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Molecular and genomic insights into multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa* causing burn wound infections in Bangladesh

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The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa* represents a significant challenge in managing nosocomial infections, particularly in vulnerable populations such as burn patients. This study provides genomic and molecular characterization of MDR and XDR *P. aeruginosa* strains isolated from burn patients at Sheikh Hasina National Institute of Burn and Plastic Surgery (SHNIBPS) in Dhaka, Bangladesh. Over an 8-month period, 110 wound swabs were collected, with 91 isolates identified as *P. aeruginosa*. Antimicrobial susceptibility testing demonstrated a multidrug-resistant pattern in 30 isolates and an extensive drug-resistant pattern in the remaining 61 isolates analyzed in this study. PCR assays detected beta-lactamase genes from all four Ambler classes, revealing a notable prevalence of bla_{NDM-1} (16.48%) and bla_{VIM-2} (31.87%), with both genes co-occurring in 3.30% of the isolates. Additionally, bla_{PER-1} (15.38%), bla_{CTX-M} (4.40%), bla_{OXA-1} (84.62%), and bla_{OXA-48} (51.65%) genes were detected. Class I integrons were detected in 84 isolates. A total of 21% of the isolates exhibited strong biofilm-forming capabilities. Key biofilm-associated genes (*pel*B, *pil*T, *rhl*B) were detected in most of the isolates. Whole genome sequence analysis of two selected XDR isolates identified different beta-lactamase genes such as bla_{PDC-98}, $bla_{PDC-374}$, bla_{OXA-50}, $bla_{OXA-677}$ and $bla_{OXA-847}$. Virulence factor genes, metal resistance genes, and prophage sequences were also identified in the analysis. The genomic epidemiology analysis of 9,055 *P. aeruginosa* strains, based on MLST data, revealed the dominance of ST235. The bla_{PDC} and bla_{OXA} genes were found to be notably prominent worldwide. The comparative genomic analysis of *P. aeruginosa* strains from Bangladesh demonstrated an expanding pangenome as well as high degree of genetic variability. The study emphasized the dynamic nature of the *P. aeruginosa* pangenome and underscored the necessity for stringent infection control measures in burn units to manage and mitigate the spread of these highly resistant strains.

Keywords *Pseudomonas aeruginosa*, Extensively drug-resistant (XDR), Burn wound infection, Bangladesh, Beta lactam resistance, Biofilm

In healthcare, managing burn wound infections is a considerable challenge, as they significantly raise patient morbidity and mortality[1]. Among the numerous pathogens responsible for these infections, *Pseudomonas aeruginosa* (*P. aeruginosa*) stands out as a particularly problematic agent, known for causing severe complications and delaying the healing process[2,3]. *Pseudomonas aeruginosa* is a Gram-negative, opportunistic pathogen

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that is commonly associated with hospital-acquired infections[4]. Its ability to thrive in diverse environments, including water, soil, and clinical settings, makes it a formidable pathogen in healthcare facilities. The emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug resistant (PDR) strains of *P. aeruginosa* has been reported worldwide, exacerbating the challenges in clinical management[5,6]. These resistant strains are often linked to prolonged hospital stays, increased healthcare costs, and higher mortality rates. The spread of these strains is facilitated by their ability to form biofilms on medical devices, such as catheters and ventilators, which protect them from both the host immune system and antibiotic treatment[5,7,8].

The rise of XDR *P. aeruginosa* represents a significant challenge in the management of nosocomial infections, particularly in vulnerable populations such as burn patients[9,10]. In burn units, where patients are highly susceptible to infections due to compromised skin integrity and immune function, outbreaks of XDR *P. aeruginosa* can lead to severe complications, prolonged hospital stays, and increased mortality rates. Infections caused by *P. aeruginosa* in burn wounds have been linked to prolonged hospital stays, increased healthcare costs, and, in severe cases, can lead to septicemia and death[11]. Over the past few decades, the extensive use and misuse of antibiotics have led to the development and spread of MDR strains of *P. aeruginosa*. Recently, the emergence of XDR strains has raised significant concerns among healthcare professionals due to their limited treatment options and potentially severe outcomes[12].

Beta-lactam antibiotics, encompassing penicillins, cephalosporins, carbapenems, and monobactams, have played a crucial role in treating bacterial infections since their discovery[13]. However, the increasing prevalence of beta-lactam antibiotic resistance has significantly compromised the effectiveness of these drugs, primarily due to bacterial production of beta-lactamase enzymes[14]. Besides, biofilm formation significantly enhances antimicrobial resistance in *P. aeruginosa* by providing a protective environment for bacterial cells[15]. The extracellular polymeric substance (EPS) matrix acts as a physical barrier, limiting antibiotic penetration. Within the biofilm, bacteria exist in various metabolic states, including dormant cells less susceptible to antibiotics targeting active growth[15,16]. Biofilms also facilitate horizontal gene transfer, spreading antibiotic resistance genes, and harbor persister cells that can survive antibiotic treatment and repopulate the biofilm. Additionally, quorum sensing regulates biofilm development and antibiotic resistance[17]. Efflux pumps play a crucial role in antimicrobial resistance in *P. aeruginosa* by actively expelling a wide range of antibiotics from the bacterial cell, thereby reducing their intracellular concentrations and effectiveness[18]. These pumps, such as MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY, span the bacterial cell membrane and outer membrane, creating a direct route for antibiotic expulsion. Integrons are primarily recognized as the genetic entities accountable for acquiring and disseminating antibiotic resistance traits across a wide range of Gram-negative clinical isolates[19]. Among the main three classes of integrons, class I type integron is more significant and widespread in *P. aeruginosa*[20]. Microbial entities can rapidly acquire genes from various organisms, which may increase their virulence or promote antimicrobial resistance[21]. Recent epidemiological studies have revealed a rising trend in antimicrobial resistance, with a notable increase in multi-drug resistant *P. aeruginosa* isolates in recent years[22,23].

Whole genome and metagenome sequencing have become pivotal in tackling the critical challenges posed by MDR, XDR, and PDR bacteria within the healthcare sector[24–27]. These sequencing technologies enable researchers to uncover extensive information, including antimicrobial resistance genes, phenotypes, virulence factors, and metabolic pathways[3,28,29]. Beyond these applications, sequencing also plays a vital role in epidemiological analysis by allowing high-resolution tracking of bacterial transmission pathways, identifying outbreak sources, and monitoring the emergence and spread of resistance traits across populations[30,31]. This comprehensive understanding is essential for developing effective strategies to combat the severe impact of these resistant organisms. In the current study, WGS approach is applied to investigate the genetic landscape of two XDR *P. aeruginosa* strain, with a specific focus on its AMR gene repertoire, mobile genetic elements, and virulence factors. Additionally, advanced applications such as phylogenomic, pangenome analysis and comparative genomics are leveraged to explore the genomic dynamics of these strain, shedding light on its relationship to other clinically significant *P. aeruginosa* isolates from Bangladesh.

In Bangladesh, the situation is particularly alarming due to the high prevalence of *P. aeruginosa* in burn units, coupled with limited resources for effective infection control and antimicrobial stewardship[3,32,33]. This study offers detailed genomic and molecular insights into MDR and XDR *P. aeruginosa* strains isolated from burn patients in a major burn unit hospital in Bangladesh, with a specific focus on *XDR* strains. By analyzing the resistance patterns, virulence factors, and genetic diversity of these isolates, we aim to understand the burden of drug resistance and pathogenicity in this critical clinical setting. The study emphasized the dynamic nature of the *P. aeruginosa* pangenome and underscored the importance of stringent infection control measures in burn units to manage and mitigate the spread of these highly resistant strains.

Results

Sample collection and isolate identification

Out of the initial 110 samples, 91 were selected for detailed analysis following preliminary assessment. From these 91 samples, 91 bacterial strains (n = 91) were successfully isolated which were responsible for causing infection in the burn wounds of the injured patients. The cohort of patients (n = 91) from whom these samples were obtained included 62 males and 29 females, representing various age groups (Supplementary Fig. 2). Through biochemical and cultural identification, the 91 isolates were presumptively identified as *P. aeruginosa*. After that, molecular identification through marker gene specific PCR confirmed and validated the isolates to be *P. aeruginosa* (Supplementary Fig. 7).

Extensive drug resistance pattern of *P. aeruginosa* in burn wound infection

The antimicrobial resistance pattern of 91 isolates of *P. aeruginosa* in 15 antibiotics of 8 categories were determined. More than 80% of the isolates showed resistance to aminoglycosides and 69 isolates showed resistance in all

three of the aminoglycosides tested such as amikacin, tobramycin and gentamicin. Besides, > 80% of the isolates showed resistance to fluoroquinolones such as ciprofloxacin and levofloxacin (Fig. 1a). Out of the 91 isolates examined in this study, 76 demonstrated resistance to meropenem, while 42 exhibited resistance to imipenem. On the other hand, all of the isolates showed sensitivity to polymyxin B and colistin. Thirty isolates demonstrated resistance to at least one agent within a minimum of three antimicrobial groups, indicating multidrug-resistance pattern. All the isolates were categorized as MDR. Notably, 61 out of the 91 isolates were categorized as XDR, highlighting their heightened resistance to a broad spectrum of drugs.

Antibiotic resistance genes investigation

Antibiotic resistance genes investigation revealed the abundance of beta lactamase producing genes. Fifteen (16.48%) isolates among the total 91 isolates of *P. aeruginosa* were detected to harboring bla_{NDM-1} type genes (Fig. 1b). On the other hand, bla_{vim-2} type genes were found in 29 isolates. The co-existence of bla_{NDM-1} and bla_{VIM-2} type genes was detected in three isolates. Besides, bla_{PER-1} and bla_{CTX-M} type genes, which are class A beta lactamase gene, was found in 14 and 4 isolates respectively. However, class D beta lactamase bla_{OXA-1} like genes were detected in 77 isolates and 47 isolates carried bla_{OXA-48} type genes. Class I Integron was detected in 84 isolates among all 91 drug resistant isolates. Efflux pump gene investigation revealed that *mex*A, *mex*C and *mex*E genes were carried by most of the test isolates (Fig. 1c). Interestingly, only one isolate was found where no *mex*A, *mex*C or *mex*E genes were detected.

