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Original Article

Coexistence of β-lactamase genes and biofilm forming potential among carbapenem-resistant *Acinetobacter baumannii* in Lahore, Pakistan

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Abstract

Introduction: Our goal was to *i*nvestigate the antimicrobial resistance due to beta-lactamase genes and virulent determinants (biofilm-forming ability) expressed by *Acinetobacter* collected from health settings in Pakistan. A cross-sectional study was conducted for the molecular characterization of carbapenemases and biofilm-producing strains of *Acinetobacter* spp.

Methodology: Two twenty-three imipenem-resistant *Acinetobacter* isolates were analyzed from 2020 to 2023.The combination disk test and modified hodge test were performed. Biofilm forming ability was determined by polystyrene tube assay. Multiplex polymerase chain reaction (PCR) for virulent and biofilm-forming genes, and 16S rRNA sequencing were performed.

Results: 118 (52.9%) carbapenem-resistant *Acinetobacter* (CR-AB) were isolated from wounds and pus, 121 (54.2%) from males, and 92 (41.2%) from 26-50-years-olds. More than 80% of strains produced β-lactamases and carbapenemases. Based on the PCR amplification of the *ITS* gene, 174 (78.0%) CR-AB strains were identified from CR-*Acinetobacter non-baumannii* (ANB). Most CR-AB were strong and moderate biofilm producers. Genetic analysis revealed the bla_{OXA-23}, bla_{TEM}, bla_{CTX-M} bla_{NDM-1} and bla_{VIM} were prevalent in CR-AB with frequencies 91 (94.8%), 68 (70.8%), 19 (19.7%), 53 (55.2%), 2 (2.0%) respectively. Among virulence genes, *OmpA* was dominant in CR-AB isolates from wound (83, 86.4%), *csuE* 63 (80.7%) from non-wound specimens and significantly correlated with bla_{NDM} and bla_{OXA} genes. Phylogenetic analysis revealed three different clades for strains based on specimens.

Conclusions: CR-AB was highly prevalent in Pakistan and associated with wound infections. The genes, bla_{OXA-23}, bla_{TEM}, $bla_{CTX-M,}$ *and* bla_{NDM-1} were detected in CR-AB. Most CR-AB were strong biofilm producers with virulent genes *OmpA* and *csuE*.

Key words: *Acinetobacter*; β-lactamases; carbapenemases; virulence genes; biofilm.

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Introduction

Acinetobacter species, including *Acinetobacter baumannii* (AB), are a significant cause of nosocomial infections. AB is a notorious opportunistic pathogen associated with various infections, including pneumonia, septicemia, and urinary tract infections [1]. These infections have become difficult to treat due to the growing prevalence of antibiotic resistance among the clinical strains of this species [1,2]. The excessive use of antibacterial drugs has made *Acinetobacter* species resistant to many available antibiotics. Multidrug resistance in AB is achieved by various mechanisms, including transforming target sites, enzymatic deactivation of drugs, decreased drug absorption by increasing efflux pumps, or reducing permeability and biofilm formation [3]. Carbapenems, the β-lactam antibiotics, have been considered to be the most effective antibiotics to cure AB infections. However, their effectiveness has been compromised due to increased resistance against these antibiotics, mediated by the emergence of β-lactamases of B and D classes [4]. The spread of carbapenem-resistant *A. baumannii* (CR-AB) and carbapenem-resistant *Acinetobacter* non-*baumannii* (CR-ANB) strains has been reported globally, leading towards a major health threat due to limited choice of treatment options [5].

AB with increased multidrug resistance and its ability to produce biofilms, allows the bacteria to persist and thrive in hospital settings on biotic and abiotic surfaces [6]. Earlier studies have documented a strong relationship between the ability of AB to form biofilms and its elevated antibiotic resistance [6,7]. Various virulence determinants are thought to be responsible for biofilm formation, such as the outer membrane protein (OmpA), chaperon usher pilus (Csu), extracellular exopolysaccharide (EPS), two-component system BfmS/BfmR, and quorum sensing system [7]. OmpA, the 38-kDa porin protein of AB, has a significant role

in biofilm development. The *CsuA/BABCDE* gene is necessary for the pilli formation to adhere to the abiotic surfaces. It has been reported that deactivating the *csuE* gene eradicates pili and biofilm production [8]. Multidrug resistance and virulence determinants significantly contribute to the severity of the infection in their host. There are limited reports on resistant gene expression and virulent determinants among CR-AB and CR-ANB from Pakistan. Moreover, resistance to a commonly prescribed antibiotic such as beta-lactam drugs is high, posing severe challenges to public health. This study aimed to assess and investigate the antimicrobial resistance (beta-lactamase genes) and virulent determinants (biofilm-forming ability) expressed by AB isolates collected from health settings in Pakistan.

