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Prevalence of Extensively and Pan-drug-resistant *Acinetobacter* spp. in Nosocomial Infections in Western Saudi Arabia

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Abstract

Multidrug resistance patterns of *Acinetobacter* spp. have led to their emergence as an important source of nosocomial infections. This study investigated the prevalence and clinical characteristics of *Acinetobacter* spp. in hospital-acquired wound and urinary tract infections. A total of 432 samples [wound swabs (210) and urine samples (222)] were analyzed for the presence of *Acinetobacter* spp. through selective culturing on MacConkey and Leeds *Acinetobacter* medium followed by identification with API 20E strips and VITEK 2 compact system. Antimicrobial susceptibility was assessed by adopting the disk diffusion method on Muller-Hinton agar, whereas the minimum inhibitory concentration procedure was carried out by using a ComASP™ Colistin test kit. Biofilm formation was examined using microtiter plates and following the crystal violet staining method. PCR was performed to amplify virulence (*lasB, bap,* and *plcN*) and antimicrobial resistance (*bla*$_{OXA-51like}$) genes. The results revealed a low prevalence of *Acinetobacter* spp. (1.85 %) where *Acinetobacter baumannii* was the predominant species. *Acinetobacter baumannii* isolates harbored *bla*$_{OXA-51-like}$ gene to exert extensive or pan-drug resistance. Most *Acinetobacter baumannii* isolates demonstrated weaker to moderate biofilm-forming capabilities and carried the *bap* gene. *Acinetobacter baumannii* isolates lacked the combination of virulence factors encoding *lasB* and *plcN* genes. *Acinetobacter baumannii* infections are rising in Saudi Arabia. The results of this study highlight the epidemiology of virulent and antibiotic-resistant *Acinetobacter* spp., particularly *A. baumannii*, in Saudi Arabia. The detailed elaboration on the diversity, virulence, and antimicrobial susceptibility patterns of *Acinetobacter* spp. in Saudi Arabia requires further in-depth molecular investigations.

Keywords: *Acinetobacter baumannii*, Wound Infections, Antimicrobial Resistance, Virulence Factors, Biofilm

INTRODUCTION

Acinetobacter genus is characterized by gram-negative, non-fermentative, and strictly aerobic bacteria, which are categorized into 38 species. *Acinetobacter lwoffii* and *A. baumannii* are the most common human pathogens. *A. haemolyticus, A. nosocomialis,* and *A. pitti* are newly emerging human pathogens of the genus *Acinetobacter*.[1-3] *Acinetobacter* species are ubiquitously found in nature with more frequent occurrence in water and soil. They are capable of surviving on dry and moist surfaces and resisting common disinfectants, which allows the growth of some *Acinetobacter* species in the hospital environment.[1,2]

Acinetobacter species are often detected in nosocomial infections, particularly in ICUs (intensive care units) where sporadic, endemic, and epidemic cases are commonly treated. *A. baumannii* is frequently implicated in various hospital-acquired infections such as secondary meningitis, bacteremia, wound and burn infections, UTIs (urinary tract infections), and infective endocarditis.[1,2,4] The bacteria enters the bloodstream in some cases leading to the occurrence of bacteremia with significantly high mortality rates of 32% to 52%. *A. baumannii*-related UTIs require continuous catheterization and antibiotic therapy. It also infects soft tissues in traumatic injuries, postsurgical wounds, and skin. *A. baumannii* infection in burn wounds complicates its treatment therapies.[2-4]

Antibiotic resistance in *A. baumannii* enhances its clinical significance and its resistance to broad-spectrum β-lactam antibiotics, cephalosporins, quinolones, aminoglycosides, and carbapenems is steadily increasing.[2,3] Therefore, the World Health Organization (WHO) has categorized *A. baumannii* as a "critical priority pathogen" to human health that necessitates urgent development of novel antibiotics.[4]

Because of the intrinsic oxacillinase (*bla*$_{OXA-51like}$) enzyme, which has over 40 sequence variants, *Acinetobacter* spp. are resistant to carbapenems. In *Acinetobacter baumannii*, the *bla*$_{OXA-51-like}$ gene is widely distributed and is thought to be an essential genetic marker for identifying *Acinetobacter* to the species level. Other types of bla genes in *Acinetobacter* may also include *bla*$_{OXA-23}$, *bla*$_{OXA-40}$, and *bla*$_{OXA-58}$. The *bla*$_{OXA-51-like}$ may hydrolyze imipenem

and meropenem (carbapenems) as well as benzylpenicillin, ampicillin, ticarcillin, and piperacillin (penicillins).[1,2,4]

Multiple virulence factors contribute to *A. baumannii* pathogenesis and associated high mortality rates, including protein secretion systems, outer membrane proteins, biofilm production, lipopolysaccharide, quorum sensing, capsule, nutrient-acquisition systems, efflux pumps, phospholipase, and elastase. These factors facilitate pathogen survival under stressed conditions and promote antimicrobial resistance.[2-4]

Little is still known about *Acinetobacter* true pathogenic potential or virulence repertory, despite a great deal of research being done on its virulence potential. *OmpA*, a member of the outer membrane proteins (OMPs), has been found to considerably contribute to the disease-causing capability of *A. baumannii*, even though it is thought that other factors may contribute to the pathogen's virulence potential. *OmpA*, the pathogen's most prevalent surface protein, is also implicated in complement resistance and biofilm development. Because *A. baumannii* may form biofilms, it can thrive continuously in unfavorable settings and conditions. In fact, it has been demonstrated that *A. baumannii* forms biofilms on abiotic surfaces, such as glass and tools used in intensive care units and/or on biological surfaces like epithelial cells. After *A. baumannii* connects to specific surfaces, the onset of biofilm development and maturation is facilitated by both pili assembly and the creation of biofilm-associated protein (BAP). Bacterial cell surfaces have BAP, which stabilizes the mature biofilm on biotic or abiotic surfaces, aiding in the growth and maturity of biofilms. Phospholipase D and C are two more important proteins that have been demonstrated to support the virulence of *A. baumannii*. Phospholipase C increases toxicity to epithelial cells, whereas phospholipase D is crucial for pathogenesis, resistance to human serum, and epithelial cell evasion.[1,2,4]