Strong biofilm formation and abundance of associated genes

After completion of the biofilm formation assay, it was found that all the isolates were biofilm former. Among them, 19 (21%) isolates were detected as SBF and 45 isolates (49%) were found to be MBF (Fig. 2a). On the other

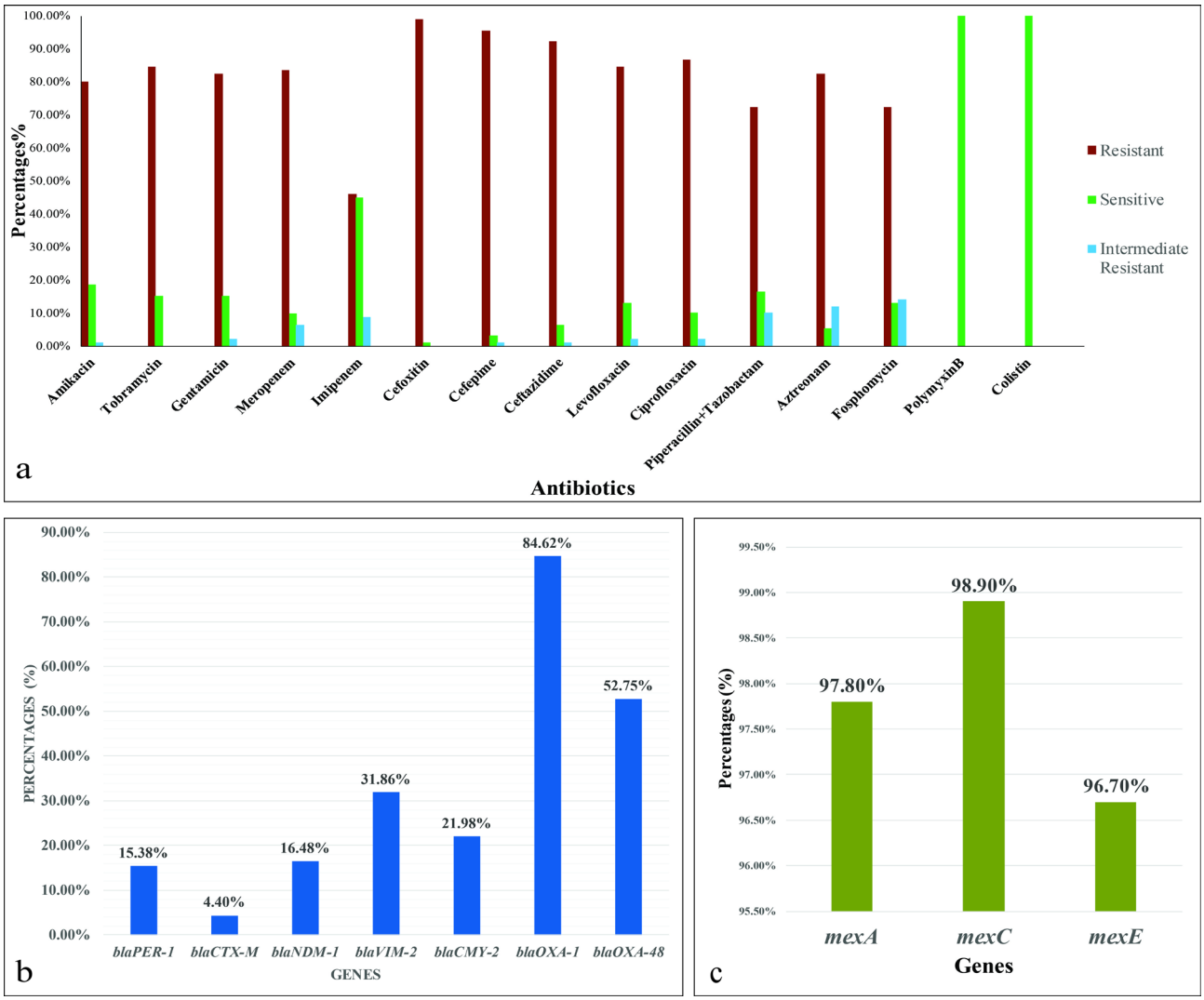

Fig. 1. Comprehensive analysis of antimicrobial resistance and genetic determinants in drug-resistant *P. aeruginosa* isolates. (**a**) Antibiotic susceptibility profile of the isolates against 14 antibiotics. (**b**) Prevalence and distribution of beta-lactamase genes harbored by the isolates. (**c**) Prevalence and distribution of efflux.

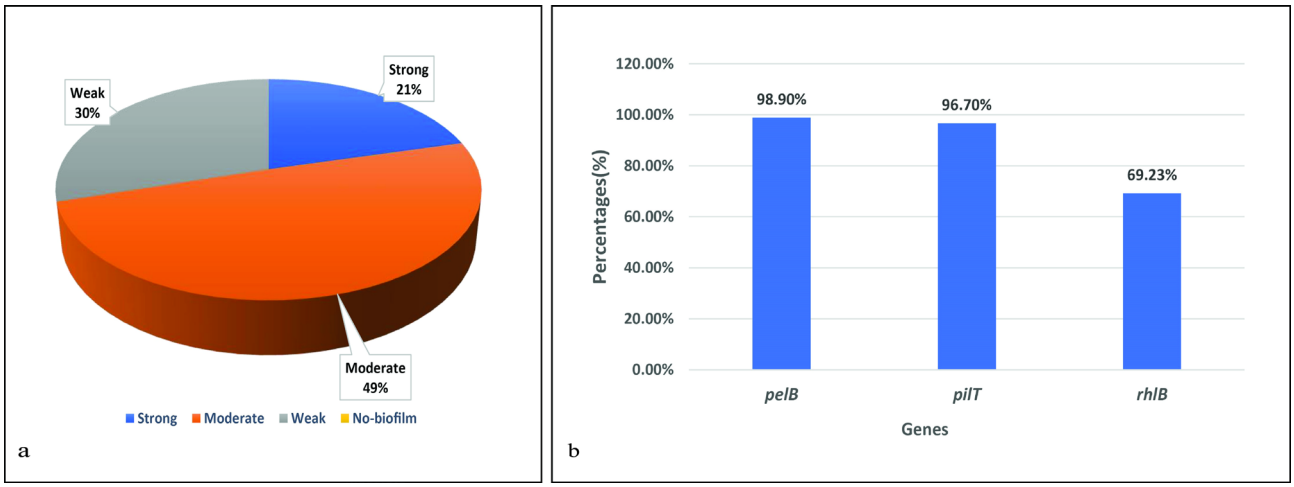

Fig. 2. Analysis of biofilm formation and associated genes in *P. aeruginosa* isolates of this study. (**a**) Distribution of biofilm-forming capacity among the isolates. (**b**) Prevalence of biofilm-associated genes *pel*B, *pil*T and *rhl*B among the test isolates.

PATRIC annotation	*Pseudomonas aeruginosa* SHNIBPS64	*Pseudomonas aeruginosa* SHNIBPS243	Prokka Annotation	*Pseudomonas aeruginosa* SHNIBPS64	*Pseudomonas aeruginosa* SHNIBPS243
Completeness of genome	96	93	Contigs	3	8
Coarse consistency	98.4	94.3	Bases	6,795,444	6,664,281
Contigs	3	8	CDS	6626	8049
genome length	6,795,444	6,664,281	rRNA	3	3
GC content	65.72342	64.98262	Repeat Region	0	4
Contig L50	1	1	tRNA	53	52
Contig N50	6,711,566	4,442,597	tmRNA	1	1
CDS	7051	8261			
CDS ratio	1.037607	1.2395936			
Hypothetical CDS	1796	3000			
tRNA	51	49			
rRNA	3	3			

Table 1. General features of the genomes through annotation with Patric Server.

hand, 27 isolates (30%) were found to be weak biofilm former. Biofilm formation and virulence associated genes investigation revealed the presence of *pel*B gene in 90 isolates among the 91 test isolates (Fig. 2b). Besides, *pil*T and *rhl*B genes were detected in 88 and 63 isolates respectively.

Whole genome sequence analysis revealed MLST, serogroup and diverse genomic features

The WGS-based identification confirmed the two selected isolates to be two different strains of *P. aeruginosa* (*P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243). The multi locus sequence typing (MLST) and serotyping indicated toward two different sequence types and serotypes of the two isolates respectively. *P. aeruginosa* SHNIBPS64 was predicted to be of multi locus sequence type 664 (MLST 664) and serotype O7. Conversely, accurate detection of the MLST for *P. aeruginosa* SHNIBPS243 was hindered due to insufficient sequence coverage, particularly in the *gua*A and *mut*L loci. But, the closest sequence type determined for the isolate was ST 3934. However, serotype determination revealed that *P. aeruginosa* SHNIBPS243 was of serotype O12. After annotation and mapping, genomes of the two isolates showed different features (Table 1 and Fig. 3).