Methodology

Study design

Acinetobacter clinical isolates were collected from three tertiary care hospitals and a Lahore diagnostic centre (with > 112 collection centres all over Pakistan) between 2020 to March 2023. Following guidelines of the Centers for Disease Control and Prevention (CDC) USA, only the isolates obtained from patients with localized or systematic infection during their stay at the hospital were considered. The Citi Lab and Research Centre Ethics Committee approved the study (Ref # 25th-12 CLRC/ 25-12).

Isolation of bacteria and antimicrobial susceptibility testing (AST)

Different specimens; such as wounds, sputum, blood, pus, urine, and nasal secretions; were collected and cultured on routine media. Biochemical tests were done to identify isolated colonies and reconfirmed by API-NE (biomerieux Marcy–l'Étoile, France). The disk diffusion method determined the antibiotic susceptibility pattern of each *Acinetobacter* isolate according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. Thirteen antibiotics, including ampicillin (AMP), aztreonam (ATM), amoxicillin + clavulanic acid (AMC), cefalexin (CEF), cefixime (CXM), *ceftriaxone* (CRO), amikacin (AK), gentamicin (CN), tetracycline (TE), ciprofloxacin (CIP), trimethoprim/sulfamethoxazole (SXT), osfomycin (FF), and Piperacillin-tazobactam (TPZ) were tested.

Phenotypic detection of carbapenemases

Based on AST results, the production of extended spectrum beta lactamases (ESBLs) and metallo beta-lactamases (MBLs) was confirmed by combination disk test (CDST) and modified Hodge test (MHT) [10]. In CDST, an imipenem disc and a combined disc of imipenem with EDTA (inhibitory supplement) were tested on a Mueller-Hinton agar plate as per the method [10]. In MHT, a meropenem disk was placed in the center of the test area on the Muller Hinton Agar (MHA) plate, and test isolates were streaked in a straight line from the edge of the disk to the edge of the plate. In the boronic acid disc test, imipenem and meropenem discs alone, and combined with aminophenyl boronic acid (APB) were tested on the same plate [10].

Biofilm formation assay

A polystyrene tube assay was performed to determine the ability of biofilm formation by each *A. baumannii* isolate. A volume of 30 µL of an overnight culture with OD_{600} = 0.1 was incubated in 1.5 mL of Mueller–Hinton broth contained in polystyrene (12 mm × 75 mm) tubes. After 48 h incubation at 37 °C, phosphate-buffered saline (PBS) was added three times to wash the adherent cells on the walls of the tubes. Crystal violet (0.02%) was added, incubated for 10 min, and discarded. Ethanol solvent was added and vortexed for 5 minutes to elute the stain from adherent cells. The optical density of each eluted solvent was measured at 580 nm using a UV-visible spectrophotometer. The assay was repeated thrice. The following formula was used to evaluate the results: ODc (optical density cut-off value) = average OD of negative control + (3 × SD of negative control) [11].

Carbapenemase gene detection

Common carbapenemase genes of AB and ANB, including bla_{IMP}, bla_{VIM}, bla_{OXA-23}, bla_{NDM-1}, bla_{CTX-M}, and bla_{TEM} were detected by polymerase chain reaction (PCR). Multiplex PCR was used using primers for carbapenems genes bla_{OXA} and bla_{TEM} after optimizing the PCR cycle (Table 1). The PCR cycle included initial denaturation at 95 °C for 5 min; then 35 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 30 seconds, and elongation at 72 °C for 1 minute; and final elongation at 72 °C for 10 minutes. The annealing temperature was 47 °C for 1 minute, followed by 55 °C for 1 minute for bla_{TEM},. Species conserved region identification gene *ITS,* and virulence genes *OmpA* and *csuE*, were also amplified. Amplification conditions for the *ITS*, *OmpA, and csuE*, genes were initial denaturation at 95 °C for 1 minute; then 35 cycles of denaturation at 95 °C for 1 minute, annealing at 56

°C for 1 minute, and extension at 72 °C for 1 minute; and finally, 72 °C for 10 minutes (Table 1).

