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Original Article

The Correlations Between Antibiotics Resistance and Biofilm Formation of *Acinetobacter baumannii* isolated from Burned Wound Patients

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ABSTRACT

One hundred and fifty samples from burned wound swabs and from both genders and different ages were included in this study. They were collected from patients in Baquba teaching hospital, Khanaqin general hospital, and outpatient clinics between the beginning of September 2023 and February 2024. Identification of A. baumannii by manual biochemical tests that used Gram staining, catalase test, oxidase test, urease test, indole test, Kligler iron agar (KIA) test, and Simmon citrate test, as well as growth at 44°C. The VITEK2 compact system includes biochemical testing for final validation. The biofilm formation capacity of the A. baumannii isolate was determined using a microtiter plate assay. ESBLs and MBLs were detected using phenotypic methods. Antibiotic susceptibility testing was performed to determine the probable resistance of A. baumannii isolates to 14 antibiotics from various classes. The results indicated that the highest incidence of infection with A. baumannii occurred in the 1-40-year-old age group, with 55 cases representing 36.7% and 36.6%. A. baumannii isolates produced 17/30 (57%) strong biofilms, 9/30 (30%) moderate biofilms, and 4/30 (13%) non-biofilms. Our results indicate that A. baumannii exhibits crucial antibiotic resistance. The recent findings indicated that all A. baumannii isolates displayed 100% MBL production, but none of the 30 isolates produced ESBL enzymes, and there was a connection between biofilm production and antibiotic resistance in these isolates. A. baumannii isolates resistant to aminoglycosides, carbapenems, and sulfonamides show a positive correlation between biofilm production and antibiotic resistance.

Keywords: A. baumannii, Antibiotics Resistance, Biofilm Formation, ESBLs, MBLs

Introduction

Burn infections provide a significant health concern, particularly in developing countries (1). Approximately 75% of people with burn injuries die from infections (2). *Acinetobacter baumannii* is often recognized as an important cause of nosocomial infections, presenting an elevated risk of mortality and serious complications for hospitalized patients, particularly in burn and intensive care units (3). *Acinetobacter baumannii* is a gram-negative bacterium that presents a threat to society by causing severe and extensive (generally nosocomial) infections with considerable mortality rates (4). This bacterium has developed multidrug resistance (MDR) in recent years, attributed to the extensive misuse of antibiotics and insufficient stewardship (5). MDR isolates are associated with a history of long

hospitalizations, catheter use, and mechanical ventilation, while immunocompromised and critically sick patients exhibit a greater susceptibility to invasive infections (6).

The new sequencing methods have transformed the detection of significant *A. baumannii* diseases, facilitating prompt identification and tailored therapy strategies based on the detection of specific resistance genes (7). *A. baumannii* is responsible for several healthcare- and community-related infections that include infections of the skin and soft tissues, infections of the urinary tract, bacteremia, pneumonia, and meningitis (4). The major virulence characteristics of *A. baumannii* are its ability to form biofilms and exhibit antibiotic resistance, which significantly contribute to bacterial survival and infection. A biofilm is a group of bacteria that are surrounded by a protective layer made of substances they produce themselves, like proteins, sugars, and DNA (9). The extracellularly produced matrix shields biofilm-encased cells, which have limited metabolic activity and increase their resistance to antibiotics and innate immune components of the host (10).

Resistance to antibiotics observed in infections with Acinetobacter has forced the use of older-generation medicines, including colistin (11). Carbapenems are the preferred drug for treating severe infections caused by MDR-*A. baumannii* (12). It is alarming that *A. baumannii* isolates are becoming more resistant to carbapenem (13). *A. baumannii* develops carbapenem resistance through various mechanisms, which include the acquisition of β-lactamases, modifications of outer protein membranes and penicillin-binding proteins (PBPs), and an overexpression of pumps for efflux (14). The production of β-lactamase enzymes by *A. baumannii* is an important factor in antibiotic resistance, frequently associated with mobile genetic elements like integrons (15). In *Acinetobacter baumannii*, the primary pathway of resistance to antibiotics in all beta-lactam classes, except monobactams, is the development of metallo-β-lactamase enzymes. Gene cassettes within integrons generally associate with MBLs, which disseminate easily among bacterial populations (16). The purpose of the study was to investigate the correlations between antimicrobial resistance and biofilm production in *A. baumannii*.