Antimicrobial resistance genes investigation revealed co-existence of different types of beta lactamase and efflux pump genes

Investigation of antimicrobial resistance genes from whole genome sequences of the two isolates revealed a numerous number and types of antimicrobial resistance genes. These genes and gene products are responsible for conferring resistance to different antibiotics. *Pseudomonas aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243 harbored around 50 and 28 antimicrobial resistance genes respectively. The co-existence of $bla_{\text{PDC-98}}$ (Class C) and $bla_{\text{OXA-50}}$ (Class D) beta lactamase producing genes were found in *P. aeruginosa* SHNIBPS64 (Fig. 4a). Along with that, two mutations (S209R, G71E) were observed in *nal*C gene (Supplementary Table 3). On

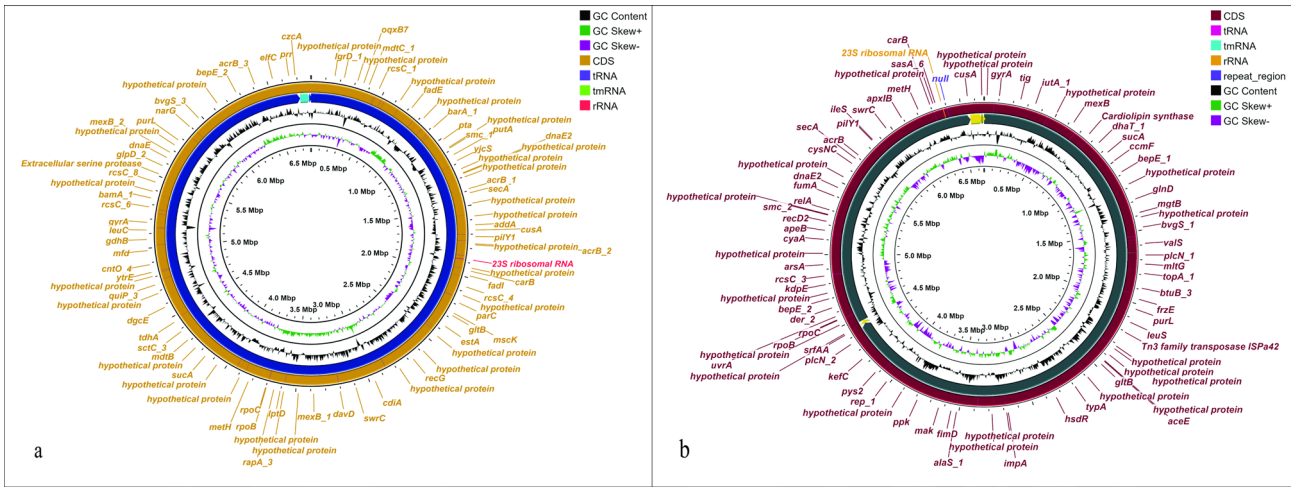

Fig. 3. Genome mapping of the whole genome sequenced isolates. (**a**) Genome map of *P. aeruginosa* SHNIBPS64. (**b**) Genome map of *P. aeruginosa* SHNIBPS243.

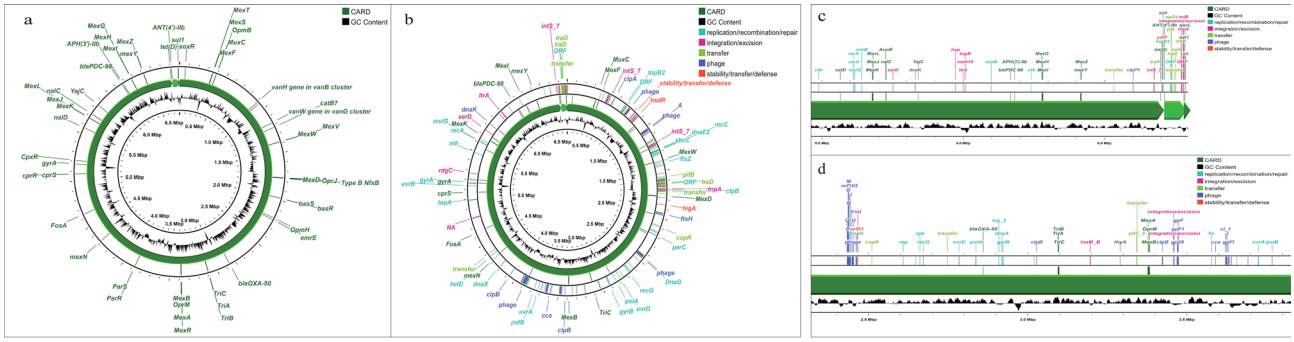

Fig. 4. Mapping of antimicrobial resistance genes and mobile genetic elements in *P. aeruginosa* SHNIBPS64. (**a**) Mapping of antimicrobial resistance genes. (**b**–**d**) Antimicrobial resistance genes and surrounding mobile genetic elements harbored by *P. aeruginosa* SHNIBPS64.

the other hand, in case of *P. aeruginosa* SHNIBPS243, co-existence of class C ($bla_{PDC-374}$) and class D ($bla_{OXA-677}$ and $bla_{OXA-847}$) beta lactamase genes were observed (Fig. 5a). No mutations in *nal*C gene was observed in this isolate (Supplementary Table 4). T83I mutation in *gyr*A gene was detected in both *P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243. Besides, both isolates harbored *sul*1 gene which is responsible for conferring resistance to sulfonamide antibiotics. Along with that, *qac*L and *qac*Delta1 genes were present in the isolates which confer resistance to disinfecting agents and antiseptics. Analysis of genes associated with efflux pumps identified various genes, including *mex*A, *mex*B, *mex*C, *mex*E etc., participating in the development of diverse efflux pumps that are recognized for their role in bestowing resistance to multiple antibiotics (Supplementary Tables 3 and 4). The efflux pump-related proteins accountable for the creation of distinct efflux pump operons are provided in the Table 2.

Mobile genetic elements investigation

Analysis revealed around 40 mobile genetic elements in case of both *P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243. Major MGEs found in *P. aeruginosa* SHNIBPS64 were ISPa100, ISPa32, ISPa1, IS6100, ISPsp1, cn_5674_ISPsp1. On the other hand, in case of *P. aeruginosa* SHNIBPS243, the major MGEs detected were ISPa37, ISPa32, ISPa6, IS6100, ISPa1, ISPa22, ISPa100, IS26, ISAs31, cn_2637_ISAs31. In depth investigation around the antimicrobial resistance genes revealed the presence of several mobile genetic elements and genes responsible for integration, excision and transfer of genetic materials (Figs. 4b and 5b). The informative genome mapping revealed that within *P. aeruginosa* SHNIBPS243, numerous antimicrobial resistance genes, including $bla_{OXA-677}$, *ant*(3″)-lla, *sul*1, and *qac*E, were flanked by mobile genetic elements such as *tni*B, *tnp*A, *tnp*M, *tnp*R, *int*S, and others which play crucial role in integration, excision and transfer of genes (Fig. 5c). Additionally, several genes and mobile genetic elements were identified near $bla_{OXA-847}$ and $bla_{PDC-374}$ (Fig. 5d,e). In the case of *P. aeruginosa* SHNIBPS64, numerous genes and elements potentially involved in the integration, excision, and transfer of genetic materials were found surrounding antimicrobial resistance genes, specifically bla_{PDC-98}, bla_{OXA-50}, *ant*(4′)-llb, *sul*1, and *aph*(3′)-llb (Fig. 4c,d). For both isolates, it is evident that numerous antimicrobial

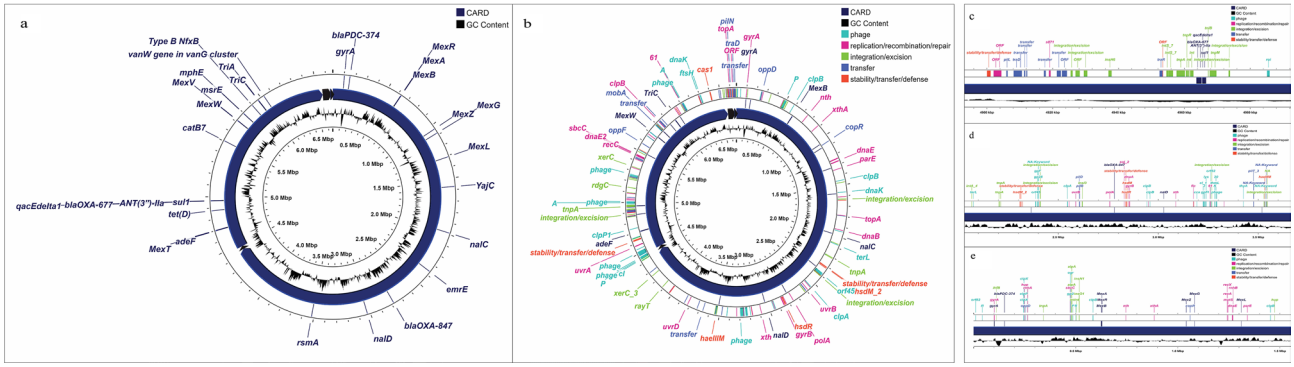

Fig. 5. Mapping of antimicrobial resistance genes and mobile genetic elements in *P. aeruginosa* SHNIBPS243. (**a**) Mapping of antimicrobial resistance genes. (**b**–**e**) Antimicrobial resistance genes and surrounding mobile genetic elements harbored by *P. aeruginosa* SHNIBPS243.

Isolate	Efflux pump operon systems and Proteins
P. aeruginosa SHNIBPS64	EmrAB-OMF, EmrAB-TolC, FloR family, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexCD-OprJ, MexCD-OprJ system, MexEF-OprN, MexEF-OprN system, MexHI-OpmD, MexHI-OpmD system, MexJK-OprM/OpmH, MexPQ-OpmE, MexVW-OprM, MexXY-OMP, QacE, Tet(G), TolC/OpmH, TriABC-OpmH
P. aeruginosa SHNIBPS243	EmrAB-OMF, EmrAB-TolC, FloR family, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexCD-OprJ, MexCD-OprJ system, MexEF-OprN, MexEF-OprN system, MexHI-OpmD, MexHI-OpmD system, MexJK-OprM/OpmH, MexPQ-OpmE, MexPQ-OpmE system, MexVW-OprM, MexXY-OMP, Tet(G), TolC/OpmH, TriABC-OpmH

Table 2. Operon systems and Proteins for efflux pumps detected in the three isolates.