16S rRNA sequencing and phylogenetic analysis

rRNA sequencing of ten representative strains were performed. The amplicons were sequenced by 1st Base (www.base.asia.com) and the assembled sequences were compared with a sequence database (GenBank) using BLAST. A phylogenetic tree was created for the maximum-likelihood analysis with bootstrap values corresponding to 1000 replications using the molecular evolutionary genetics analysis (MEGA 11, NJ) software [10,13].

Statistical analysis

The frequencies and percentages of categorical variables were calculated. Pearson correlation test (two tailed), using SPSS (IBM SPSS Statistics 23.0) was applied to assess the relationship of antimicrobial resistance and virulence genes.

Results

Distribution of isolates and screening of CR-AB

A total of 223 strains included in the study were imipenem-resistant *Acinetobacter*. More than half of the study strains were isolated from wound swab/pus specimens (118, 52.9%), followed by blood (43, 19.2%), urine (28, 12.5%), respiratory secretions (21, 9.4%), and others (13, 5.8%). The distribution of infection by age group were 26–50 years (92, 41.2%), followed by 0–25 years (67, 30%), 51–75 years (51, 22.8%), and > 75 years (13, 5.8%) (Table 2).

Table 2. Distribution of isolates according to different parameters.

Study parameter	n (%)
Strains screened	413
ImpR	223 (53.9)
Demographic Data	
Gender-based distribution	
Male	121 (54.2)
Female	102 (45.7)
Age-wise distribution	
0–25	67 (30.0)
26–50	92 (41.2)
51–75	51 (22.8)
> 75	13 (5.8)
Sample Source	
Wound/Pus/swab/discharge	118 (52.9)
Respiratory secretions	21 (9.4)
Blood	43 (19.2)
Urine	28 (12.5)
Others	13 (5.8)
Phenotypic detection tests	
MHT	196 (87.8)
CDST + EDTA	190 (85.2)
CDST + PBS	188 (84.3)

ImpR: imipenem resistant strains; MHT: modified Hodge test; CDST + EDTA: combined disk synergy test with ethylenediaminetetraacetic acid; CDST + PBS: combined disk synergy test with phosphate buffered saline.

Phenotypic detection of CR-AB

Penicillin and cephalosporins had the highest resistance rate for isolates from wound and non-wound specimens. The resistance rate of bacteria isolated from non-wound specimens were AMP (91%), ATM (89%), AMC (90%), CEF (89%), CXM (90%), CRO (89%), AK (64%), CN (70%), CIP (67%), SXT (86%) FF (91%) and TPZ (66%) (Figure 1A). Similar trends of antibiotic resistance were noticed for strains isolated from wound samples. CEF, CXM, and CRO were mostly non-susceptible. Half of the strains were resistant to TE (50%) (Figure 1B). Colistin was the least resistant drug with 99% sensitivity in both groups. Overall, the strains' resistance rates were > 55% against all tested antibiotics. β-lactamases production was

Table 1. Primers sequences with amplicon sizes.

Gene	Primer sequence (5'-> 3')	Amplicon size (bp)	Reference
***bla*$_{IMP}$**	Forward-GGAATAGAGTGGCTTAAYTCTC Reverse-GGTTTAAYAAAACAACCACC	232	[12]
***bla*$_{VIM}$**	Forward-GATGGTGTTTGGTCGCATA Reverse-CGAATGCGCAGCACCAG	390	[12]
***bla*$_{NDM}$**	Forward-GGTTTGGCGATCTGGTTTTC Reverse-CGGAATGGCTCATCACGATC	621	[12]
***bla*$_{OXA}$**	Forward-GCGTGGTTAAGGATGAACAC Reverse-CATCAAGTTCAACCCAACCG	438	[12]
***bla*$_{CTX-M}$**	Forward-GACGATGTCACTGGCTGAGC Reverse-AGCCGCCGACGCTAATACA	500	[13]
***bla*$_{TEM}$**	Forward- ATGAGTATTCAACATTTCCG Reverse- CTGACAGTTACCAATGCTTA	862	[14]
ITS	Forward- CATTATCACGGTAATTAGTG Reverse- AGAGCACTGTGCACTTAAG	208	[15]
OmpA	Forward- GTTAAAGGCGACGTAGACG Reverse- CCAGTGTTATCTGTGTGACC	578	[15]
csuE	Forward- CATCTTCTATTTCGGTCCC Reverse- CGGTCTGAGCATTGGTAA	168	[15]
16S rRNA	Forward- AGAGTTTGATCCTGGCTCAG- Reverse- GGTTACCTTGTTACGACTT	1400	[16]

Table 3. Distribution of different *bla* genes among the CR-AB and CR-ANB.