Antibiotic-resistant *Acinetobacter baumannii* infections are rising in Saudi Arabia, however, only a few studies have documented their prevalence in various cities along with antimicrobial susceptibility patterns.[5-9] Thus, the elucidation of *Acinetobacter baumannii* epidemiology in Saudi Arabia requires further in-depth investigation. Therefore, the current study investigated *Acinetobacter* spp. prevalence, antibiotic susceptibility profiles, and virulence factors associated with wound and UTI infections in hospitalized patients in Makkah, western Saudi Arabia.

MATERIALS AND METHODS

Sample collection
A total of 432 clinical samples [wound swabs (210) and urine samples (222)] of hospitalized patients were collected (October 2021 to January 2022) from four hospitals in Makkah, Saudi Arabia. The wound swab samples were collected using sterile swabs with Amies and charcoal transport media (Zhejiang Runlab Technology Co., Ltd., China) whereas urine samples were collected in sterile urine sample bottles. An ice box was used to transfer the samples to the laboratory without direct exposure to the sunlight. Microbiological examinations were initiated on the sampling day.

Isolation and identification of *Acinetobacter* spp.
MacConkey agar (Oxoid, UK) and Leeds *Acinetobacter* selective agar plates (HiMedia, India) were used to culture the samples (wound swabs and urine) for 24 h at 37°C.[10-13] *Acinetobacter* spp. were identified by using API 20E strips (bioMerieux, France), and further confirmed by VITEK 2 Compact System (bioMerieux).[14,15]

Antimicrobial susceptibility profiles of *Acinetobacter* spp.
Kirby-Bauer disk diffusion method and Clinical and Laboratory Standard Institute's (CLSI) guidelines were followed to perform antimicrobial susceptibility tests of *Acinetobacter* spp.[16,17] The choice of antimicrobial agent tested in this study was based on the recommendations and guidelines of CLSI clinical breakpoints for *Acinetobacter* spp.[17] Briefly, McFarland standard suspension (0.5) of each confirmed *Acinetobacter* spp. isolate was prepared followed by spreading onto Mueller-Hinton agar (HiMedia) plates and incubation (18-24 h) at 37°C along with antibiotic disks. Antimicrobial susceptibility test involved 14 antibiotics (Oxoid, Basingstoke, UK) belonging to 8 antimicrobial classes[17] including

Piperacillin (100 μg), Ampicillin-Sulbactam (10/10 μg) [penicillins], Ciprofloxacin (5 μg), Levofloxacin (5 μg) [fluoroquinolones], Ceftazidime (30 μg), Cefepime (30 μg) [cephalosporins], Gentamicin (10 μg), Tobramycin (10 μg), Amikacin (30 μg) [aminoglycosides], Imipenem (10 μg), Meropenem (10 μg) [carbapenemes], Trimethoprim-Sulfamethoxazole (1.25/23.75 μg) [folate pathway antagonists], and Tetracycline (10 μg) [tetracyclines]. Colistin [lipopeptides] susceptibility was assessed by adopting the broth dilution method and calculating minimum inhibitory concentration (MIC) according to the CLSI recommendations.[17] ComASP™ Colistin test kit (Liofilchem Diagnostics, Italy) was used for the assay by following manufacturers' guidelines.[18,19] Briefly, the McFarland (0.5) standard was prepared, and sterile saline was used to dilute the suspension (1:2 (v/v)) for the preparation of solution (A). Solution B was prepared by adding solution A (0.4 ml) in a tube containing Mueller Hinton II broth. Solution B aliquot (100 μl) was dispensed into each well of a microtiter plate containing different concentrations of antibiotics. The inoculated microtiter plates were incubated (16-20 h) at 36 ± 2°C. CHROMagar ESBL (Saudi Laboratory Prepared Media – SPLM, Saudi Arabia) was used to detect extended-spectrum beta-lactamase (ESBL) *Acinetobacter* spp. phenotypes after the incubation of plates (24 h) at 37°C.[20]

Calculation of multiple antibiotic resistance index (MARI)

The number of antibiotics facing isolate resistance was divided by the total number of tested antibiotics to calculate the multiple antibiotic resistance index (MARI).[21] MARI ≥ 0.2 depicts the origin of isolates from frequent antibiotic usage areas whereas MARI ≤ 0.2 presents the bacterial origin from less frequent antibiotic usage areas.[22]

Biofilm-forming capability of *Acinetobacter* spp.

A modified 96-well microtiter plate method was followed to assess the biofilm formation capability of *Acinetobacter* spp.[23,24] Briefly, overnight nutrient broth (HiMedia) cultures were diluted in a fresh medium (10^8 CFU, equal inoculum level with 0.5 McFarland standard).