Phage	Category	Score	Closest phage	Gene number
(i) *Pseudomonas aeruginosa* SHNIBPS64				
PASHNIBPS64_1_19	Active	0.99	Pseudomonas phage MD8	60
PASHNIBPS64_2_20	Active	0.98	Pseudomonas phage F10	44
PASHNIBPS64_3_71	Active	0.86	Pseudomonas phage phi1	93
PASHNIBPS64_4_72	Active	0.95	Pseudomonas phage YMC11/02/R656	57
PASHNIBPS64_5_73	Active	0.94	Pseudomonas phage YMC11/02/R656	28
PASHNIBPS64_6_116	Active	0.87	Erythrobacter phage vB_EliS_R6L	25
PASHNIBPS64_6_117	Active	0.85	Erythrobacter phage vB_EliS_R6L	16
(ii) *Pseudomonas aeruginosa* SHNIBPS243				
PASHNIBPS243_1_59	Active	0.97	Pseudomonas phage YMC11/02/R656	59
PASHNIBPS243_2_60	Active	0.86	Pseudomonas phage YMC11/02/R656	58
PASHNIBPS243_3_78	Active	0.82	Pseudomonas phage phi3	13
PASHNIBPS243_4_80	Active	0.94	Marinobacter phage PS3	23
PASHNIBPS243_5_81	Active	1.00	Pseudomonas phage YMC11/02/R656	48
PASHNIBPS243_6_97	Active	0.86	Pseudomonas phage phi1	59
PASHNIBPS243_6_115	Active	0.88	Pseudomonas phage JBD68	66

Table 3. Investigation of prophage in the genome of *P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243.

resistance genes are encircled by mobile genetic elements accountable for the excision, integration, and transfer of genes (Figs. 4 and 5).

Detection of prophage in the genome sequences

Prophage investigation in the two test isolates revealed numbers of phage sequences and associated genes integrated into the bacterial genomes (Supplementary Fig. 5). Further in-depth investigation revealed several active phage sequences integrated into the genomes of the two bacterial isolates (Table 3). Most of the prophage sequences identified were of different Pseudomonas phages. Notably, the phage integration analysis uncovered the integration of a Marinobacter phage sequence in the genome of *P. aeruginosa* SHNIBPS243. This phage, belonging to the Podoviridae family, was not found in its entirety; however, a significant portion comprising 23 genes was integrated into the bacterial genome. Additionally, *P. aeruginosa* SHNIBPS64 exhibited integration of Erythrobacter phage sequences. An intriguing aspect of this analysis was the detection of genes associated

with antimicrobial resistance within the prophage regions integrated into the bacterial genomes. Specifically, in *P. aeruginosa* SHNIBPS243, the *msr*E gene was identified within the prophage sequence region (Supplementary Fig. 6b). Similarly, in *P. aeruginosa* SHNIBPS64, the *par*R and *par*S genes were found to be integrated within the prophage sequences as detected by the VirSorter tool (Supplementary Fig. 6a).

Virulence factor and metal resistance genes investigation

The virulence factor genes identified in the *P. aeruginosa* isolates SHNIBPS64 and SHNIBPS243 were categorized into several key functional groups (Supplementary Tables 5, 6 and Supplementary Fig. 3). Both isolates contained genes related to adherence, with SHNIBPS64 having 42 flagella-related genes (e.g., *fla*G, *fle*N, *fli*C) and 23 type IV pili genes (e.g., *fim*T, *pil*A, *pil*Q), while SHNIBPS243 had 32 flagella-related genes (e.g., *fla*G, *fle*N, *fli*C) and 21 type IV pili genes (e.g., *fim*T, *pil*A, *pil*Q). Antimicrobial activity genes included phenazine biosynthesis genes in both isolates, with SHNIBPS64 having nine (e.g., *phz*A1, *phz*S) and SHNIBPS243 having seven (e.g., *phz*A1, *phz*S). Antiphagocytosis-related genes were found in both isolates, with SHNIBPS64 containing 12 alginate biosynthesis genes (e.g., *alg*D, *alg*G) and SHNIBPS243 containing 10 (e.g., *alg*D, *alg*G). Both isolates had genes for rhamnolipid biosynthesis (e.g., *rhl*A, *rhl*C in SHNIBPS64; *rhl*A, *rhl*B in SHNIBPS243). Enzymatic activity genes were noted in both isolates, with hemolytic phospholipase C (*plc*H), non-hemolytic phospholipase C (*plc*N), and phospholipase C (*plc*B) present in SHNIBPS64, and additionally phospholipase D (*pld*A) in SHNIBPS243. Iron uptake mechanisms included genes related to pyoverdine biosynthesis (e.g., *pvd*A, *pvd*Q in SHNIBPS64; *pvd*E, *pvd*N in SHNIBPS243), one pyoverdine receptor gene (*fpv*A) in both isolates, and pyochelin biosynthesis genes (e.g., *pch*A, *pch*R in SHNIBPS64; *pch*A, *pch*E in SHNIBPS243). Protease-related virulence factors were represented by alkaline protease (*apr*A), elastase (*las*A, *las*B in SHNIBPS64), and protease IV (*prp*L) in both isolates. Quorum sensing included genes for acylhomoserine lactone synthase (*hdt*S) and both *las*I/*las*R and *rhl*I/*rhl*R systems in SHNIBPS64, and hdtS and *rhl*I/*rhl*R system in SHNIBPS243. The secretion system category featured genes for *Pseudomonas syringae* TTSS effectors, Hcp secretion island-1 type VI secretion system, and *Pseudomonas aeruginosa* TTSS translocated effectors in SHNIBPS64, and the Hcp secretion island-1 type VI secretion system and *Pseudomonas aeruginosa* TTSS translocated effectors (e.g., *exo*S, *exo*T, *exo*Y) in SHNIBPS243. Toxin production was indicated by exotoxin A (*tox*A) and hydrogen cyanide production genes (*hcn*A, *hcn*B, *hcn*C) only in SHNIBPS64. Immune evasion included genes related to capsule formation present only in SHNIBPS64. Comparative analysis showed that both isolates had 185 virulence factor genes in common. The findings highlighted the extensive virulence potential of both isolates, with SHNIBPS64 exhibiting a broader spectrum of virulence factors, including additional toxin production and immune evasion genes. Metal resistance genes investigation also revealed several genes responsible for conferring resistance against different metals (Supplementary Table 7).

Subsystems and metabolic pathways investigation

The comparative subsystem category distribution analysis between *P. aeruginosa* SHNIBPS64, *P. aeruginosa* SHNIBPS243 and *P. aeruginosa* PAO1 (Reference Strain) revealed differences across various metabolic and functional categories (Supplementary Fig. 4 and Supplementary Table 8). Carbohydrate metabolism is markedly more prevalent in SHNIBPS64, displaying 304 features, whereas SHNIBPS243 has 229 features. In terms of protein metabolism and cofactor synthesis, both isolates showed relatively similar counts, with SHNIBPS64 having 215 and 189 features, respectively, compared to 203 and 185 in SHNIBPS243. Membrane transport features were higher in SHNIBPS64, with 184 features, while SHNIBPS243 has 152 features. Fatty acids, lipids, and isoprenoids metabolism exhibited a slight increase in SHNIBPS243, showing 142 features compared to 131 in SHNIBPS64. Additionally, respiration-related features were higher in SHNIBPS64 (126 features) compared to SHNIBPS243 (109 features). Significant disparities were observed in categories such as iron acquisition and metabolism, with SHNIBPS64 showing 108 features versus 51 in SHNIBPS243 and PAO1. Notably, virulence, disease, and defense mechanisms are more prevalent in SHNIBPS64, with 85 features compared to 67 in SHNIBPS243. On the other hand. The reference strain *P. aeruginosa* PAO1 contained only 62 features in the virulence, disease, and defense mechanisms category. Further, the distribution of features in other metabolic processes, such as the metabolism of aromatic compounds, nucleosides and nucleotides, and RNA metabolism, was slightly higher in SHNIBPS64 compared to SHNIBPS243. Categories such as sulfur metabolism, potassium metabolism, motility, and chemotaxis also show higher counts in SHNIBPS64.

The metabolic pathways of *P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243 were compared to elucidate their distinct metabolic capabilities (Supplementary Table 9). The primary metabolic pathways identified in both strains include the citrate cycle (TCA cycle), glycolysis/gluconeogenesis, and the pentose phosphate pathway. These strains also engage in various amino acid metabolism processes, such as those involving alanine, aspartate, and glutamate. Additionally, they share pathways for the metabolism of fatty acids, pyruvate, and butanoate. Notable secondary metabolite pathways include isoquinoline alkaloid biosynthesis and novobiocin biosynthesis, shared by both strains. However, *P. aeruginosa* SHNIBPS243 exhibits additional secondary metabolite pathways, such as tropane, piperidine, and pyridine alkaloid biosynthesis, indicating a broader metabolic versatility. The xenobiotics biodegradation pathways further highlight the differences between the strains. Both SHNIBPS64 and SHNIBPS243 possess pathways for the degradation of caprolactam, gamma-hexachlorocyclohexane, 2,4-dichlorobenzoate, benzoate via hydroxylation, fluorobenzoate, toluene and xylene, atrazine, bisphenol A, and ethylbenzene. Unique to SHNIBPS243 are pathways for the degradation of styrene, naphthalene and anthracene, tetrachloroethene, and DDT, alongside drug metabolism pathways involving cytochrome P450 and other enzymes and geraniol degradation.

Genome-wide beta-lactam resistance gene pool investigation

The investigation into the genome-wide beta-lactam resistance gene pool in *P. aeruginosa* isolated from both human and non-human sources reveals significant differences in the abundance and diversity of resistance genes (Fig. 6a,b). The analysis included 6574 (n = 6574) *P. aeruginosa* whole genomes, encompassing the two isolates from this study. Among these, 6408 (n = 6408) genomes were from human sources, and 166 genomes (n = 166) were from non-human sources. *P. aeruginosa* isolates from human sources, indicated a diverse array of beta-lactam resistance genes. Overall, the bla_{PDC} and bla_{OXA} genes are notably prominent in both human and non-human isolates, with the highest abundance observed in human isolates. In non-human isolates, these genes also exhibit significant abundance. Other resistance genes such as bla_{VIM}, bla_{NDM}, bla_{SIM}, bla_{IMP}, bla_{GES}, bla_{KPC}, bla_{SPM}, bla_{ADC}, bla_{CMY}, and bla_{DHA} are present in human isolates but at much lower abundances. These genes are minimally represented or nearly absent in non-human isolates. Additionally, genes like bla_{PER}, bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{VEB}, bla_{CARB}, and bla_{AMPc} appear in human isolates with minimal representation, while in non-human isolates, bla_{ACT}, bla_{IMP}, bla_{VIM}, bla_{CTX-M}, bla_{TEM}, and bla_{GES} are present but with very low counts. The comparative analysis suggests that *P. aeruginosa* from human sources harbors a more extensive and abundant repertoire of beta-lactam resistance genes compared to those from non-human sources.