***bla* class**	***bla* gene**	**Wound/pus (n = 118)**		**Others (n = 105)**	
		CR-AB n (%) *ITS*+ = 96 (81.3%)	**CR-ANB n (%) *ITS*- = 22 (18.6%)**	**CR-AB n (%) *ITS*+ = 78 (74.2%)**	**CR-ANB n (%) ITS- = 27 (25.7%)**
Class A	bla_{TEM}	68 (70.8)	4 (18.1)	35 (44.8)	3 (11.1)
	bla_{CTX-M}	19 (19.7)	2(9.0)	13 (16.6)	4 (14.8)
Class B	bla_{VIM}	2(2.0)	0 (0)	0 (0)	0 (0)
	bla_{IMP}	0 (0)	0 (0)	0 (0)	0 (0)
	bla_{NDM-1}	53 (55.2)	3 (13.6)	40 (51.2)	2 (7.4)
Class D	bla_{OXA}	91 (94.8)	10 (46.5)	69(88.4)	10 (37)
Virulent genes					
	OmpA	83 (86.4)	9 (40.9)	64(82.0)	9(33.3)
	CsuE	74 (77.0)	12 (54.5)	63 (80.7)	10 (37)

CR-AB: carbapenem-resistant *Acinetobacter baumannii*; CR-ANB: carbapenem-resistant *Acinetobacter non baumannii*.

confirmed by MHT, CDST + EDTA, and CDST + PBS in 87.8%, 85.2%, and 84.3% of isolates, respectively.

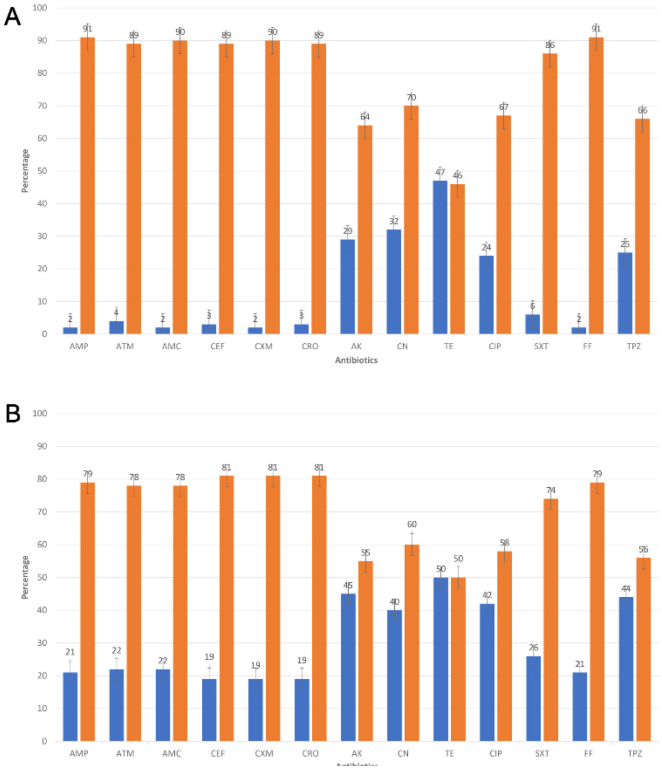
Molecular characterization of resistance/virulence genes and biofilm determinants

Based on the amplification of *ITS* gene by PCR, 174 (78.0%) CR-AB strains were identified and 49 (21.9%) strains were from CR- *Acinetobacter non-baumannii* (ANB). Out of 174 CR-AB, 96 (55%) were wound isolates, and 78 (44.8%) non-wound specimens. ANB were also grouped into isolates from wound (22, 44.9%) and non-wound samples (27, 55%). Genetic analysis of CR-AB from wound revealed that Class D bla_{OXA-23} was the most frequent among the investigated genes (91, 94.8%), followed by bla_{TEM} (68, 70.8%), $bla_{NDM-1,}$ (53, 55.2%), bla_{CTX-M} (19, 19.7%), and bla_{VIM} (2, 2%) strains. bla_{IMP} was not detected in any of the tested isolates. Among CR-AB from non-wound specimens, numbers (percentages) of bla_{OXA-23}, bla_{NDM-1}, bla_{TEM}, and bla_{CTX-M} were 69 (88.4%), 40 (51.2%), 35 (44.8%), and 13 (16.6%), respectively. bla_{VIM} and bla_{IMP} were not detected in any strain. The virulence genes *OmpA* and *csuE* were more than 77% in CR-AB and less than 55% in the ANB group (Table 3).