Inoculum aliquots (20 μl) were separately transferred to the wells of a flat uncoated 96-well microtiter plate (Corning incorporated, life sciences, USA) along with fresh broth (180 μl) followed by incubation (24 h) at 37°C. Distilled water was used to wash microtiter plates thrice before staining with crystal violet solution (1%, 200 μl). The plates were again rinsed with distilled water thrice to remove unbound cells. Then, plates were dried and ethanol (95%, 150 μl) was added before measuring the absorbance (590 nm) in a microplate reader (BioTek Synergy 2 Multimode Plate Reader, USA). The assay was performed in triplicate to obtain consistent results. Biofilm formation capabilities of *Acinetobacter* spp. were calculated and categorized as strong (S), moderate (M), and weak (W) according to the formula mentioned in Table 1.[23,24]

Molecular detection of virulence factors-encoding genes and antimicrobial resistance in *Acinetobacter* spp.

AllPrep Bacteria kit (Qiagen, USA) was used to extract *Acinetobacter* spp. DNA. DNA quantity and quality were measured Spectrophotometrically (Denovix DS-11 Spectrophotometer, USA). The PCR amplification of genes [*plcN* (466 bp), $bla_{OXA-51-like}$ (353 bp), *bap* (560 bp), and *lasB* (300 bp)] was carried out using DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, USA). Primer sequences, product sizes, and annealing temperatures of the genes are listed in Table 2.[25] A Veriti Thermal Cycler (Thermo Fisher Scientific) was used for the PCR amplification, and the reaction mix (25 μl) consisted of 2X master mix (12.5 μl), forward and reverse primers (10 μM, 2.0 μl), DNA (10 ng/μl, 1.0 μl), and nuclease-free water (9.5 μl). PCR conditions were separately adjusted for each gene as $bla_{OAX-51-like}$ [initial denaturation (95°C, 3 min), 35 cycles of denaturation (95°C, 30 sec), annealing (55°C, 30 sec), and extension (72°C, 1 min) followed by a final extension (72°C, 7 min)], *bap* [initial denaturation (95°C, 3 min), 35 cycles of denaturation (95°C, 30 sec), annealing (58°C, 30 sec), and extension (72°C, 1 min) followed by a final extension (72°C, 7 min)], *plcN* [initial denaturation (95°C, 3 min), 35 cycles of denaturation (95°C, 30 sec), annealing (55°C, 30 sec), and extension (72°C, 1 min) followed by a final extension (72°C, 7 min)],

and *lasB* [initial denaturation (95°C, 3 min), 35 cycles of denaturation (95°C, 30 sec), annealing (55°C, 30 sec), and extension (72°C, 1 min) followed by a final extension (72°C, 7 min)]. Agarose gel electrophoresis verified the quality and size of PCR amplification products. The gel (2%) was prepared using ultrapure Agarose (Cleaver Scientific, UK) and TBE buffer (1X) followed by the addition of SYBR-safe DNA stain (Invitrogen, USA). PCR products (4 μl) were loaded into the wells of the gel. A DNA ladder of 100-1000 bp (Thermo Fisher) served as a reference to estimate the size of PCR products. The gel was run at 100 mV for 30 minutes and DNA fragments were observed in a UV Trans-illuminator (Gel Documentation and imaging system).[25]

Statistical analysis

SPSS Statistics (SPSS version 21.0) was used for the statistical analysis. Pearson correlation coefficient revealed the linear association between *Acinetobacter* spp. biofilm forming capability and multidrug resistance.

Control strains

The controls were comprised of *Acinetobacter baumannii* BAA-747, a clinical reference isolate obtained from Al-Borg Medical Diagnostic Laboratories, Jeddah, Saudi Arabia, and *Escherichia coli* ATCC® 25922™.

RESULTS

A total of 432 clinical samples [wound swabs (210) and urine (222)] were examined, which revealed the presence of *Acinetobacter* spp. in only seven wound swabs (3.33%), and one urine sample (0.45%). The overall *Acinetobacter* spp. incidence was noted as 1.85% among 432 clinical samples. The bacterial growth on MacConkey plates was mostly characterized by lactose-fermenting bacteria (*Klebsiella* and *E. coli*), and a few non-lactose fermenting bacteria (*Pseudomonas* and/or *Acinetobacter*). The Leeds *Acinetobacter* medium differentiated *Acinetobacter* spp. and *Pseudomonas* spp., which were further confirmed by API 20E strips and the VITEK 2 compact system. API 20E strips identified all isolates as *Acinetobacter* spp. The VITEK 2 compact system recognized six *Acinetobacter baumannii* isolates (AB1, AB2, AB3, AB6, AB7, and AB8) from wound swabs whereas one isolate of wound origin was identified as *Acinetobacter haemolyticus* (AB5). The only isolate from a urine sample was identified as *Acinetobacter junii* (AB4).

Antimicrobial susceptibility profiles of *Acinetobacter* spp.

Seven out of eight wound swab isolates (88%) exhibited resistance to three or more antimicrobial agents (Table 3). The urine isolate was susceptible to all tested antimicrobial agents except colistin. Overall, the highest

Table 1. Biofilm formation in *Acinetobacter* spp.

Biofilm formation	Strong	Moderate	Weak
BF= AB - CW	0.200-0.299	0.100-0.199	<0.100
BF= AB ÷ CW	4.00-5.99	2.00-3.99	<2.00

BF: biofilm, AB: stained attached bacteria, CW: stained control wells

Table 2. Primers for antimicrobial resistance and virulence factors encoding genes in *Acinetobacter* spp.