Materials & Methods

Study design and duration

The cross-sectional approach was conducted at Baquba teaching hospital, Khanaqin general hospital and out patients clinics, namely in the burn department and nursing clinic, from September 2023 to February 2024.

Patients and sample collection.

One hundred and fifty clinical sterile cotton swabs transport media (Amie's) were collected from burn wound infections. Patient information, such as name, age, gender, reason of burn, degree of burn, rate of burning and treatment, was recorded using a questionnaire form. The swabs were cultured promptly after collection for diagnosis.

Bacteriological isolation and identification

All samples were inoculated onto blood agar and MacConkey agar and incubated at 37°C for 24 hours. Identification of A. baumannii using manual biochemical assays, including Gram staining, Catalase test, Oxidase test, Urease test, Indole test, Kligler iron agar (KIA) test, Simmon citrate test, and growth at 44ºC. Biochemical assays are incorporated into the VITEK2 compact system for the final confirmation.

Antibiotic susceptibility test

Antibiotic susceptibility testing was conducted to evaluate the potential resistance of A. baumannii isolates to 14 antibiotics from various classes: Polymyxin (PB), Ticarcillin/Clavulanate (TCC), Cefotaxime (CTX), Piperacillin/Tazobactam (PTZ), Ceftazidime (CAZ), Cefepime (CPM), Imipenem (IMI), Meropenem (MEM), Doxycycline (DXT), Tetracycline (TE), Ciprofloxacin (CIP), Ceftriaxone (CTR), Colistin (CS), and Trimethoprim/Sulfamethoxazole (TMP/SMX). All isolates went through antibiogram testing according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2023), utilizing the Kirby-Bauer disk diffusion method with disks (Bioanalyse, Turkey) on Mueller-Hinton agar. After measuring the inhibition zones in millimeters using the zone inhibition ruler, the results were compared with the guidelines provided by the National Committee for Clinical Laboratory Standards (17).

Biofilm formation

Biofilm formation capacity of A.baumannii isolates was determined using amicrotitre plate assay as describe previously (18). A.baumannii isolates were grown overnight in brain heart infusion broth (BHIB) containing (1%) glucose at 37°c. Free cells were removed and biofilm were washed three times with normal saline and fixed with 200 µl of 0.1% crystal violet for 20 min at room temperature. Crystal violet was dissolved using 95% ethanol for 20 min and the absorbance was measured at 630 nm. Biofilm formation was categorized as follows: non-biofilm-forming (OD < ODc); moderate (2xODc < OD < 4xODc); strong (4xODc < OD). The reported values represent the averages of three measurements taken over three consecutive days.

Phenotypic Detection of ESBLs Isolates

The detection of ESBLs in the isolates was performed utilizing the double-disk synergy test (DDST). Bacteria suspended on Muller-Hinton agar were incubated with ceftazidime (30 µg), aztreonam (30 µg), cefepime (30 µg), cefotaxime (30 µg), and ceftriaxone (30 µg) alone with Augmentin (amoxicillin 20 µg, clavulanate acid 10 µg) placed at the center, with a 20 mm distance between them on an inoculated agar plate. An ESBL was thought to be present when the edge of the cephalosporin disk inhibition zone clearly extended toward the disk containing clavulanate (19).

Phenotypic detection of MBLs

To make a suspension of A. baumannii, a single colony was added to 5 ml of normal saline and mixed until it matched a 0.5 McFarland standard after 24 hours of incubation. Approximately 0.1 cc of the bacterial suspension was subsequently applied using a sterile cotton swab onto Muller Hinton agar plates and allowed to dry at ambient temperature. Two imipenem discs (10 µg) were placed 20 mm apart on a Muller Hinton agar plate, followed by the addition of 5 µl of pre-prepared EDTA to one of the imipenem discs, and incubated overnight at 37° C. The positive effect for MBL development was shown by an increased zone of bacterial inhibition surrounding the imipenem + EDTA disc, measuring nearly 7 mm or higher, in contrast to the disc free of EDTA (20).

Ethical approval

The study was done following the ethical guidelines derived from the Declaration of Helsinki. The procedure was conducted after obtaining verbal and analytical consent from the patients. The local ethics committee approved the study protocol and the patient information and consent form under document number 587 on September 18, 2023.