Pangenome analysis, epidemiological pattern and pathogenicity profiling

The pangenome analysis of *P. aeruginosa* strains with all the 12 genomes available from Bangladesh revealed significant genetic diversity, characterized by a relatively small core genome comprising 3,670 genes (27%) and a substantial proportion of accessory genes (soft core, shell, and cloud genes collectively making up 72.9%) (Fig. 7a–c). The phylogenetic tree and heatmap highlight the varied evolutionary relationships and gene distributions across the strains (Fig. 7a). The pie chart and gene count summary further emphasize the predominance of accessory genes, with 4991 soft core genes (37%) and 4900 shell genes (36%), underscoring the open nature of the pangenome. This extensive accessory gene pool indicates ongoing gene acquisition, contributing to the species' adaptability and genetic plasticity. The gene alignment plot corroborates these findings by showing significant homologous regions and structural variations among the strains (Fig. 7d). Collectively, these results suggest that *P. aeruginosa* maintains a highly dynamic and open pangenome, which likely enhances its environmental resilience and pathogenic potential.

The prediction of the global distribution of *P. aeruginosa* strains based on the publicly available MLST data revealed highest concentration in regions such as North America, Europe, China, Brazil, and Australia (Fig. 8a). The genomic epidemiology analysis of 9,055 *P. aeruginosa* strains, based on MLST data, reveals a complex population structure with notable prevalence of specific sequence types (STs) (Fig. 8b). The analysis identifies ST 235, ST 253, ST 111, ST 395, ST 308, ST 244, ST 175 and ST 274 as the most prevalent, indicating their widespread dissemination and clonal expansion across various regions. ST 235, ST 253 and ST 111 are particularly dominant, suggesting a significant role in the global epidemiology of *P. aeruginosa*.

The pathogenicity analysis of various *P. aeruginosa* strains isolated in Bangladesh highlighted significant differences in their pathogenic profiles (Fig. 9a). Notably, *P. aeruginosa* SHNIBPS64, *P. aeruginosa* SHNIBPS243, *P. aeruginosa* SRS1 and *P. aeruginosa* SHNIBPS206 exhibited the relatively higher pathogenicity. In contrast, strains like *P. aeruginosa* TG523, *P. aeruginosa* DMC-20C, *P. aeruginosa* DMC-30b *and P. aeruginosa* MZA4A showed moderate pathogenicity prediction, whereas *P. aeruginosa* DMC-27b had a lower pathogenicity. In terms of matched pathogenic families, *P. aeruginosa* DMC-30b and *P. aeruginosa* TG523 showed the highest number of pathogenic families, indicating a broad range of pathogenic mechanisms (Fig. 9b). Conversely, despite their high pathogenicity scores, *P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243 matched fewer experimental pathogenic families.

Discussion

The analysis of 91 *P. aeruginosa* strains isolated from burn wound infections provided important insights into its prevalence in clinical settings. The successful isolation of these strains underscored the significant burden of *P. aeruginosa* in burn wound infections, highlighting its role as a major causative agent. The consistently high isolation rate observed highlighted the critical role of *P. aeruginosa* as a dominant etiological agent in burn wound infections and underscored the importance of robust infection control practices. There are several reports of the high prevalence of *P. aeruginosa* in clinical settings[34–37]. This alarming high prevalence also emphasizes the need

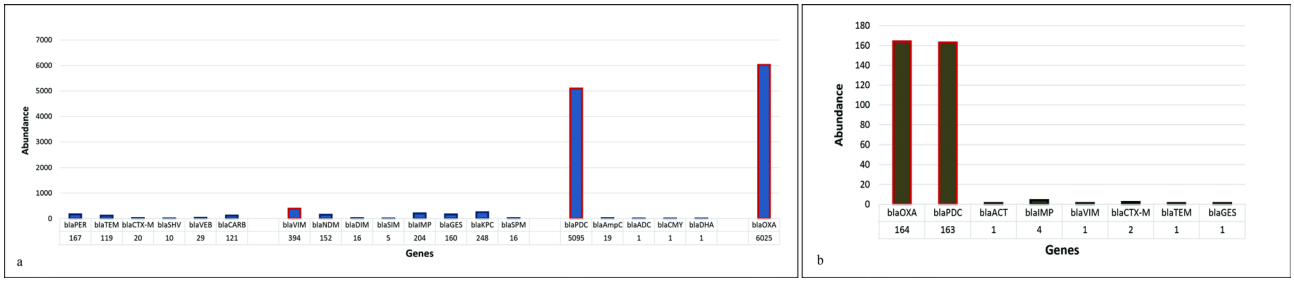

Fig. 6. Genome wide beta lactam resistance genes analysis of 6,574 publicly available *P. aeruginosa genome* worldwide. (**a**) Distribution of beta lactam resistance genes of *P. aeruginosa* isolated from human sources. (**b**) Distribution of beta lactam resistance genes of *P. aeruginosa* isolated from non-human sources.

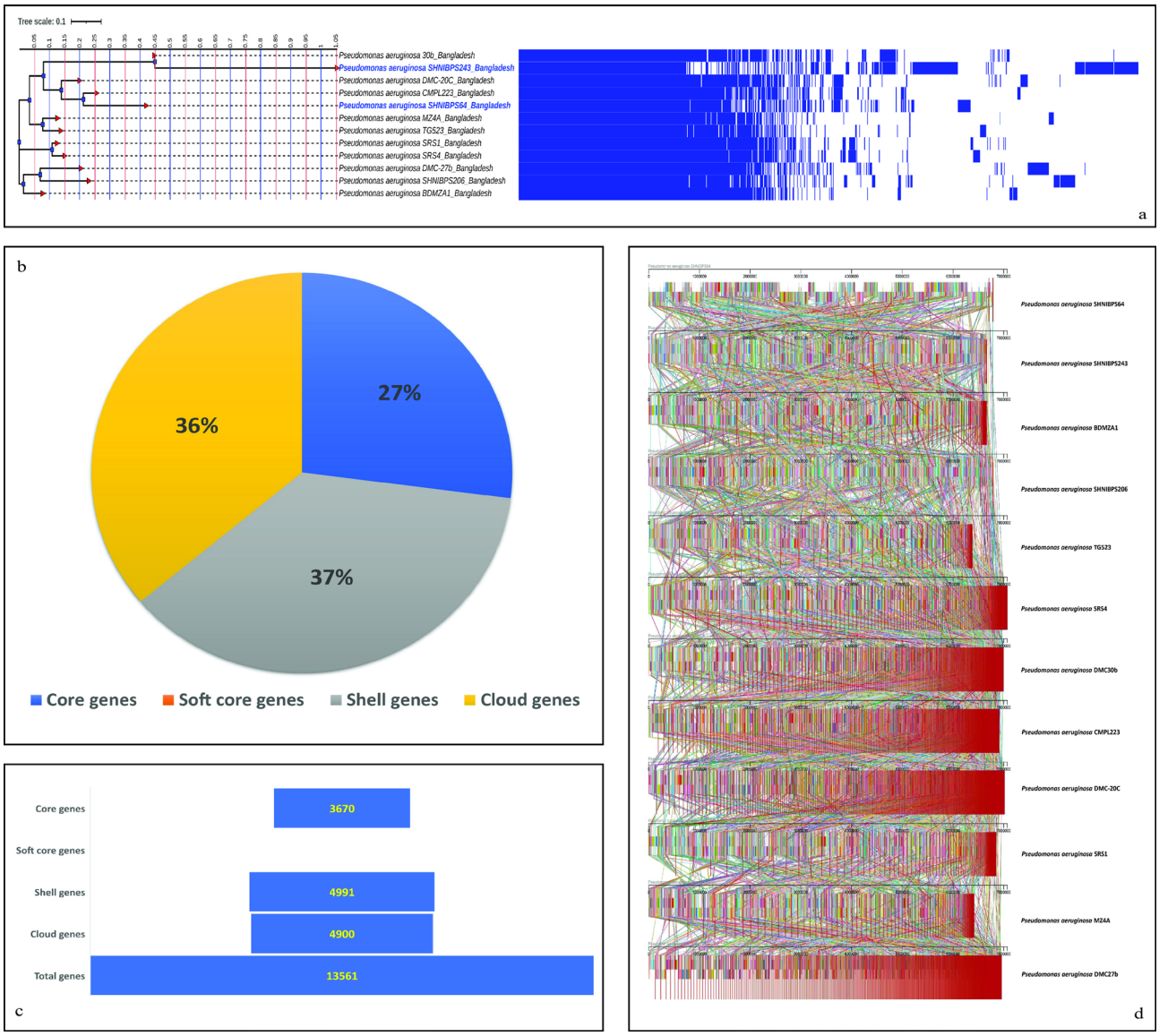

Fig. 7. Pangenome and Comparative genomic analysis of all the publicly available *P. aeruginosa* genomes from Bangladesh. (**a**) Pangenome and phylogenomic analysis based on core genome alignment, illustrating the genetic relationships among the isolates. (**b**,**c**) Pangenome composition highlighting the distribution of core, soft core, shell, and cloud genes across the isolates. (**d**) Genome alignment using Mauve depicting the extensive genomic rearrangements and variability in the genomes.

for ongoing surveillance and effective antimicrobial stewardship to mitigate the risk of outbreaks and manage antibiotic resistance associated with this opportunistic pathogen.