Gene primer	Sequence 5'→3'	Product size (bp)	Annealing temperature
bla$_{OXA-51-like}$	5'-TAATGCTTTGATCGGCCTTG-3' 5'-TGGATTGCACTTCATCTTGG-3'	353	55°C
bap	5'-ATGCCTGAGATACAAATTATTGCCAAGGATAATC-3' 5'-AGGTGCTGAAGAATCATCATCATTAC-3'	560	58°C
plcN	5'-GTTATCGCAACCAGCCCTAC-3' 5'-AGGTCGAACACCTGGAACAC-3'	466	55°C
lasB	5'-GGAATGAACGAAGCGTTCTC-3' 5'-GGTCCAGTAGTAGCGGTTGG-3'	300	55°C

Table 3. Antimicrobial resistance profiles of *Acinetobacter* spp.

Isolates	Sample	Clear zone diameter (mm)													MIC
		CAZ	FEP	IPM	MEM	SAM	PRL	CN	SXT	AK	CIP	LEV	TOB	TE	CL†
AB6	WS	13 (R)	12 (R)	12 (R)	10 (R)	14 (I)	8 (R)	19 (S)	0 (R)	21 (S)	0 (R)	18 (S)	20 (S)	0 (R)	0.5 (S)
AB7	WS	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	1 (I)
AB5	WS	25 (S)	21 (S)	14 (R)	0 (R)	0 (R)	0 (R)	17 (S)	0 (R)	0 (R)	0 (R)	0 (R)	22 (S)	0 (R)	16 (R)
AB3	WS	0 (R)	0 (R)	12 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	2 (I)
AB2	WS	0 (R)	16 (I)	27 (S)	29 (S)	0 (R)	19 (I)	0 (R)	0 (R)	14 (R)	0 (R)	0 (R)	0 (R)	0 (R)	16 (R)
AB1	WS	0 (R)	0 (R)	12 (R)	0 (R)	11 (R)	0 (R)	12 (R)	0 (R)	21 (S)	0 (R)	0 (R)	20 (S)	0 (R)	0.5 (S)
AB4	UR	24 (S)	27 (S)	35 (S)	31 (S)	30 (S)	29 (S)	25 (S)	25 (S)	22 (S)	27 (S)	27 (S)	30 (S)	26 (S)	16 (R)
AB8	WS	0 (R)	0 (R)	9 (R)	7 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0.5 (S)
Total resistance (%)		6 (75)	5 (63)	6 (75)	6 (75)	6 (75)	6 (75)	5 (63)	7 (88)	5 (63)	7 (88)	6 (75)	4 (50)	7 (88)	3 (38)

CAZ: Ceftazidime, FEP: Cefepime, IPM: Imipenem, MEM: Meropenem, SAM: Ampicillin-Sulbactam, PRL: Piperacillin, CN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, AK: Amikacin, CIP: Ciprofloxacin, LEV: Levofloxacin, TOB: Tobramycin, TE: Tetracycline, CL: Colistin

†: MIC-based antimicrobial susceptibility testing against colistin using ComASP™ Colistin test kit. All other AST was carried out by the Kerby-Baur disc diffusion method and measured in millimeters (mm)

S: susceptible, I: intermediate, R: resistant

WS: Wound swabs, UR: Urine samples

Interpretations of antimicrobial susceptibility testing were based on the CLSI clinical breakpoints [17] for *Acinetobacter* spp.

Table 4. Correlation between antimicrobial resistance and biofilm formation in *Acinetobacter* spp.

Isolate	Species	Resistant pattern	Number of classes	Biofilm formation	$bla_{oxa\text{-}51\text{-}like}$	MARI
AB6	*A. baumannii*	CAZ, FEP, IPM, MEM, SAM, PRL, SXT, CIP, TE	6	Weak	+	0.43
AB7	*A. baumannii*	CAZ, FEP, IPM, MEM, SAM, PRL, CN, SXT, AK, CIP, LEV, Tob, TE, CL	8	Weak	+	0.6
AB5	*A. haemolyticus*	IPM, MEM, SAM, PRL, SXT, AK, CIP, LEV, TE, CL	7	Weak	-	0.5
AB3	*A. baumannii*	CAZ, FEP, IPM, MEM, SAM, PRL, CN, SXT, AK, CIP, LEV, Tob, TE, CL	8	Strong	+	0.6
AB2	*A. baumannii*	CAZ, FEP, SAM, PRL, CN, SXT, AK, CIP, LEV, Tob, TE, CL	7	Weak	+	0.5
AB1	*A. baumannii*	CAZ, FEP, IPM, MEM, SAM, PRL, CN, SXT, CIP, LEV, TE	7	Moderate	+	0.5
AB4	*A. junii*	CL	1	Moderate	+	0.072
AB8	*A. baumannii*	CAZ, FEP, IPM, MEM, SAM, PRL, CN, SXT, AK, CIP, LEV, Tob, TE	7	Weak	+	0.5
Pearson correlation			r = -0.1351 (no significance)			

CAZ: Ceftazidime, FEP: Cefepime, IPM: Imipenem, MEM: Meropenem, SAM: Ampicillin-Sulbactam, PRL: Piperacillin, CN: Gentamicin, SXT: Trimethoprim-Sulfamethoxazole, AK: Amikacin, CIP: Ciprofloxacin, LEV: Levofloxacin, Tob: Tobramycin, TE: Tetracycline, CL: Colistin, MARI: Multiple Antibiotic Resistance Index
Pearson correlation examined the association between biofilm formation and multidrug resistance in *Acinetobacter* spp.

Table 5. Biofilm Formation by *Acinetobacter* spp.