Results and Discussion

The distribution of ages and genders among patients infected with A. baumannii

The distribution of affected patients according to age and sex is shown in Table1. Among the infected patients ages less than 1 year to over 60 years, the lowest incidence occurred in the > 60 age group, with 7 cases (4.7%), while the highest incidence was observed in the 1-40 age group, with 55 cases (36.7% and 36.6%). The table indicates a higher occurrence among males, 76 (50.7%), compared to females, 74 (49.3%), with statistical significance (P<0.05).

Table 1. Distribution of A. baumannii according to age and gender.

Characteristic	Males, N (%) 76 (50 .7 %)	Females, N (%) 74 (49 .3 %)	Total, N (%) 150 (100 %)
Age (year)			
< 1 year	4 (2.7 %)	2 (1.3%)	6 (4 %)
1 - 20	39 (26 %)	16 (10 .7%)	55 (36.7 %)
21 - 40	17 (11. 3%)	38 (25. 3 %)	55 (36. 6%)
41 - 60	12 (8 %)	15 (10 %)	27 (18 %)
> 60	4 (2 .7 %)	3 (2 %)	7 (4. 7 %)

Identification and isolation of isolates of A. baumannii

All isolates displayed the appearance of Gram-negative coccobacilli, with occasional diplococci colonies. On MacConkey agar, the isolates of A. baumannii presented as small, lactose-intolerant, and pale colonies; on blood agar, they manifested as opaque, creamy, and non-hemolytic colonies. The ability of all A. baumannii isolates to grow at 44ºC was demonstrated by their favorable growth results.

This test was used to differentiate A. baumannii, which can grow at this temperature, from other Acinetobacter species that cannot. The oxidase, indole, and urease generation tests yielded negative findings for every isolate; however, the catalase and citrate utilization tests yielded positive results. Kligler iron agar exhibited an alkaline slant, no alteration at the bottom, and tested negative for H_2S without gas generation (Figure 1). Biochemical test results are presented in Table 2. According to Table 3, there were 30 (20%) positive growths of A. baumannii, 52 (34.7%) other bacteria, and 68 (45.3%) no growth of bacteria.

Table 2. A. baumannii biochemical test

Test	Result
Lactose fermentation	(- ve)
Gram stain	(- ve)
Catalase test	(+ ve)
Oxidase test	(- ve)
Citrate utilization	(+ ve)
Growth at 44c	(+ ve)
Hemolysis	(- ve)
Urease test	(- ve)
Indole Production test	(- ve)
Kligler iron agar KIA	(K / K)
Growth on blood agar	(+ ve)
H2S, gas production	(- ve)

Table 3. Isolation of Acinetobacter baumannii isolates

Positive bacterial growth		**Negative bacterial growth**	**Total**
A. baumannii	**Other bacteria**	**No bacterial growth**	
30 (20%)	52 (34 . 7 %)	68 (45 . 3%)	150 (100 %)

