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Biofilm formation on multi, extensively, and pan-drug resistance in nosocomial ESKAPE pathogens among Iraqi patients

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ABSTRACT

The study investigates the prevalence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) profiles among ESKAPE pathogens. It examines the role of biofilm formation in the development of antimicrobial resistance. This cross-sectional study, conducted between May 2024 and March 2025, analyzed 546 clinical specimens collected from patients aged 1–85 years (mean ± SD = 36.07 ± 18.97) who were admitted to intensive care, ENT, internal medicine, gynecology, surgery, and burn units across several Iraqi hospitals, including Ramadi, Al-Fallujah, Heet, Ghazi Al-Hariri, Burns Specialist, Al-Yarmouk, and Al-Hakeem. Bacterial identification was performed using the VITEK 2 Compact system, and antimicrobial susceptibility testing was performed according to CLSI (2024). Biofilm formation was assessed qualitatively using the microtiter plate method and quantitatively using sodium alginate beads. Of the 546 isolates, 331 (60.62%) were identified as ESKAPE pathogens; among these, 51.0% were MDR, 32.9% were XDR, and 9.4% were PDR. Biofilm formation was observed in 86.9% of the isolates. Notably, *Staphylococcus aureus* showed a significant association between MDR and strong biofilm formation (OR = 6.04, p = 0.018), whereas *Klebsiella pneumoniae* exhibited a weaker correlation (OR = 0.77, p = 0.015). The microtiter plate method (86.9%) demonstrated higher sensitivity than the alginate bead assay (82.4%), particularly for *Enterobacter* spp. (p = 0.002). The findings highlight the high prevalence of MDR, XDR, and PDR ESKAPE pathogens, particularly *A. baumannii and K. pneumoniae*, in Iraqi hospitals, underscoring the need to integrate antimicrobial resistance profiling and biofilm evaluation into infection control and therapeutic management.

Keywords: *Biofilm formation, ESKAPE pathogens, Infection, Multidrug resistance*

1 INTRODUCTION

Antimicrobial resistance (AMR) poses a serious global public health challenge, threatening the effective treatment of infections caused by bacterial pathogens. Among the most concerning are the ESKAPE pathogens *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp., which are major contributors to multidrug resistance (MDR) in hospitals [1]. These bacteria "escape" the effects of available antibiotics through various mechanisms, including gene sharing, enzymatic drug degradation, modification of target sites, reduced membrane permeability, and genetic mutations that compromise antibiotic efficacy [2]. ESKAPE pathogens are a primary cause of hospital-acquired infections (HAIs) and are particularly dangerous for im-

munocompromised individuals, hospitalized patients, and those with underlying health conditions. Their prevalence is strongly linked to antibiotic misuse and overuse, which promote resistance and lead to chronic and complicated infections. Consequently, infections caused by these organisms are associated with prolonged hospital stays, increased morbidity and mortality, and substantial health care costs. The challenge is even greater in low- and middle-income countries (LMICs), where health care systems are often under-resourced, and surveillance data on drug-resistant ESKAPE pathogens remain limited [3].

A key factor in the persistence of these pathogens is their ability to form biofilms, structured microbial communities that adhere to surfaces and are embedded in a self-produced extracellular matrix of polysaccharides, proteins, and extracellular DNA [4]. Bacteria alternate between two growth phases: the planktonic phase, in which cells float freely, and the sessile phase, in which they attach and form biofilms. Biofilm development involves five stages: (i) initial adhesion to surfaces, (ii) irreversible attachment and exopolymer formation, (iii) maturation and matrix production, (iv) formation of micro- and macrocolonies, and (v) dispersal of cells to new sites [4]. Biofilm-associated infections, such as those affecting surgical wounds, burns, urinary catheters, and ventilators, represent a significant clinical problem because biofilms confer enhanced resistance to antibiotics and host immune defenses [5]. Within biofilms, antimicrobial penetration is restricted, and antibiotics primarily target planktonic cells, leaving sessile cells protected [6]. Microcolonies within biofilms exhibit much higher resistance levels than their planktonic counterparts, making infections difficult to eradicate and prone to recurrence [7]. For instance, *Pseudomonas aeruginosa* produces exopolysaccharides that support complex biofilm architecture and enhance antibiotic resistance, contributing to its role as a major nosocomial pathogen [8].