Biofilm formation in A. baumannii

Out of 174, strong biofilm-formers CR-AB were the most common (90, 51.7%), followed by moderate and weak biofilm formers (58 (33.3%) and 17 (9.7%), respectively). Only 9 (5.1%) of strains were non-biofilm forming. *OmpA* (85, 48.8%), and *csuE* (72, 41.3%), were mainly detected in strong biofilm formers (Table 4).

Figure 1A. Graphical representation of AST trends in non-wound samples; **B.** Graphical representation of AST trends in wound samples. Orange indicates resistance; blue indicates sensitivity.



AMP: ampicillin; ATM: aztreonam; AMC: amoxicillin-clavulanic acid; CEF: cefixime; CTX: cefotaxime; CRO: ceftriaxone; AK: amikacin; CN: gentamycin; TE: tetracycline; CIP: ciprofloxacin; SXT: septran; FF: Fosfomycin; TZP: tazobactam-piperacillin.

Table 4. Correlation of biofilm-related genes and biofilm formation (n = 174).

Biofilm formation	**Isolates/biofilm formation %**	**Biofilm related genes Isolates/gene %**	
		OmpA	***CsuE***
Non-biofilm	9/5.1	3/1.7	4/2.2
Weak biofilm	17/9.7	15/8.6	12/6.9
Moderate biofilm	58/33.3	54/31.0	49/28.1
Strong biofilm	90/51.7	85/48.8	72/41.3

CR-AB harbours a combination of genes

All the genes (bla_{OXA-23}, bla_{TEM}, bla_{CTX-M}, $bla_{NDM,}$ and bla_{VIM}) were amplified from one of the strains. The combination of bla_{OXA-23}+bla_{TEM} and bla_{OXA-23} +bla_{NDM-1} was present in 82 (47.1%) and 40 (23%) strains, respectively. Other combinations were bla_{CTX-M}+bla_{NDM-1}, bla_{TEM}+$bla_{NDM,}$ and bla_{TEM}+bla_{CTX-M} detected in 18 (10.34%), 17 (9.77%), and 15 (8.62%) strains respectively. Only one strain with bla_{OXA-23}+bla_{VIM} was detected. However, the coexistence of virulence genes *OmpA* and *csuE* in biofilm-forming CR-AB was detected in 139 (79.9%) strains (Table 5). Statistical analysis showed that the presence of bla_{NDM} and bla_{OXA} genes were correlated with the presence of bla_{OMP} and bla_{CsuE} in study strains (p value < 0.05) (Table 5, 6).

16S rRNA sequencing

The neighbor-joining tree was constructed by 16S rRNA gene sequence data isolated from different specimens. One of the clades showed a close relationship between strains isolated from blood and urine. Among the three clades, two clades were generated for non-surgical wound and pus samples, and post-surgical wound and pus samples (Figure 2).

Discussion

AB is labelled as a "red alert" pathogen because of the development of antibiotic resistance to all the available antibiotics and the ability to survive in harsh environmental conditions through biofilm formation. Previous studies revealed that the drug-resistant AB had evolved their target sites for antibiotics and efflux pumps and enzymatically degraded the antibiotics of β lactam and aminoglycoside families [7]. Here, we assessed the virulent determinants and biofilm-forming ability in CR-AB isolates collected from health settings in Pakistan. Infections were more common among people in the age group 26–50 years and males. Our results revealed that most isolated AB strains were more resistant to cephalosporins and carbapenems. Numerous studies have reported the high prevalence of CR-AB in Pakistani clinical settings [17-20]. A study showed 7% of AB strains isolated from clinical samples in Lahore with high levels of resistance to all antibiotics, including carbapenems [17,18]. Shahid *et al*., also found that more than 50% of AB isolates were non-susceptible to aminoglycosides [19]. Moreover, the percentages of imipenem and meropenem-resistant AB strains have risen globally from 39.0% and 30.1% in 2005 to 72.3% and 71.5% in 2021, respectively [20].

Table 5. Coexistence of *bla* and carbapenemases genes.