Isolate Code	Species	Origin	Strong	Moderate	Weak
AB 1	*Acinetobacter baumannii*	Wound		0.162	
AB 2	*Acinetobacter baumannii*	Wound			0.058
AB 3	*Acinetobacter baumannii*	Wound	0.236		
AB 4	*Acinetobacter junii*	Urine		0.163	
AB 5	*Acinetobacter haemolyticus*	Wound			0.032
AB 6	*Acinetobacter baumannii*	Wound			0.17
AB 7	*Acinetobacter baumannii*	Wound			0.048
AB 8	*Acinetobacter baumannii*	Wound			0.077
Total (%)			1 (12.5 %)	2 (25 %)	5 (62.5 %)

resistance rates (88%, n = 8) were noted against ciprofloxacin, tetracycline, and trimethoprim/sulfamethoxazole followed by 75% (n = 8) resistance to ceftazidime, imipenem, meropenem, ampicillin/sulbactam, piperacillin, and levofloxacin (Table 3). Approximately, 63% of *Acinetobacter* spp. demonstrated resistance to cefepime, gentamicin, and amikacin. The lowest *Acinetobacter* spp. resistance (38%) was noted against colistin (Table 3).

Seven wound-swab *Acinetobacter* spp. isolates (88%, n = 8) exhibited multidrug-resistance (MDR) patterns (resistance to three or more antimicrobials) (Table 4). Notably, *Acinetobacter baumannii* (AB3) and *Acinetobacter baumannii* (AB7) exerted resistance against all the 14 tested antibiotics of eight different antimicrobial classes including colistin, which suggests them as pan-drug-resistant (PDR) isolates. It was followed by *Acinetobacter baumannii* (AB1 and AB2) and

Acinetobacter haemolyticus (AB5), which were resistant to 11, 12, and 10 antibiotics of seven classes, respectively. These results categorized them as extensively drug-resistant (XDR) isolates (Table 4).

Five ESBL-positive *Acinetobacter* spp. (63%) isolates from wound swabs grew on CHROMagar ESBL and harbored the $bla_{OXA-51-like}$ gene as well. $bla_{OXA-51-like}$ gene-containing *Acinetobacter junii* of urine sample was susceptible to all the antibiotics except colistin and remained ESBL-negative on CHROMagar ESBL (Table 4). MARI of all wound swabs *Acinetobacter baumannii*

and *A. haemolyticus* ranged between 0.4 and 0.6 depicting their origin from frequent antibiotic usage areas. Contrarily, 0.07 MARI of *A. junii* of UTI revealed their origin from areas with lesser usage of antibiotics.

Biofilm-forming capability of *Acinetobacter* spp. and its correlation with multidrug-resistance

Biofilm formation of all eight *Acinetobacter* spp. was examined using microtiter plates with crystal violet staining. Table 5 depicts that a substantial number of *Acinetobacter* spp. (87.5 %, n = 8) demonstrated either moderate (25%, n = 8) or a weak (62.5%, n = 8) biofilm-forming capability. Pearson correlation coefficients revealed a non-significant (r = -0.1351) relationship between MDR and biofilm formation (Table 4 and Figure 1). Notably, there was no association between biofilm formation and resistance to a particular drug. Seven *Acinetobacter* spp. of wound swabs contained biofilm-association protein (*bap*)-encoding genes (Table 6).

Prevalence of virulence factors-encoding genes in *Acinetobacter* spp.

PCR amplification of virulence factors-encoding genes revealed that seven out of eight (87.5 %) *Acinetobacter baumannii* (wound swabs) and *Acinetobacter junii* (UTI) harbored biofilm-association protein (*bap*) and $bla_{OXA-51-like}$ (class D oxacillinase) genes (Table 6 and Figure 2 and 3). Elastase (*lasB*) and phospholipase C (*plcN*)-encoding genes were detected in only one isolate (12.5 %) of wound swab origin that was identified as *Acinetobacter haemolyticus* (AB5). This isolate
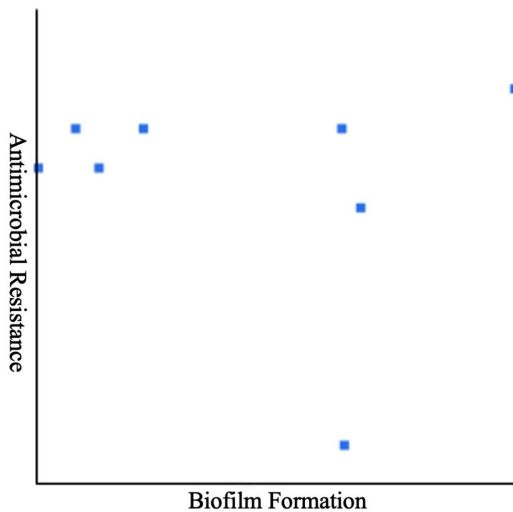
Figure 1. Pearson correlation between biofilm formation in *Acinetobacter* and multidrug resistance, no significance was observed

Table 6. Prevalence of virulence factors and antimicrobial resistance encoding genes in *Acinetobacter* spp.

Isolate Code	Species	Source	$bla_{OXA-51-like}$	bap	plcN	lasB
AB1	*Acinetobacter baumannii*	WS	+	+	-	-
AB2	*Acinetobacter baumannii*	WS	+	+	-	-
AB3	*Acinetobacter baumannii*	WS	+	+	-	-
AB4	*Acinetobacter junii*	UR	+	+	-	-
AB5	*Acinetobacter haemolyticus*	WS	-	-	+	+
AB6	*Acinetobacter baumannii*	WS	+	+	-	-
AB7	*Acinetobacter baumannii*	WS	+	+	-	-
AB8	*Acinetobacter baumannii*	WS	+	+	-	-

WS: wound swabs, UR: Urine samples, *bap*: Biofilm-associated protein gene, *plcN*: Phospholipase C gene, *lasB*: Elastase gene, $bla_{OXA\ 51-like}$: Class D β-lactamase resistance gene

did not contain $bla_{\text{OXA-51-like}}$ and *bap* genes (Table 6 and Figure 2 and 3).