The extensive drug resistance observed in *P. aeruginosa* isolates from burn wound infections highlighted a critical public health challenge. The high resistance rates to aminoglycosides and fluoroquinolones, as well as the significant resistance to carbapenems, reflect the adaptability and resilience of *P. aeruginosa* in clinical settings, particularly in burn units where patients are highly susceptible to severe infections. Interestingly, the isolates demonstrated higher resistance to meropenem compared to imipenem. This finding contrasts with several previous studies in which Gram-negative bacilli exhibited greater susceptibility to meropenem than to imipenem[38,39]. The universal sensitivity to polymyxin B and colistin provides a potential treatment pathway, although their use must be carefully managed due to toxicity concerns. The detection of various beta-lactamase genes, such as $bla_{\text{NDM-1}}$, $bla_{\text{VIM-2}}$, $bla_{\text{PER-1}}$, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{OXA-48}}$, highlighted the complexity of resistance mechanisms in these isolates. This diversity of resistance genes reflects the genetic adaptability of the isolates and their ability to counteract a broad range of beta-lactam antibiotics[40,41]. The co-existence of multiple beta-lactamase genes in some strains suggests a heightened capacity for resistance, making infections harder to treat. Class I integrons play a significant role in the dissemination of resistance genes, highlighting the dynamic nature of genetic exchange in *P. aeruginosa*. The widespread presence and overexpression of efflux pump genes, including *mexA*, *mexC*, and *mexE*, significantly contributes to the multidrug resistance phenotype by

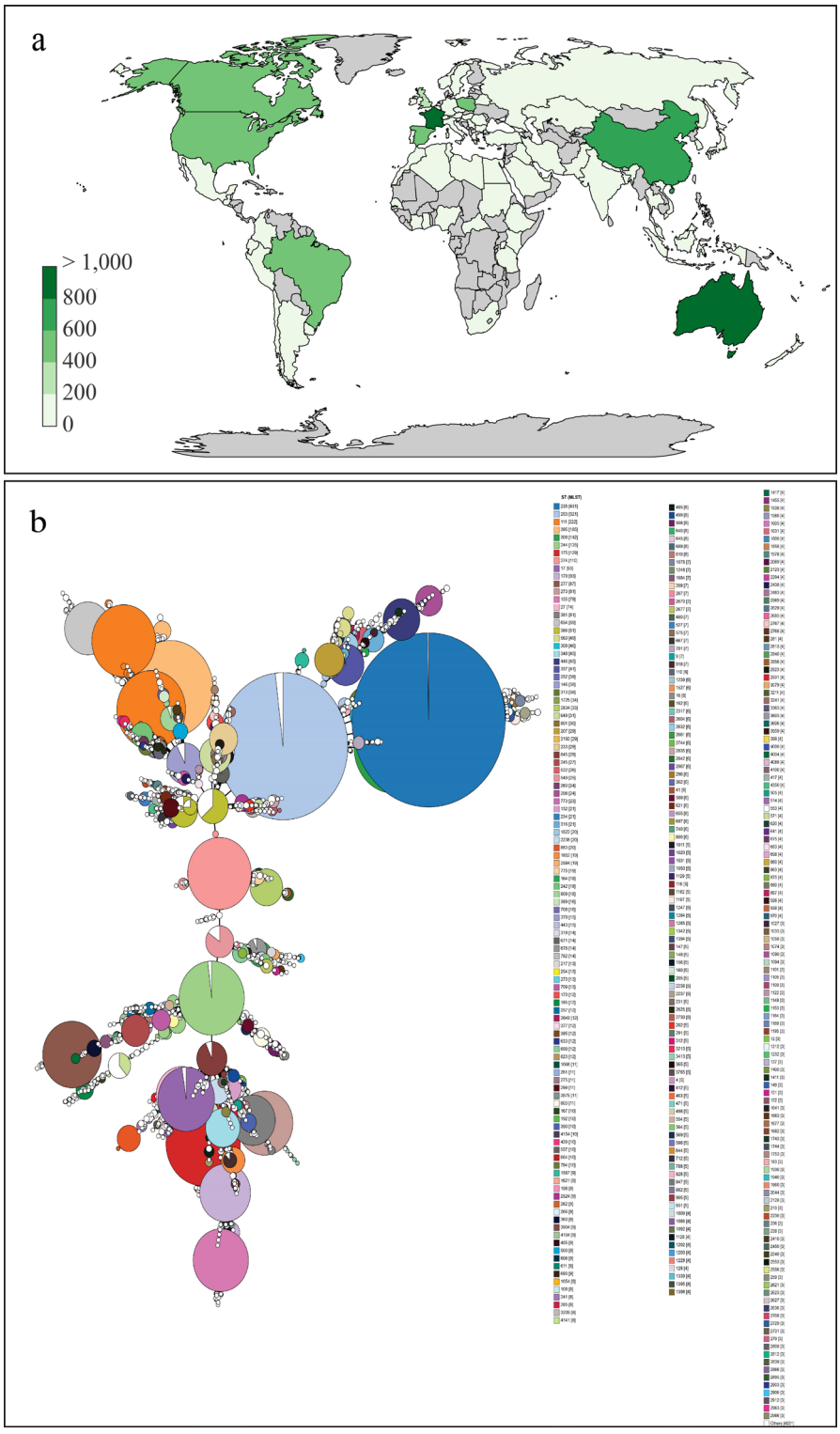

Fig. 8. Global distribution and MLST based epidemiological analysis of *P. aeruginosa* strains. (**a**) Geographic distribution of *P. aeruginosa* strains across the world, with countries shaded according to the number of strains sampled. (**b**) Global epidemiological analysis based on MLST of *P. aeruginosa* strains worldwide.

actively expelling a broad range of antibiotics from bacterial cells[42]. These efflux pumps reduce the intracellular concentration of antimicrobial agents, thereby diminishing their efficacy This mechanism, combined with other resistance determinants, complicates therapeutic interventions.

The strong biofilm formation observed in the *P. aeruginosa* isolates from burn wound infections presents a significant challenge for treatment and patient outcomes. The investigation into biofilm-associated genes

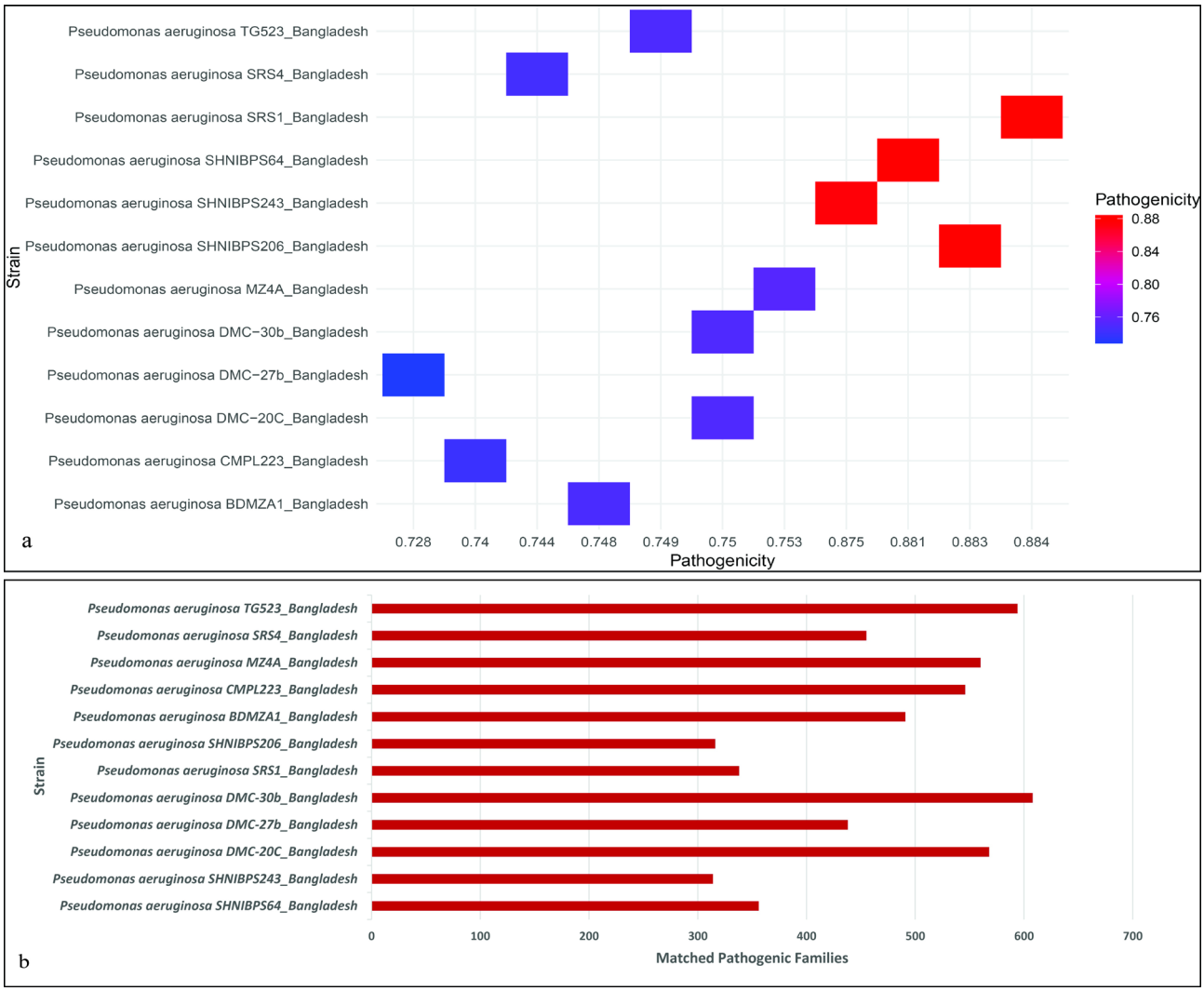

Fig. 9. Comparative analysis of pathogenicity and matched pathogenic families among different Pseudomonas aeruginosa strains isolated in Bangladesh. (**a**) Pathogenicity scores of the strains, with a color gradient indicating the level of pathogenicity (blue to red, from lower to higher pathogenicity). The strains are listed along the y-axis, with their corresponding pathogenicity scores on the x-axis. (**b**) Number of matched pathogenic families for each strain, represented as horizontal bars. Strains are listed along the y-axis, and the number of matched pathogenic families is shown on the x-axis.

revealed a high prevalence of *pel*B, *pil*T, and *rhl*B genes among the isolates. The *pel*B gene, detected in 90 of the 91 isolates, plays a crucial role in the production of the polysaccharide matrix that constitutes the biofilm, enhancing structural integrity and protection. The presence of *pilT* and *rhlB* genes highlighted their role in biofilm formation and its regulation. These genes contribute to key processes such as surface attachment and biofilm maintenance, which are critical for bacterial survival and persistence. PilT is involved in type IV pilus-mediated motility, which is essential for initial surface attachment and biofilm development[43], while *rhl*B is part of the rhamnolipid biosynthesis pathway, contributing to biofilm maturation and maintenance[44]. In the context of burn wound infections, the strong biofilm-forming capacity of *P. aeruginosa* exacerbates the clinical management of these infections. Biofilms serve as reservoirs for bacterial cells, protecting them from antibiotic penetration and immune clearance[45]. This makes eradication difficult, often leading to recurrent infections and increased morbidity. The correlation between biofilm formation and the presence of specific virulence genes suggests that these isolates are well-equipped to establish and maintain chronic infections in burn wounds.