***bla* genes**	**CR-AB isolates n = 174 (%)**
bla_{OXA-23}, bla_{NDM-1}	40 (23.0)
bla_{OXA-23}, bla_{NDM-1}, and bla_{TEM}, $bla_{VIM.}$ bla_{CTX-M}	1 (0.57)
bla_{TEM}, bla_{NDM-1}	17 (9.77)
bla_{OXA-23}, bla_{TEM}	82 (47.1)
bla_{OXA-23}, bla_{VIM},	1 (0.57)
bla_{TEM}, bla_{CTX-M},	15 (8.62)
bla_{CTX-M}, bla_{NDM-1}	18 (10.34)
Coexisting virulent genes (*OmpA* + *csuE*)	139 (79.9)

CR-AB: carbapenem-resistant *Acinetobacter baumannii*.

Colistin has been reported as one of the best choices of drug with sensitivity rate 96.2%, similar to our findings [17]. Our results were concordant with a previous study in which more than 80% of CR-AB strains were also confirmed for carbapenemase production [21]. Based on the species conserved region of identification *ITS,* 174 strains were identified as AB and 49 as ANB species. When investigating *bla* genes in both groups, we detected the presence of bla_{OXA-23} in most CR-AB. Previous literature has revealed the prevalence of oxacillinases producing CR-AB carrying bla_{OXA-23} in Pakistan [18,22]. Indeed, *the blaOXA-23* gene belonging to ST2 resides on plasmids responsible for the acquisition and diffusion of carbapenem-resistance genes in CR-AB. In addition, strong promotor sequences of *ISAba1* and *ISAba4* upstream of bla_{OXA-23} contribute to dissemination in the environment [22]. This study highlights the seriousness of the matter. The bacterial species of our region are becoming resistant to almost all antibiotics, and we are left with little to no choice of antibiotic-based treatments.

Regarding the coexistence of carbapenemase genes, the most common combination was bla_{OXA} + $bla_{TEM,}$ followed by bla_{OXA} + $bla_{NDM.}$ These combinations make the primary resistance profile of any strain. The coexistence of bla_{OXA} and bla_{TEM} in CR-AB was detected by Han *et al*. in China using reverse

Table 6. Pearson correlation of *bla* and carbapenemases genes with virulence genes.

AMR genes	***bla*$_{OMP}$**	**Significance (2-tailed)**	***bla*$_{CsuE}$**	**Significance (2-tailed)**
***bla*$_{TEM}$**	0.946	0.054	0.863	0.137
***bla*$_{CTXM}$**	0.755	0.245	0.545	0.455
***bla*$_{VIM}$**	0.629	0.371	0.479	0.521
***bla*$_{NDM}$**	1.000**	0.000	0.958*	0.042
***bla*$_{OXA}$**	1.000**	0.000	0.962*	0.038

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed). AMR: antmicrobial resistant genes.

transcriptase quantitative PCR (RT-qPCR) [23]. In this study, most biofilm-forming strains carried virulence genes *OmpA* and *csuE*. *OmpA* and *csuE* that encode porin proteins and pili, enabling the bacteria to adhere effortlessly to several medical devices and host epithelial cells. So, it plays a crucial role in infection invasion and persistence in hospital settings. Therefore, catheter-related infections are associated with AB [24–26]. Several studies investigated the biofilm formation ability associated with antibiotic resistance in AB. Boiofilms can reduce antibiotic absorption into bacterial cells, and bacteria can thrive under harsh environments with multidrug resistance [27]. Scientists have also claimed that carbapenem-resistant strains can produce more biofilm than carbapenem-susceptible strains [28]. Positive correlation of *csuE* gene with the presence of *ISAba1* suggested that biofilm formation enhances the virulence of strains [29]. These findings predict that the infections of CR-AB and CR-ANB strains are difficult to treat due to resistance to all antibiotics. The virulence of these strains has increased many folds due to survival in harsh conditions and increased resistance to therapies.

This study has a few limitations. Firstly, the sample size was small. Secondly, strains were analyzed for only carbapenemases production. Antibiotic resistance may also be due to other proteins, such as efflux pumps and synergistic or antagonistic mechanisms.

Conclusions

The study identified a high frequency of CR-AB. bla_{OXA-23} was the most prevalent, followed by bla_{NDM-1}. bla_{CTX-M} and bla_{TEM}. A combination of different genes was also observed to be responsible for limiting the therapeutic options. A high percentage of virulent determinants *OmpA* and *csuE* contributed to pathogenesis of isolates. This also highlighted the biofilm-forming potential, which enhanced antimicrobial resistance in AB and transmission in clinical isolates in our health setting.

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Authors' contributions

MA: experiments, data analysis; AA: strains collection and screening; KN: experiment and data analysis; SS: supervision, SR: conceptualization, investigation, supervision, funding acquisition, validation.

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