DISCUSSION

Immunocompromised hospitalized patients are more prone to *Acinetobacter* spp. infections. This study investigated *Acinetobacter* spp. incidence in wound swabs and urine samples of hospitalized patients in multiple centers. Overall *Acinetobacter* prevalence was quite low (1.85%, n = 432) in both types of samples. Most *Acinetobacter* spp. isolates (87.5%, n = 8) were recovered from wound swabs whereas only one isolate was identified in urine samples. *Acinetobacter baumannii* was the most prevalent (75%) species in wound swabs. *Acinetobacter*

haemolyticus was also recovered from the wound swabs whereas *Acinetobacter junii* was the only identified isolate in urine samples. The clinical prevalence of *Acinetobacter* spp., particularly *A. baumannii*, varies among countries, cities, and regions of the same country. For example, *A. baumannii* clinical isolation rates range between <1% to < 30% in Europe, whereas an average clinical isolation rate of only 0.7% has been reported in North America. Recently, an average clinical *Acinetobacter baumannii* isolation rate of 4.6% has been reported in the Middle East.[26] However, a study investigated *Acinetobacter* spp. incidence in 11 hospitals in five Saudi Arabian cities (Makkah, Medina, Tabuk, Jeddah, and Riyadh) and reported its comparatively low prevalence (3.9%, n = 124).[8] Thus, the low clinical prevalence
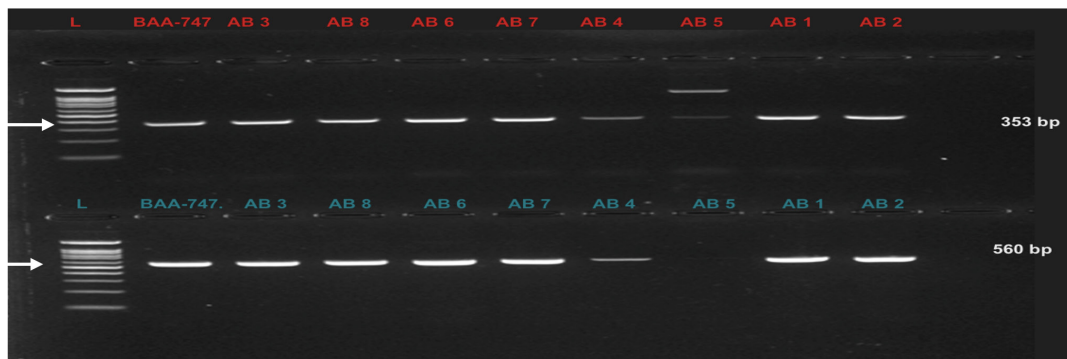
Figure 2. Upper row: $bla_{\text{OXA-51-like}}$ gene (253 bp) in *Acinetobacter* spp. Lane (L) represents DNA marker (Merck KGaA, Darmstadt, Germany), Lane (BAA-747) represents positive control, and Lanes AB1-AB8 represent *Acinetobacter* spp. Bottom row: *bap* virulence gene (560 bp) in *Acinetobacter* spp. Lane (L) represents DNA marker (Merck KGaA, Darmstadt, Germany), Lane (BAA-747) represents positive control, and Lanes AB1-AB8 represent *Acinetobacter* spp.
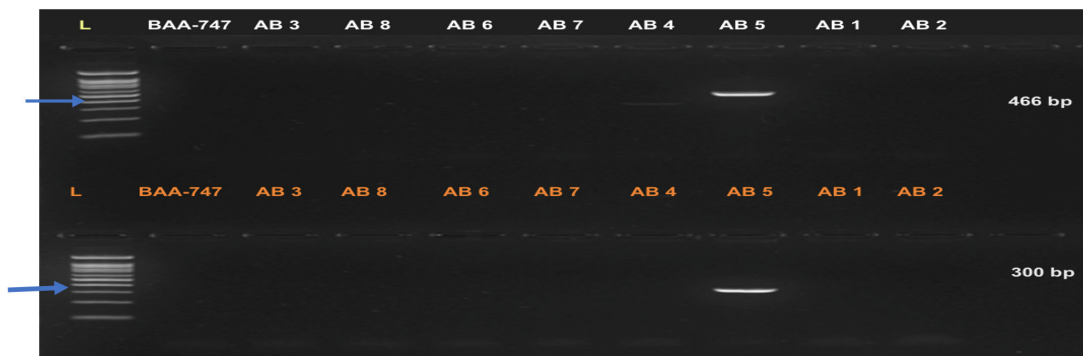
Figure 3. Upper row: *plcN* gene (466 bp) in *Acinetobacter* spp. Lane (L) represents DNA marker (Merck KGaA, Darmstadt, Germany), Lane (BAA-747) represents positive control, and Lanes AB1-AB8 represents *Acinetobacter* spp. Bottom row: *lasB* gene (300 bp) in *Acinetobacter* spp. Lane (L) represents DNA marker (Merck KGaA, Darmstadt, Germany), Lane (BAA-747) represents positive control, and Lanes AB1-AB8 represents *Acinetobacter* spp.

of *Acinetobacter baumannii* during this study is in line with the previous global and Saudi Arabian reports.[8,26,27]