Given these challenges, addressing AMR among ESKAPE pathogens requires a multifaceted approach that includes enhanced infection control, antibiotic stewardship, and biofilm-targeted therapies. This study, therefore, aims to investigate the prevalence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) clinical isolates of ESKAPE pathogens in Iraqi hospitals. It will also assess the impact of biofilm formation on different levels of antimicrobial resistance and evaluate the associated risks. The findings will contribute to the development of effective antimicrobial stewardship policies and strategies to prevent and treat ESKAPE-related infections in Iraq.

2 MATERIALS AND METHODS

2.1 Study design and collection of samples

This cross-sectional study analyzed 546 clinical specimens collected from patients aged 1–85 years (mean ± SD = 36.07 ± 18.97). Patients were admitted to ICUs, ENT, internal medicine, gynecology, surgery, and burn units across several Iraqi hospitals, including Ramadi, Al Fallujah, Heet, Ghazi Al-Hariri, Burns Specialist, Al Yarmouk, and Al-Hakeem, between May 2024 and March 2025. Patient information, including age, sex, admission date, and antibiotic use, was recorded using a structured survey. Only the first sample from each patient was analyzed, except in cases where species differed in resistance, samples were collected more than 7 days apart, or samples originated from different sites; duplicate urine samples were excluded if corresponding blood cultures were identical. Clinical specimens included pus, urine, cerebrospinal fluid, respiratory samples (sputum, tracheal aspirates, or bronchoalveolar lavage), ear swabs, vaginal swabs, and blood samples from patients with various infections. All samples were processed within 1 h of collection. Blood cultures were incubated using the BacT/ALERT® 3D system (bioMérieux, France) for 5–21 days, while other specimens were cultured on brain heart infusion (BHI) agar (HiMedia, India) or MacConkey agar (HiMedia, India) at 37 °C for 24–48 h. Microorganisms were initially identified through Gram staining (HiMedia, India) and standard biochemical tests. Final identification was confirmed using the VITEK® 2 Compact system (bioMérieux, France) with GN/GP ID cards. Bacterial suspensions were standardized to 0.50–0.63 McFarland in 0.45% saline using the DensiCHECK Plus system (bioMérieux, France) [9].

2.2 Antimicrobial susceptibility testing

Frozen bacterial isolates stored in brain heart infusion broth with 20% glycerol (HiMedia, India) at -80 °C were thawed and streaked on blood agar plates (HiMedia, India) to obtain isolated colonies, which were incubated at 37 °C for 18–24 h. For antimicrobial susceptibility testing, 4–5 well-isolated colonies were suspended in 0.45% sterile saline (HiMedia, India) and adjusted to a 0.50-0.63 McFarland standard ($1.5 \times 10_8$ CFU/mL) using the DensiCHECK Plus system (bioMérieux, France) [10]. Gram-negative bacteria were tested using AST 419 cards (VITEK® 2, bioMérieux, France), Gram-positive bac-

teria using AST P580 cards (VITEK® 2, bioMérieux, France), and ESBL/metallo-β-lactamase production was screened using NO45 cards (VITEK® 2, bioMérieux, France). All cards were incubated in the VITEK® 2 Compact system (bioMérieux, France) at 35–37 °C, which automatically determined MICs according to CLSI 2023 guidelines and classified isolates as sensitive (S), multidrug-resistant (MDR), extensively drug-resistant (XDR), or pan-drug-resistant (PDR) [2]. Quality control was performed using ATCC reference strains: *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *A. baumannii* ATCC 19606, and *E. faecalis* ATCC 29212.