Whole genome sequencing of two XDR *P. aeruginosa* isolates (*P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243) isolated from burn wound infections in this study revealed their detailed genomic characteristics. The identification of two different sequence types and serotypes underscored the variability within *P. aeruginosa* populations in clinical settings. This diversity is crucial in understanding the adaptability and pathogenic potential of these strains. The presence of multiple beta-lactamase genes and efflux pump genes in both isolates aligns with existing literature on the mechanisms *P. aeruginosa* employs to evade antibiotic treatment[42,46,47]. The co-existence of class C and class D beta-lactamases, as well as mutations in resistance-associated genes such as

*nal*C and *gyr*A, contributes to a robust defense against a wide range of antibiotics[48–50]. These genetic arsenals may complicate treatment regimens, particularly in burn patients who are already vulnerable to severe infections. The detection of *sul*1, *qac*L, and *qac*Delta1 genes, conferring resistance to sulfonamides and disinfectants, further illustrates the resilience of these strains in hospital environments where such agents are frequently used. This resilience can lead to persistent infections and outbreaks, making infection control measures more challenging. Efflux pump genes, including *mex*A, *mex*B, *mex*C, and *mex*E, are well-documented contributors to multidrug resistance in *P. aeruginosa*[42,51]. Their presence in these isolates highlights the importance of efflux mechanisms in the survival and proliferation of this pathogen in hostile environments, such as burn wounds treated with multiple antibiotics.

The integration of active phage sequences into the genomes of *P. aeruginosa* strains SHNIBPS64 and SHNIBPS243 provides significant insights into the genomic plasticity and potential adaptive advantages conferred by prophages. The presence of Pseudomonas, Marinobacter, and Erythrobacter phages underscores the role of horizontal gene transfer in enhancing bacterial fitness. Notably, the Marinobacter phage integration in SHNIBPS243, with a significant portion of 23 genes, and the Erythrobacter phage sequences in SHNIBPS64 highlight cross-genus phage integration. A critical finding is the detection of antimicrobial resistance genes within these prophage regions. The *msr*E gene in SHNIBPS243 and the *par*R and *par*S genes in SHNIBPS64 suggest that phage integration can disseminate resistance traits, enhancing bacterial survival under antibiotic pressure. This phage-mediated transfer of resistance genes underscores the role of prophages in the evolution of antibiotic resistance. These insights emphasize the need to consider phage dynamics in developing therapeutic strategies against *P. aeruginosa***.** Targeting phage-bacterium interactions and the mobilization of resistance genes via prophages could be pivotal in mitigating antimicrobial resistance and managing infections caused by these resilient pathogens.

The subsystem feature distribution underscores the metabolic and functional versatility of *P. aeruginosa* SHNIBPS64 compared to *P. aeruginosa* SHNIBPS243, particularly in areas related to metabolism, iron acquisition, virulence, and stress responses, which could have significant implications for their adaptability and pathogenicity. The diverse metabolic capabilities are essential for the bacteria's energy production, biosynthesis, and overall survival in nutrient-limited environments like burn wounds. The analysis of xenobiotics biodegradation pathways highlights the ability of both strains to degrade a wide range of environmental pollutants and toxic compounds. The capability to metabolize diverse xenobiotics suggests that these isolates can survive in environments with high levels of chemical contaminants, including those found in hospital settings. The presence of pathways for drug metabolism and other enzymes, alongside unique pathways for the degradation of complex xenobiotics indicates their potential to resist multiple antimicrobial agents. This metabolic flexibility and the ability to detoxify harmful compounds may contribute to their persistence and the difficulty in eradicating from hospital environments. The high counts of iron acquisition and virulence features suggest that these strains are well-equipped to acquire essential metals and deploy effective pathogenic strategies, which are critical for their growth and infection in iron-limited host environments. The enhanced virulence mechanisms further highlighted their potential to effectively colonize and persist in burn wound sites, evading host immune defenses.

The open pangenome of *P. aeruginosa* strains from Bangladesh, as evidenced by the large number of accessory genes, implied continuous horizontal gene transfer events and genomic rearrangements. This characteristic is particularly concerning from a clinical perspective, as it may facilitate the emergence of new virulent strains with enhanced resistance to antibiotics and other therapeutic interventions[52]. The observed genetic diversity also reflected the pathogen's ability to occupy different ecological niches, thereby increasing its survival and propagation potential. The phylogenetic tree and gene distribution heatmap underscored the evolutionary relationships and gene content variability among the strains, suggesting that even within a localized region, *P. aeruginosa* exhibits considerable genetic heterogeneity. This diversity could be a response to selective pressures in the environment, including exposure to different antimicrobial agents and competition with other microbial species. Overall, the findings underscore the importance of monitoring the genetic evolution of *P. aeruginosa*. Understanding the dynamics of its pangenome can provide crucial insights into its pathogenic mechanisms and inform the development of more effective treatment strategies and public health interventions. The open nature of its pangenome highlights the need for continuous surveillance and research to mitigate the risks associated with this adaptable and potentially hazardous pathogen.

The comparative analysis of pathogenicity among various *P. aeruginosa* strains isolated from Bangladesh revealed significant heterogeneity in their virulence profiles, with implications for clinical management and therapeutic interventions. The high pathogenicity scores observed in *P. aeruginosa* SHNIBPS64 and *P. aeruginosa* SHNIBPS243 suggested these strains possess potent virulence factors capable of causing severe infections. Interestingly, despite their high pathogenicity, these strains exhibited a lower number of matched pathogenic families. This discrepancy between high pathogenicity scores and a lower number of matched pathogenic families suggest that these strains possess highly potent pathogenic mechanisms that may not be reflected solely by the number of matched pathogenic families. These strains may rely on a few highly effective pathogenic mechanisms rather than a broad array of virulence factors. The results also suggest that these isolates may possess numerous proteins associated with unknown or uncharacterized pathogenic families that are not included in the current database. This presents a significant opportunity for further research to identify and characterize these proteins, which could enhance our understanding of the mechanisms and dynamics of their pathogenicity, particularly in causing wound infections and various hospital-acquired infections. Furthermore, such research could open new aspects for identifying therapeutic targets and developing novel treatments to combat these highly pathogenic organisms.

There is an urgent need to implement robust infection control strategies, ensure continuous surveillance, and accelerate the development of novel antibiotics or alternative therapeutic approaches. Understanding the

mechanisms underlying resistance and targeting specific pathways, such as beta-lactamase activity and efflux pumps, could enhance the effectiveness of existing treatments. Moreover, the high prevalence of resistance genes calls for ongoing research into innovative strategies to combat these highly adaptable and pathogenic organisms, ensuring better outcomes for patients with burn wound infections.

Conclusion

This study provides molecular characterization of MDR and XDR *P. aeruginosa* strains isolated from burn patients in Bangladesh. Additionally, it offers a comprehensive genomic analysis of two representative XDR strains, leveraging whole-genome sequencing to elucidate their genetic features and resistance mechanisms. The identification of high prevalence of beta-lactamase genes, efflux pump genes, biofilm associated genes, class I integron highlighted the formidable nature of these pathogens as well as underscored the multifaceted mechanisms driving antimicrobial resistance and virulence in these isolates. Whole-genome sequencing and pangenome analysis revealed considerable genetic diversity, pathogenicity, and an expanding pangenome in *P. aeruginosa*. Also, ST235 identified as a globally dominant sequence type. So, there is a critical need for robust infection control measures and antimicrobial stewardship programs to combat the dissemination of highly resistant strains in burn units and beyond. Future research should broaden the scope of genomic surveillance to include diverse healthcare settings and environmental reservoirs across Bangladesh.

Materials and methods

The diagram of the brief methodology of this study is depicted in supplementary Fig. 1.

Sample collection

During 8 months, from October, 2021 to June, 2022; around 110 wound swabs were collected from infected wound of critically injured burn patients who were admitted in Sheikh Hasina National Institute of Burn and Plastic Surgery (SHNIBPS), Dhaka, Bangladesh. Informed consent was obtained from the patients. All methods were carried out in accordance with relevant guidelines and regulations. The experimental protocols and ethical permission for the study were approved by the Ethical Review Committee of Faculty of Biological Sciences, University of Dhaka (Ref. No. 191/Biol. Scs.). The samples were collected during two different sessions- October–November, 2021 and May–June, 2022. After preliminary analysis, 91 samples were subjected to further analysis. The wound swabs collected from the patients were first cultured on MacConkey agar (MAC) and Mueller Hinton Agar (MHA) plates in the Microbiological Laboratory of SHNIBPS in order to find out the predominating organisms in the sample and also to omit the possibility of biasness. Then the predominant isolated colonies were inoculated on nutrient broth carefully and brought to The Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka in a cooler with icepacks (below 4 °C) and processed within a few hours. The organisms were streaked on nutrient agar (NA) plates for the generation of single colonies and preliminary screening was also carried out on cetrimide agar plates.