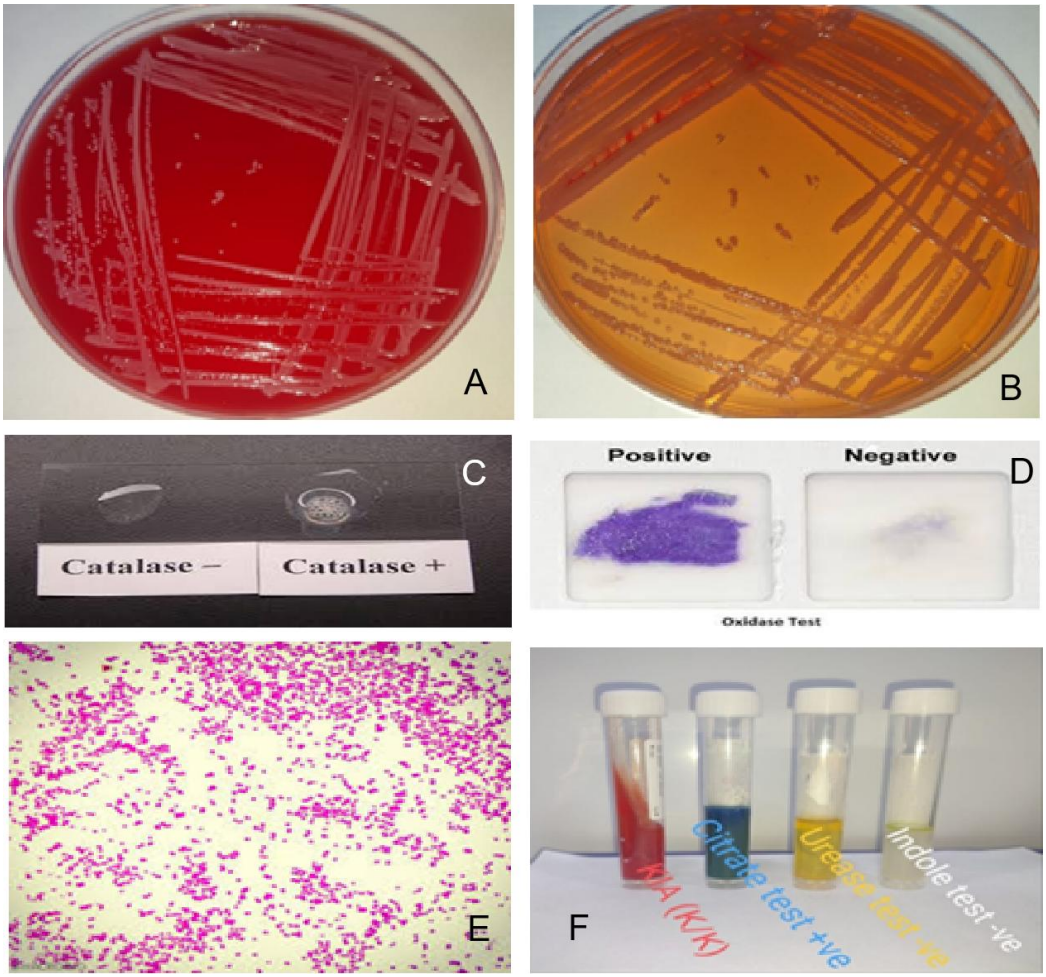

Figure 1. Isolation of *A. baumannii* A (Blood agar), B (MacConkey agar), C (Catalase test), D (Oxidase test), D (Gram stain), E (Biochemical tests)

A. baumannii Antimicrobial Susceptibility Test

The results revealed that A. baumannii clinical isolates are much more resistant to the antibiotics studied, as shown in figure 2, which displays the antibiogram profile of the isolates., while all isolates were sensitive to Colistin (cs) and Polymyxin (PB). They further indicate that isolates which vary in their susceptibility to the antibiotics.

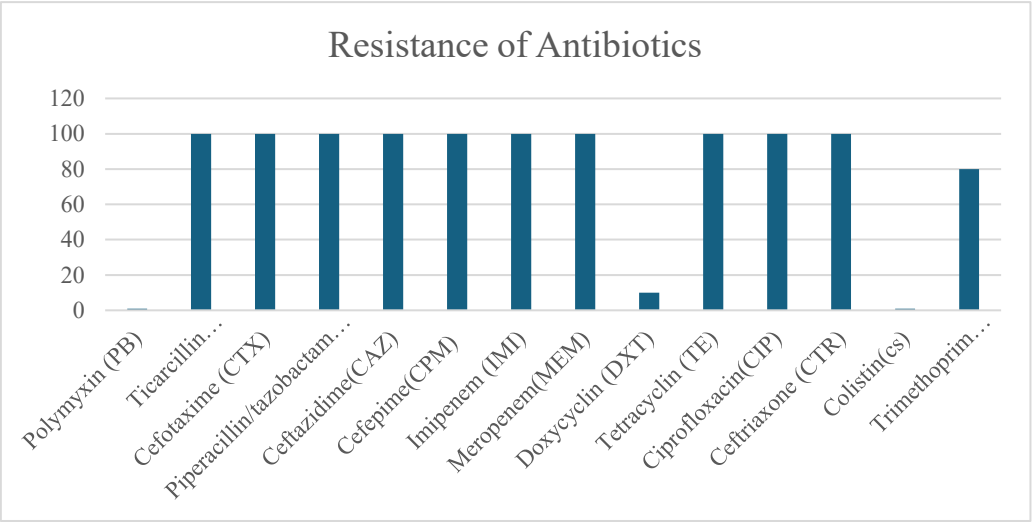

Figure 2. The proportion of *A. baumannii* isolates demonstrating resistance to 14 antimicrobial agents Phenotypic MBL and ESBL Detection

For determination of phenotypic MβL production among isolates, MIC test strips containing imipenem along with EDTA were used. All A. baumannii isolates 100% were MBLs producing as shown in figure 3 and 4. The DDST screening method was used to identify ESBL-producing isolates phenotypically. A total of 30 isolates showed that all A. baumannii isolates did not produce ESBL enzymes.

