye infections were harbouring $bla_{\text{NDM-1}}$. Before 2014, no isolate showed presence of $bla_{\text{NDM-1}}$ in *P. aeruginosa* in India. However, it might be possible that $bla_{\text{NDM-1}}$ could be harbouring in Indian isolates before 2014, but due to lower number of genomes submitted, actual picture of $bla_{\text{NDM-1}}$ circulation could not be figured out. Horizontally transferable $bla_{\text{VIM-2}}$ in co-existence with $bla_{\text{NDM-1}}$ is reported to reduce susceptibility rates and increase MIC values [34]; however, in the present study, no isolate was found co-harbouring $bla_{\text{VIM-2}}$ and $bla_{\text{NDM-1}}$.

The emergence of bla_{KPC} gene was first found in *P. aeruginosa* in 2009 in the United States [35]. In our study, only one isolate, MGL-108, was found to co-harbour $bla_{\text{KPC-2}}$ and $bla_{\text{VEB-5}}$. There is a report of $bla_{\text{KPC-2}}$ in *P. aeruginosa* in Germany [36]. Interestingly, they also had found it to be ST235. $bla_{\text{DIM-1}}$ carbapenemase was first reported in *P. stutzeri* in 2003 and is known to have broad-spectrum activity against cephalosporins and carbapenems [37]. In this study, $bla_{\text{DIM-1}}$ was found to be in co-existence with $bla_{\text{NDM-1}}$, and ST1203 was associated with it. $bla_{\text{PAC-1}}$ is reported to be associated with ST664 [38], but in our study, along with ST664, $bla_{\text{PAC-1}}$ was found to be associated with ST235, ST534, ST316, ST1047, ST308, and ST244. Carbapenemases and ESBLs, which are known to provide resistance towards carbapenem drugs were found in a lower number of isolates. This suggests that carbapenemases and ESBLs mediated resistance in *P. aeruginosa* in India might not be at the peak alarming rate. But increasing high-risk clones of *P. aeruginosa* can lead to increased circulation of high-risk clones in India.

In this study, resistance against β-lactams was due to the expression of the chromosomally encoded class C and D bla_{PDC} and bla_{OXA}. bla_{PDC} being chromosomally encoded, all isolates (collected as well as submitted) showed the presence of bla_{PDC}, and more than 24 variants of bla_{PDC} were found in Indian isolates. Since the isolates ranged from collection year 1995 to 2022; this variation is naturally possible. Many structural modifications of bla_{PDC} leading to changes in the β-lactam resistance profile are reported as evidenced by the report of > 400 bla_{PDC} variants. The huge number of allelic variants accounts for a

Fig. 5 iTOL representation of AMR genes found in clinical *P. aeruginosa* from India. Isolates name highlighted with green were collected by lab, nodes highlighted with red color indicates isolate belonging to high-risk clone, circles filled with red color shows presence of carbapenemases, circles filled with purple color marks the presence of ESBLs, circles filled with dark yellow color marks presence of narrow spectrum beta-lactamases, circles with mulberry color shows the presence of Class-C beta-lactamases in isolate and circles filled with blue color shows presence of Class-D oxacillinases. Circles filled with green color shows presence of aminoglycosides resistance genes and circles filled with light blue colour shows presence of macrolide resistance genes. Unfilled circles marks the absence of respective gene. Along with that, year of isolation, sequence type, serotype, site of origin and name of state of isolation is shown. iTOL online tool was used to create this image (Color figure online)

highly polymorphic enzyme capable of tolerating amino acid substitutions, insertions, and deletion. Experiments on long-term evolution on a single patient, expanding the analysis over the course of 26-years of a *P. aeruginosa* hypermutator lineage in the CF lung, led to the selection of novel bla_{PDC} allelic variants with enhanced cephalosporin resistance [39]. Since, most Indian isolates co-harboured either bla_{OXA} or bla_{NDM} with bla_{PDC}, the actual

contribution of bla_{PDC} in providing resistance is difficult to comment.

aph(3')-IIb (detected in all isolates) is a chromosomally encoded aminoglycoside phosphotransferase though it does not provide resistance against aminoglycosides antibiotics such as gentamicin, amikacin, and tobramycin [40]. Other aminoglycosides modifying enzymes such as *aac(3)-Ic*, *aac(3)-IId, aac(6')-Ib, aac(6')-Ii, aph(3')-Ib, aph(3')-VIa, ant(2')-Ia, aadA, aadA2, aadA5, aadA6,* and *aadA11* were occasionally found in our study. Further, it is known that aminoglycosides modifying enzymes are sufficient to cause aminoglycosides resistance and have been shown to have independent as well as additive effects with other aminoglycoside resistance determinants in *P. aeruginosa* [41].

The drawback of the present study is that the resistance categories (Susceptible/MDR/XDR/PDR) for clinical genomes submitted to BV-BRC databases were not available, so that exact comparison for antibiotic resistance profiles with antibiotic resistance genes cannot be co-related. Another important analysis about the presence of resistance genes on plasmid or chromosome was not possible due to limitations of available tools. We were unable to detect plasmids from sequencing data using PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/) tool as it lacks *Pseudomonas* database. We further accessed ICEFinder (https://bioinfo-mml.sjtu.edu.cn/ICEfinder/ICEfinder.html) tool to check the presence of gene on plasmid or chromosome, but unfortunately short-read sequencing restricts this analysis. A substantial quantity of genomic data were available from southern India, while only a limited number of genomes were available from western India, and even less genomes were present from both northern and eastern India. To fill this gap, a Pan-India study can be carried out with significant number of isolates from various infection sites from different regions in India.

Conclusion

To the best of our knowledge, this is the first whole genome sequence-based study undertaken to know the genetic diversity prevailing among resistance genes in pathogenic *P. aeruginosa* in India. We found that carbapenemases and ESBLs are circulating in lower number in India which could be a time being relief with respect to tackling antimicrobial resistance. However, ten isolates exhibiting a combination of the third most prevalent sequence type, ST1203, and the second most common serotype, O5, possessed dual carbapenemase genes (bla_{NDM-1} and bla_{DIM-1}), as well as, ST773, ST1601, and ST534, which possess bla_{NDM-1}, were found in few isolates, but they can be potent high-risk clones in future. No additional bla_{OXA} were identified in the collected susceptible and multidrug-resistant isolates, which may serve as a target for beta-lactamase detection in extensively drug-resistant and pandrug-resistant infections. The significant diversity of antimicrobial resistance genes observed in Indian *P. aeruginosa* underscores the necessity of regional surveillance. This finding will facilitate future research in comprehending the epidemiology of high-risk clones of *P. aeruginosa*.

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Data Availability Data will be available upon request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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