Cultural and molecular identification

Phenotypic methods were employed for the preliminary identification of the isolate, involving the examination of cultural and morphological traits, along with various biochemical tests[53] (Supplementary Table 1). Since our primary focus was on screening for *P. aeruginosa*, the isolates underwent cultivation on cetrimide agar as an initial step in the cultural screening process. Further, a series of biochemical tests and microscopy were carried out for presumptive identification. On the other hand, rapid molecular approach through PCR based system included genus (*opr*I) and species (*opr*L) specific primers (Supplementary Table 2) for the rapid and specific detection of *P. aeruginosa*[54]. The DNA of *Pseudomonas aeruginosa* ATCC 27853 was used as positive control to validate the results.

Antimicrobial susceptibility testing, antimicrobial resistance genes and integron investigation

Antibiotic susceptibility testing for the test isolate was conducted using the Kirby Bauer method[55], employing 14 antibiotics recommended for *P. aeruginosa* in accordance with the CLSI (Clinical and Laboratory Standards Institute, 2021) guidelines[56]. In case of colistin, MIC (Minimum Inhibitory Concentration) was carried out in order to find out the antimicrobial susceptibility pattern. Gene specific PCR was carried out for the detection of some major genes responsible for the production of beta lactamase which confer resistance to beta lactams. According to the Amber classification[57], there are mainly four classes of beta lactamases. In this study, we have tried to detect some particular pre-targeted genes (Supplementary Table 2) from each class in order to investigate their occurrence. We conducted PCR under specified conditions, adhering to the defined annealing temperature for each primer (Supplementary Table 2). In this study, the existence or occurrence of class I integron in the isolates were investigated. For detecting the presence of class I integons, PCR using specific primers (Supplementary Table 2) was carried out.

Biofilm formation assay and associated genes investigation

The biofilm-forming capacity of the isolates was evaluated using the crystal violet biofilm formation assay (CV assay)[58]. All samples were cultured in Luria broth medium at 37 °C with shaking for 24 h, followed by a 100-fold dilution in Luria broth. After that, 100 µL of a diluted culture of the test isolate was introduced into Thermo Scientific™ 96-Well Microtiter Microplates. The diluted samples were incubated on plates under static conditions at 37 °C for 24 h. Following the incubation period, planktonic cells were then removed, and the wells containing biofilms were washed three times with distilled water. After drying, the biofilm was treated with 125 µL of a 0.1%

crystal violet solution in water. To quantify the extent of biofilm formation, UV absorbance at 600 nm was measured using a microplate reader (GloMax Explorer, Promega), with a 30% acetic acid in water solution serving as the reference. Biofilm formation ability was categorized using the following standard formula: OD ≤ ODcut = Non-biofilm-former, ODcut < OD ≤ 2 × ODcut = Weak biofilm-former, 2 × ODcut < OD ≤ 4 × ODcut = Moderate biofilm-former, OD > 4 × ODcut = Strong biofilm-former and ODcut = ODavg (average of OD's) of negative control + 3 × standard deviation of ODs of negative control. Here OD means optical density of the samples in CV assay. The biofilm associated genes *pel*B, *pil*T, and *rhl*B were detected for the screening for genes relevant to biofilms through gene specific PCR. The details about the primers used are listed in Supplementary Table 2.

Efflux pump associated genes investigation

Several efflux pump genes and related operons are responsible for conferring resistance to a wide range of antibiotics in *P. aeruginosa*. Among those the resistance-nodulation-division (RND) family of efflux pumps, which exist as a tripartite system and contain a polyspecific substrate binding pocket are the major types. Primer pairs specific for internal *mex*A, *mex*C and *mex*E genes were used to amplify the corresponding genes, for MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux system. The primer sequences and annealing temperature are listed in Supplementary Table 2. PCR products were visualized followed by agarose gel electrophoresis.

Whole genome sequencing, assembly and annotation

Two XDR isolate which were collected in two different sessions were subjected to whole genome sequencing and analysis. These two isolates were chosen from two different collection periods, representing the temporal scope of the study. Additionally, both isolates were chosen based on their carbapenem-resistance and strong biofilm-formation pattern, which are significant phenotypic traits associated with persistence and resistance. The DNA of the two selected isolates were extracted using Genomic DNA Purification kit for gram negative bacteria (New England Biolabs, UK) following protocol of Genomic DNA purification from gram negative bacteria (NEB#T3010). The whole genome sequencing of the target isolates was performed under Illumina platform using Illumina Miniseq sequencing system at Bangladesh Council of Scientific and Industrial Research (BCSIR). The generated raw FASTQ files were evaluated for quality through FastQC (v0.11)[59]. Following that, the adapter sequences and low quality ends per reading were trimmed using Trimmomatic (v0.39)[60]. After trimming, the high-quality reads were subjected to de novo assembly using SPAdes v3.15.4[61]. After assembly, scaffolding was carried out through Multi-CSAR tool[62] using multiple reference genomes. Completeness of the scaffolded genome was checked using CheckM[63]. The scaffolded genomes were undergone annotation through Prokka[64], RAST [65], eggNOG[66] and Prokaryotic genome annotation pipeline (PGAP)[67,68] of NCBI. The graphical map of the circular genomes were generated through Proksee server[69]. The whole genome sequence based identification of the isolates were carried out using k-mer based algorithm through LINbase server[70] and KmerFinder tool of Center for Genomic Epidemiology[71]. The identification was validated based on ANI (Average Nucleotide Identity). The serotype of the strains were investigated using PAst (v1.0) tool Center for Genomic Epidemiology[72].

WGS based antimicrobial resistance genes, efflux pump genes, metal resistance genes and virulence factor genes investigation

Antimicrobial resistance genes (ARGs) were investigated through CARD Resistance Gene Identifier server[73]. Additionally, all efflux pump-related genes and operons were investigated through annotated using Prokka[64]. Metal resistance genes were identified using BacMet[74], and virulence factor genes were analyzed with the VFDB tool[75]. The pathogenicity of the two bacterial isolates towards human hosts was predicted using the PathogenFinder (v1.1) web tool from the Center for Genomic Epidemiology[76]. Presence of integrons of the two isolates were investigated using IntegronFinder tool[77] of the same platform.

Further the worldwide epidemiology and genome-wide analysis of the beta-lactam resistance gene pool included 6,574 (n = 6574) *P. aeruginosa* whole genomes available in the NCBI[78] and BV-BRC[79] databases, encompassing the two isolates from this study. Among these, 6,408 (n = 6408) genomes were from human sources (Supplementary Table 10), and 166 genomes (n = 166) were from non-human sources (Supplementary Table 11). The analysis was conducted using good-quality genome sequences, while poor-quality genomes were excluded to ensure the reliability and accuracy of the results. The whole genome sequences were first downloaded in FASTA format. After that, the CARD Resistance Gene Identifier (CARD:RGI) was used to analyze the antimicrobial resistance genes from the genomic data. The default parameters were used during the analysis.

Subsystems analysis, mobile genetic elements and prophage investigation

Subsystems and functional metabolic pathways were analyzed using the RAST and PATRIC servers. The biosynthetic gene clusters were determined through antiSMASH7.0 tool. The MGEs from the isolate's whole genome were analyzed using the MobileElementFinder tool[80] and mobileOG-db[81]. Phage integration within the bacterial genome was examined using the PHAge Search Tool Enhanced Release (PHASTER)[82] and the Prophage Hunter tool[83].

Pangenome analysis, pathogenicity profiling and MLST based epidemiological pattern investigation

A pangenome and comparative genome analysis was conducted to assess the diversity of the target strain. This analysis aimed to identify the presence and absence variations (PAVs) of genes or gene families among strains circulating in Bangladesh by analyzing all the whole genomes publicly available from Bangladesh. 12 whole genome sequences were involved in the pangenome analysis including the two target strains of this study. The assembled FASTA files of the genomes were retrieved from BV-BRC[79]. The genomes were aligned and visualized

using Mauve software package[84] which does alignment of conserved genomic sequence with rearrangements. Genome annotation was performed using Prokka[64], and the resulting gff3 files were used for pangenome analysis with Roary[85], a tool that rapidly builds large scale pan genomes identifying the core and accessory genes. We tried to get a detailed overview of the organism's pangenome status and to assess the genomic diversity among the Bangladeshi strains. Pathogenicity profiling and prediction of the isolates' potential to infect human hosts were performed using PathogenFinder (v1.1)[76], a web-based tool developed by the Center for Genomic Epidemiology. The resulting data were subsequently analyzed and visualized utilizing the Tidyverse package in RStudio[86].

Further, MLST analysis was carried out using MLST 2.0 server[87] and PubMLST server[88]. The genomic epidemiology and global distribution of nine thousand fifty-five (n = 9,055) *P. aeruginosa* strains, based on their MLST data, were analyzed using PubMLST. A grapetree was constructed to illustrate the relative distribution and prevalence of different sequence types of *P. aeruginosa* strains.

Data availability

The genome sequence data were submitted in NCBI under the BioProject ID PRJNA1150750. The accession no. of the two isolate *Pseudomonas aeruginosa* SHNIBPS64 and *Pseudomonas aeruginosa* SHNIBPS243 are SRR30335767 and SRR30335766 respectively. Besides, the other genomes used for secondary analysis were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and BV-BRC (https://www.bv-brc.org/)

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Author contributions

M.M.R., M.R.I. and S.M.M. developed the hypothesis and designed the study. S.M.M., M.I.M., M.H.H. and S.K.S. carried out the laboratory experiments. M.R.I., I.I., and N.N.R. assisted in laboratory experiments. S.M.M. performed the whole genome sequence analysis and computational analysis. M.R.I., F.F., K.A. assisted in the computational analysis. S.M.M. prepared the first draft of the manuscript. M.M.R, I.I., N.N.R., M.R.I., S.K.S, J.F.M. and A. critically reviewed the manuscript. M.M.R. supervised the whole study. All the authors approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

Ethical permission to conduct the experimental protocols was given and verified by the Ethical Review Committee of Faculty of Biological Sciences, University of Dhaka (Ref. No. 191/Biol. Scs.). We confirm that all methods were performed in accordance with the relevant guidelines and regulations. Informed consent was taken from the patients for sample collection, conduct research with the samples and to publish the outcome.

Additional information

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