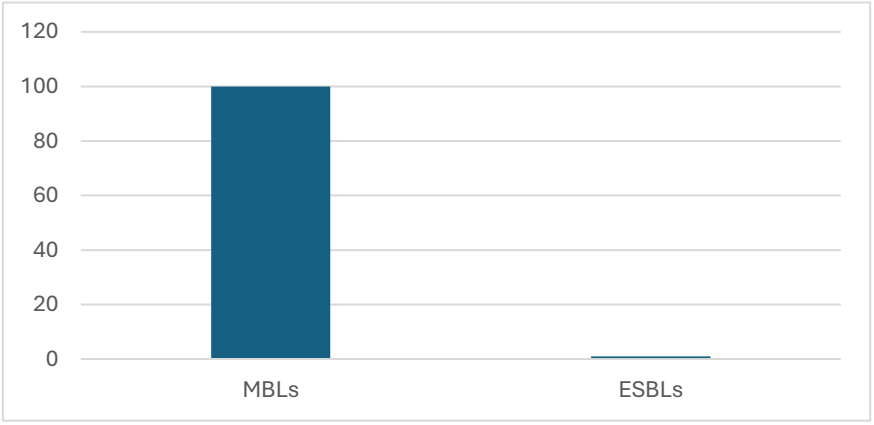

Figure 3. The Percentage of Production MBLs and ESBLs of *A. baumannii* isolates

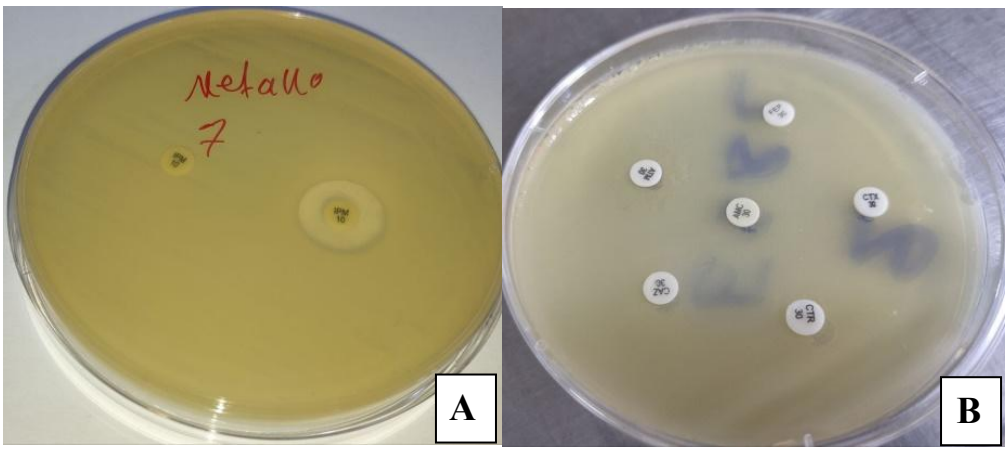

Figure 4. Phenotypic A(MBLs) and B (ESBLs) detection

Results of Biofilm production

The study reviewed the ability of 30 A. baumannii isolates to form biofilms and compared the production of biofilms between the different isolates after they were incubated in polystyrene microtiter wells. The results showed 17/30 (57%) formed strong biofilms , 9/30 (30%) formed moderate biofilms , 4/30 (13%) non biofilm producers in our observations (Figure 5 and 6).