The low incidence of *Acinetobacter* spp., particularly *Acinetobacter baumannii*, could be attributed to its opportunistic nature as it mainly infects immunocompromised patients.[4] Moreover, a recent study has linked clinical *Acinetobacter baumannii* prevalence with the overall antibiotics applications in clinical settings.[26] Pearson correlation revealed that lower antibiotic resistance rates in a particular city or region lead to lower *Acinetobacter* spp. clinical isolation rates in that area. However, *Acinetobacter* spp. prevalence rises with the overall increase in antibiotic resistance, which highlights a high risk of more *Acinetobacter*-linked nosocomial infections in the future.[26]

During this study, eight *Acinetobacter* spp. isolates were recovered from clinical samples, and *Acinetobacter baumannii* was the most prevalent species. All six *Acinetobacter baumannii* (75%, n = 8) isolates and one *Acinetobacter haemolyticus* (12.5%, n = 8) isolate were recovered from the wound swabs whereas only one *Acinetobacter junii* (12.5%, n = 8) isolate was detected in urine samples. Aedh *et al.*[28] have also reported the highest prevalence of *Acinetobacter baumannii* (98.4%, n = 124) among samples of 12 Saudi Arabian hospitals.[8] Similar findings have been reported in other parts of the world.[1,27] Gupta *et al.*[27] also reported the predominance of *Acinetobacter baumannii* (72%) among 111 isolates of *Acinetobacter* spp. *Acinetobacter baumannii* is known to cause 80% of total *Acinetobacter*-related infections.[29]

The predominant prevalence of *Acinetobacter baumannii* than other *Acinetobacter* spp. could be attributed to its virulence determinants such as biofilm formation, proteinase and gelatinase secretion to damage host tissues, phospholipase C secretion to enhance the toxicity to epithelial cells, and the presence of intrinsic class D oxacillinase.[1,29,30] The presence of *Acinetobacter haemolyticus* and *Acinetobacter junii* was quite low, and both have never been reported in Saudi Arabia in wound infections (*A. haemolyticus*) and UTIs (*A. junii*). However, a similar low incidence of *A. haemolyticus* and *A. junii*-associated infections has been reported

in other studies.[27,30,31] The occurrence of *A. haemolyticus*-associated wound infection is less frequent, whereas *A. junii* is considered a rare pathogen of UTIs.[27,31] Thus, the results of this are in accordance with the previous reports.

The overuse of clinical antibiotics in medical, agriculture, and veterinary applications contributes to the global rise in antimicrobial resistance (AMR).[32] The detection of multidrug, extensively, and pan-drug-resistant *Acinetobacter baumannii* in nosocomial infections has significantly increased.[1] During the current study, seven out of eight *Acinetobacter* spp. demonstrated multidrug-resistance patterns, which included six *Acinetobacter baumannii* isolates and one *Acinetobacter haemolyticus* isolate. Furthermore, two of the six *Acinetobacter baumannii* were resistant to all the tested antibiotics of eight antimicrobial classes, which established these isolates as pan-drug-resistant (PDR). Three *Acinetobacter baumannii* were resistant to 10 or more antibiotics of seven antimicrobial classes, which confirmed their extensively drug-resistant (XDR) nature. Multidrug-resistant *Acinetobacter baumannii* has been reported in Saudi Arabia with the rising incidence of XDR and MDR *Acinetobacter baumannii* isolates as well.[7,28,33-35]

A study in Makkah City revealed MDR patterns in 90% of the isolated *Acinetobacter baumannii*.[36] An investigation in Jeddah reported a rise in MDR *Acinetobacter baumannii* (55% to 67%) and PDR *Acinetobacter baumannii* (20% to 33%) from 2010 to 2013.[37] Similarly, an increasing trend of PDR and XDR *Acinetobacter baumannii* occurrence has been observed in Europe, the USA, and other regions.[38-40] MDR patterns of *Acinetobacter* species could be attributed to their intrinsic chromosomal acquisition of genes encoding resistance to carbapenems and cephalosporins, and point mutations for resisting colistin, quinolones, aminoglycosides, and tertracyclines. Moreover, the acquisition of transposons, plasmids, and other mobile genetic elements facilitates the transfer of more resistance genes to *Acinetobacter* spp., particularly *A. baumannii*.[41]

Broad-spectrum beta-lactam combinations (ampicillin-sulbactam), and cephalosporins (cefepime and ceftazidime) are considered the first line of drugs for treating

Acinetobacter baumannii. However, *Acinetobacter baumannii* presented high resistance to these antibiotics during this study, which is in line with the previous reports in Saudi Arabia[7,8,28,33] and other countries.[4,26,38,40] Therefore, fluoroquinolones, carbapenems, and aminoglycoside have become the first choice for MDR-*Acinetobacter baumannii* infection treatment.[4,26] *Acinetobacter* spp. (*Acinetobacter baumannii* and *A. haemolyticus*) of wound swabs exhibited significant resistance (75%, n=8) to carbapenems (imipenem and meropenem) during this study. *Acinetobacter baumannii* isolates were found to have intrinsic $bla_{OXA-51-like}$ genes.[42] The incidence of carbapenem-resistant *Acinetobacter baumannii* is increasing in Saudi Arabia and other countries. *Acinetobacter baumannii* also exerted higher resistance against fluoroquinolones followed by aminoglycosides and cephalosporins, which is similar to the previous reports in Saudi Arabia and other countries.[4,7,8,26,28,33,38,40] Therefore, polymyxins (colistin) tetracyclines have become the second line of choice for countering MDR *Acinetobacter baumannii* infections.[4,26] *Acinetobacter baumannii* isolates detected in this study were also significantly resistant to colistin, which is considered a last treatment option despite its hazards to kidneys and other side effects.[1] Therefore, treatment of PDR and XDR *Acinetobacter* spp. infected wounds are becoming a challenging task, which necessitates the development of novel therapeutic options and the implementation of strict infection control strategies.