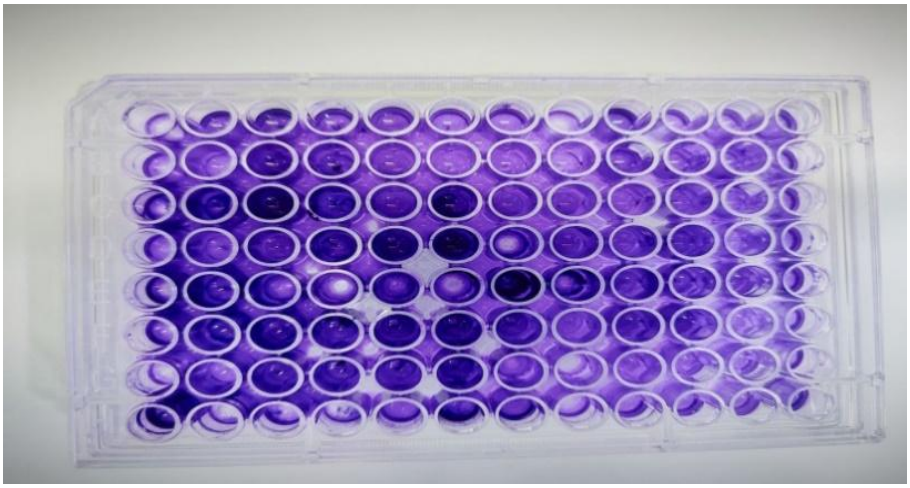

Figure 5. Polystyrene Microtiter Wells

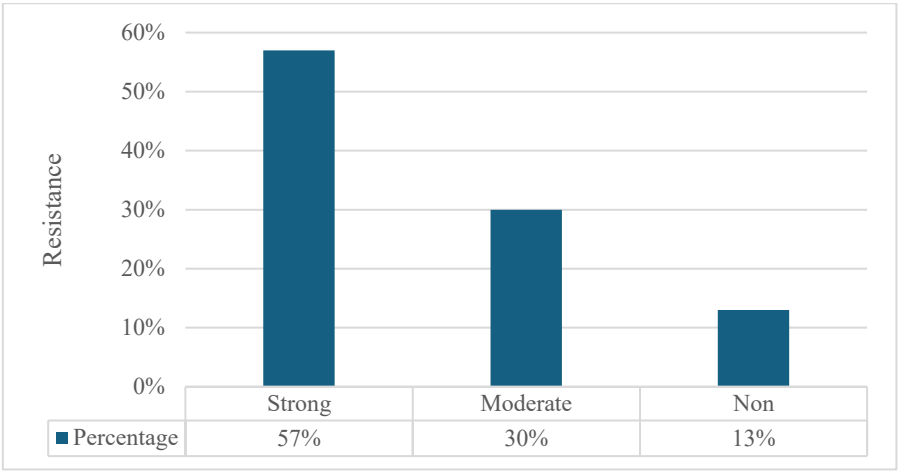

Figure 6. Biofilm Production of *A. baumanni*

The Relationship Between Antibiotic Resistance and Biofilm in A. baumannii

The results shown in table 4 showed the relation of biofilm produced by A. baumannii and antibiotic resistant as follow: Strong producer (5.9% MDR and 94.1% XDR), Moderate producer (0% MDR and 100% XDR), Non producer (25% MDR and 75% XDR) and the total was MDR (2 (6.7%)) and XDR (28 (93.3%)).

Table 4. Demonstrate the Correlation Between Biofilm Formation and Antibiotic Resistance in *A. baumannii* Isolates

Biofilm		**Antibiotic resistant**	
Types	**No.%**	**MDR No.%**	**XDR No.%**
Strong producer	17 (56.7%)	1 (5.9%)	16 (94.1%)
Moderate producer	9 (30%)	-	9 (100%)
Non producer	4 (13.3%)	1 (25%)	3 (75%)
Total	30 (100%)	2 (6.7%)	28 (93.3%)

Discussion

The results showed in table 1 shown the highest incidence infected with *A. baumannii* was among (1- 40) years old age group by 55 (36.7% and 36.6%). Several studies agree with the current results. Mohammed *et al* (21), recorded from a total of 150 patients, *A. baumannii* was among the age group of 1-45 years. For sex patients, 162 (79.4%) were male and 42(20.58%) were female. These results agree with the current results. Another study agrees with present results done by Hussein *et al* (22) who revealed that among the infected patients of age, the lowest incidence appeared in the 71-80 years age group (3.48%), while the highest incidence was observed in the 31-40 years age group (25.22%). The data indicate a higher incidence among males (66.96%) compared to females (33.04%), with statistical significance ($P<0.05$).

The study findings indicated that a higher percentage of males are affected throughout a majority of age groups, and the expected age-related variation in gender among patients infected with *A. baumannii* was absent in this cohort (23). A significant result suggests that the age of 49 years may serve as a critical barrier, with male predominance most evident in individuals aged 49 years or younger. The disparity in occurrence between women and men may be attributed to the typically higher activity levels of men, resulting in increased opportunities for exposure to environments harboring bacteria (24). Previous research indicated that Acinetobacter infections were more prevalent in males (25). This may be related to differences in immune responses between males and females. Sex differences are visible in different species. Furthermore, men were more likely to be affected by hospital-acquired infections, which may be caused by higher hospitalization rates, especially in older age groups (21).

The current results showed in Table 3 shown 30 (20%) infected with *A. baumannii*, these results agree with Abdullah and Merza (26). who pointed 41 (6.8 %) isolates of *A. baumannii* were obtained from a total of 603 clinical samples of burn swab. *Acinetobacter baumannii* is a severe, aggressive bacterial infection that causes critical nosocomial diseases, especially in burn patients (27). A rising incidence of hospital-acquired illnesses has been documented globally. However, the correlation between *A. baumannii* isolates from various hospital settings and patients has received little attention (28). The following research conducted by Radhi et al. (29) indicated that the majority of samples collected were from burns (48.1%). Ghaima (30), who reported the distribution of various clinical samples suggested that the majority were obtained from burn victims. These findings could result from *A. baumannii's* capacity to persist for prolonged durations on hospital equipment and its potential for developing treatment resistance. The variations in prevalence rates may result from changes in study design, methodology, and study time. From the figure 2 the current results high resistance of *A. baumannii* against antibiotics. Several results agree with the current results, Shenkutie *et al* (31) reported that the majority of *A. baumannii* isolates from burn swabs exhibited significant antibiotic resistance. *Acinetobacter baumannii* has emerged as a significant focus of scientific research due to its considerable antibiotic resistance. This resistance results in an elevated mortality rate, as strains resistant to antimicrobial drugs provide considerable difficulties for physicians and healthcare professionals in eradicating both hospital- and community-acquired diseases. Moreover, these developing resistance bacteria pose a significant challenge for patients in burn units (32). Antibiotic resistance has risen due to the acquisition of mobile genetic components, including transposons, plasmids, and integrons, leading to a rise in multidrug-resistant (MDR) strains (33). Carbapenem-resistant *Acinetobacter baumannii* (CR-Ab) constitutes a global issue, and the rise of carbapenem resistance is a serious concern. Carbapenems are used as a last option to treat infections caused by multidrug-resistant gram-negative bacteria. These bacteria often demonstrate resistance to all commonly used antibiotics (34). Consequently, infections caused by pathogenic multi-drug-resistant *A. baumannii* (MDR-Ab) are challenging to eliminate. Plasmid-mediated resistance results in widespread drug resistance outbreaks. *A. baumannii* is also referred to as XDR-Ab (35). Abduljabar and Mawlood (36) observe that *A. baumannii* has become a major hospital infectious agent due to the prevalence of multidrug-resistant strains, recently recognized as a significant contributor to hospital-acquired infections (HAIs), with death rates ranging from 8 to 35%, depending on the infection type and strain, along with increasing healthcare costs and prolonged hospitalizations (37).

Mortality rates have been increasing gradually due to the emergence of multidrug-resistant pathogens, especially *Acinetobacter species*. Infections correlate with elevated mortality and morbidity in burn patients (38). Despite the majority of clinical isolates exhibiting resistance to several antimicrobials, multidrug-resistant isolates have appeared because of the widespread use of broad-spectrum drugs around the world (21). Mechanisms of antimicrobial resistance refer mainly to regulation of antibiotic transportation through bacterial membranes, alteration of the antibiotic target site, and enzymatic modifications resulting in antibiotic neutralization, virulence factors that may affect antibiotic susceptibility profiles and confer drug resistance are also being discussed (39). From the figure 3, the current results revealed all A. *baumannii* isolates 100% were MBL producing while from total of 30 isolates, 100% *A. baumannii* isolates identified that not produce ESBL enzymes, these results agree with Abd El-Baky (40). Carbapenemases constitute three categories of β-lactamases. The three types include Ambler class A and D carbapenemases (which use serine) and class B carbapenemases (which rely on zinc), with the class B ones being inhibited by substances like EDTA, making them metallo-β-lactamases (MBLs) (41). Metallo-β-lactamase (MBL) enzymes can hydrolyse all β-lactam antibiotics except monobactams. Genes that encode these enzymes may be mediated by plasmids or chromosomes. The main types of MBL enzymes are grouped into families called VIM, IMP, SPM, GIM, SIM, and NDM (42). Conversely, the formation of ESBLs is regarded as a significant resistance mechanism, and their connection with MBLs