Multiple studies have investigated the virulence potential of *Acinetobacter baumannii*, but still further in-depth studies are required. Several factors might contribute to the *Acinetobacter baumannii* pathogenicity including biofilm synthesis, efflux pumps, protein secretion systems, and phospholipases.[43] This study investigated various virulence determinates of *Acinetobacter baumannii*, *A. haemolyticus*, and *A. junii* such as biofilm-forming capability, bap-encoding genes, phospholipase C (*plcN*), and elastase (*lasB*). The biofilm formation was noted in all the *Acinetobacter* isolates. However, most isolates demonstrated weak biofilm-forming capability except one isolate of *Acinetobacter baumannii* from wound infections, which exhibited strong biofilm-forming efficiency. Bala *et al*.[44] have also

reported the inability or weaker biofilm-forming capability of 75 *Acinetobacter baumannii* isolates. *Acinetobacter* spp. biofilms on abiotic and biotic surfaces are associated with persistent and chronic infections, antibiotic resistance, and survivability in hospital environments.[44]

Biofilm formation could be crucial in the rising emergence of MDR pathogens. However, Pearson correlation (r = -0.1351) analysis during this study did not present any correlation between biofilm-forming capability and MDR *Acinetobacter* spp. Avila-Novoa *et al*.[45] have also reported that there is no relationship between biofilm-forming capability and MDR development in *Acinetobacter baumannii* that was revealed by genotypic and phenotypic methods. Contrarily, Rao *et al*.[46] have associated a higher tendency of biofilm formation in *A. baumannii* with MDR. Moreover, they noted higher antibiotic resistance patterns in biofilm-producing isolates than the isolates that do not produce biofilm. Similarly, another study has also established an association between the biofilm-forming capability of *Acinetobacter baumannii* and MDR profiles.[47] Therefore, it is difficult to draw definitive conclusions regarding the biofilm formation and its association with MDR development in *Acinetobacter* spp.

The majority of *Acinetobacter* spp. isolates presented a weak biofilm-forming capability. However, the *bap* gene was amplified from these isolates, which has been linked to biofilm formation and maturation.[43] However, all *Acinetobacter* spp. do not express the *bap* gene and their biofilm-forming capability does not solely rely on this gene. Outer membrane protein A (*ompA*) and pili assembly system (*csu*) are also known to contribute to biofilm formation.[48] Aliramezani *et al*.[25] have also noted that all *bap* gene-harboring *Acinetobacter baumannii* isolates did not exhibit strong biofilm-forming capabilities.

Elastase and phospholipases are well-documented *Acinetobacter baumannii* virulence factors.[49] *plcN* gene-encoded Phospholipase C hydrolyzes phospholipids to spread infection in host tissues whereas *lasB* gene-encoded elastase degrades elastin to damage the host's defense mechanisms and tissues.[25] During this study, *Acinetobacter baumannii* isolates did not contain both of these genes (*lasB* and *plcN*) except *Acinetobacter haemolyticus*. Different studies have

reported varying carriage of *lasB* and *plcN* genes in clinical isolates of *Acinetobacter baumannii*. Aliramezani *et al*.[25] demonstrated the presence of the *plcN* gene in 20% (n = 100) *Acinetobacter baumannii* isolates, whereas the *lasB* gene was noted only in 4% of isolates in Iran. Kareem *et al*.[50] reported the detection of the *plcN* gene in 23.3% (n = 30) isolates, whereas the *lasB* gene was found in 53.33% of *Acinetobacter baumannii* isolates in Iraq. The combined presence of both genes (*plcN* and *lasB*) might enhance the *Acinetobacter baumannii* virulence.[50]

CONCLUSION

This study demonstrates lower *Acinetobacter baumannii* incidence in nosocomial wound and UTI cases in western Saudi Arabia. Nonetheless, some isolates exhibited PDR, XDR, and MDR against tested antibiotics. However, a weak biofilm-forming capability was noted in most *Acinetobacter baumannii* isolates even in the presence of the *bap* gene. *Acinetobacter baumannii* mostly lacked the combined presence of the *plcN* and *lasB* genes except for one isolate. This study first time reports the incidence of a virulent and MDR *Acinetobacter haemolyticus* that is associated with wound infections. *Acinetobacter junii* was isolated from urine samples, which exhibited colistin resistance. *Acinetobacter baumannii* infections are increasing in Saudi Arabia. Thus, the results of this study shed more light on the epidemiology of virulent and antibiotic-resistant *Acinetobacter* spp., particularly *A. baumannii*, in Saudi Arabia. Further molecular-level studies could reveal more details about the diversity, virulence, and antimicrobial susceptibility patterns of *Acinetobacter* spp. in Saudi Arabia.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION
HHA, HAM and LAN conceptualized the study. HHA, NAO, KE, and HAM applied methodology. HSAI-G, SA and MMSA investigated the study. HSAI-G, LAN, NAO performed data curation. HSAI-G, LAN and HAM performed formal analysis. KE and IA collected the resources. HHA and HSAI-G wrote original draft. HHA and IA wrote, reviewed and edited the manuscript. HAM, LAN and HHA performed supervision. All authors read and approved the final manuscript for publication.

FUNDING

DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT
This study was reviewed and approved by the Department of Biology Postgraduate and Research Ethics Committee and also approved by the Faculty of Applied Science Postgraduate and Research Ethics Committee, approval number (342140314434/52962) on 27 October 2021. All urine and wound samples analysed in this study were anonymous, and only the gender of the sample provider was disclosed. Personal, clinical and epidemiological data related to these samples were not provided or disclosed during the study.

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