amplifies the resistance level (43). Stanimirova (44) found that out of 43 *A. baumannii* samples, 42 (97.7%) tested positive for metallo-β-lactamases (MBLs). Numerous studies support our findings (45); the observed differences in the prevalence of ESBL- and MBL-producing *A. baumannii* strains probably result from variations among the patients studied and differing rates of antibiotic use in hospitals.

The results showed in figure 6 revealed 57% of *A. baumannii* produce strong biofilm formation, the results agree with Asaad (46), who found that among all *A. baumannii* isolates examined for biofilm formation, 66 (70.1%) were classified as strong biofilm producers, whereas 28 (29.9%) were identified as non-biofilm producers. *A. baumannii*, an important emerging pathogen linked to nosocomial infections, is known for its ability to form biofilms (47). Biofilm formation enables *A. baumannii* to persist on dry surfaces, potentially prolonging its presence in hospitals and increasing the risk of hospital infections and outbreaks (44). From Table 4, Several studies have identified a correlation between biofilm formation and antibiotic resistance in *A. baumannii* isolates. The biofilm formation in MDR and XDR *A. baumannii* clinical isolates is associated with severe nosocomial infections, which has been recognized as a global health crisis (48). Monfared (49) demonstrated that ≥ 70% of *A. baumannii* isolates displayed resistance to cephalosporins, aminoglycosides, carbapenems, and fluoroquinolones, frequently used in clinical settings. Furthermore, the prevalence rates of MDR and XDR isolates in this study were 28% and 73%, respectively. The global prevalence rates of multidrug-resistant clinical isolates have been documented, ranging from 21% to 95%. Carbapenem resistance has been identified as a critical characteristic of XDR *A. baumannii*, although determining its prevalence remains challenging and not fully addressed. (50).

This study found that biofilm-producing isolates were reported to have a greater incidence of MDR and XDR and a higher rate of resistance to individual antibiotics than non-biofilm-producing isolates (46). In prior studies, it has been observed that biofilm formation is positively related to antibiotic resistance in *A. baumannii* isolates that are more resistant to aminoglycosides, carbapenems, and sulphonamides (51). The hospital environment's continuous exposure to significant selective pressure leads to *A. baumannii* developing acquired resistance to several antibiotic classes and subclasses through many mechanisms., including efflux pumps, porin expression, antibiotic target mutations, and drug-inactivating enzymes (52).

The increased drug resistance of bacteria linked to biofilms can be explained by several mechanisms, including the diminished diffusion of antibiotics into the biofilm, the presence of persistent cells that reduce growth rates, the low metabolic activity of cells located deep within the biofilm, enhanced horizontal transfer of resistance genes due to cellular proximity, and an elevated mutation rate in response to stress (53). A positive correlation has been established between biofilm formation and antimicrobial resistance in *A. baumannii*; however, a study revealed a negative relationship between biofilm development and meropenem resistance in nosocomial *A. baumannii* isolates. The creation of biofilms may affect antibiotic susceptibility and lead to clinical failure, even when the administered dosage is within the sensitive range (54).

Conclusion

Our findings revealed an elevated rate of antibiotic resistance in the clinical isolates of *A. baumannii* examined. The frequency of β-lactamase-producing isolates and their association with serious illnesses is rising internationally at a worrying rate. Phenotypically detecting MBLs and studying the resistance profiles of these isolates is essential for monitoring drug resistance in hospital settings. In *A. baumannii* isolates exhibiting greater resistance to the carbapenems, aminoglycosides, and sulfonamides, there is a link between the ability to form biofilms and antibiotic resistance.

Conflict of Interest

The authors declare that there is no competing of interests.

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