2.3 Microtiter plate assay (MPA)

Biofilm formation was assessed using sterile flat-bottomed 96-well polystyrene plates (Corning®, USA) [11]. Bacterial species were cultured on BHI agar (HiMedia, India) at 37 °C for 24 h, and 4–5 colonies were suspended in 5 mL BHI broth (HiMedia, India) to a 0.5 McFarland standard (1.5×10^8 CFU/mL). Then, 20 µL of this suspension was added to 180 µL of BHI with 5% glucose (HiMedia, India) in 96-well plates, with 200 µL of sterile broth in control wells. Plates were incubated overnight at 37 °C. Wells were washed with PBS (Sigma-Aldrich, USA), air-dried, fixed with methanol (Merck, Germany), stained with 0.1% crystal violet (Sigma-Aldrich, USA), washed, and resolubilized with 33% acetic acid (Merck, Germany). Optical density (OD) was measured at 570/630 nm using a Microplate ELISA Reader (Rodan, USA). Biofilm capacity was classified as non, weak, moderate, or strong based on OD values relative to negative controls. Each assay was performed in triplicate [12, 13].

2.4 Sodium alginate beads technique

2.4.1 Preparation of alginate beads

Sodium alginate (Sigma-Aldrich, USA), extracted from brown algae, was dissolved in deionized water at a concentration of 4 g/100 mL. Calcium chloride ($CaCl_2$; Merck, Germany) was prepared by dissolving 29.4 g in 100 mL of deionized water, yielding a 2.65 M solution. For bead formation, 200 µL of 4% sodium alginate solution was mixed with 20 µL of 2.65 M $CaCl_2$ in each well of a 96-well plate (Corning®, USA), followed by an additional 20 µL of 2.65 M $CaCl_2$. The calcium ions chelate the alginate, inducing gelation [14]. Plates were covered with Parafilm (Bemis, USA) and incubated at 60 °C for 4 h to solidify the beads. The resulting beads, shaped by the wells of the 96-well plate, could be stored at 4 °C for up to 1 week [15].

2.4.2 Alginate beads mediated biofilm formation

To perform the experiments, the 200 µL alginate beads (Sigma-Aldrich, USA) were collected in 96-well plates, washed in 48-well plates using distilled water (dH_2O), and transferred into new 48-well plates. Overnight-inoculated bacterial cultures were diluted to approximately 10^4 CFU/mL, and 600 µL of this dilution was added to each well containing the beads. Incubation was performed at 37 °C with shaking at 150 rpm to promote biofilm development. All conditions were performed in triplicate, and sterility controls were included to ensure experimental validity [16].

2.4.3 Dislodging sessile cells from beads

To determine bacterial growth on the alginate beads, each sample was transferred into a 15 mL Falcon tube containing 2 mL of dissolving buffer, prepared from a 10× stock solution containing 5.3 g Na_2CO_3 and 5.2 g citric acid per 100 mL of distilled water. Tubes were vortex-mixed for 10 s, followed by serial 10-fold dilution of the resulting suspensions (6–8 times) in BHI broth using a 96-well plate [17]. Bacterial enumeration was performed using the Miles–Misra method, whereby 20 µL aliquots of the various dilutions were plated on BHI agar and incubated overnight (up to 48 h) at 37 °C. The bacterial concentration (CFU/mL) was determined using the formula N = (C × df)/V, where C is the number of colonies (2-30 counted), df is the dilution factor, and V is the actual plated volume (0.02 mL for 20 µL aliquots).

To evaluate growth on alginate beads, the beads were immersed in 2 mL of dissolving buffer prepared from a 10× stock (5.3 g Na_2CO_3 and 5.2 g citric acid per 100 mL distilled water). The mixture was crushed, rotated, and vortexed for 10 s, then subjected to 6-8 serial 10× dilutions in BHI broth. Using the Miles-Misra method, 20 µL aliquots were plated on BHI agar and incubated at 37 °C for ≤48 h. CFU/mL was calculated as N = (C × df)/V, where C represents the average colony count (from 2–30 colonies), df is the dilution factor, and V is the actual plated volume (0.02 mL for 20 µL). Low-cost, simple, and reliable, but labor-intensive [18, 19].

2.5 Statistical analysis

Statistical analysis used the mean, percentage, frequency, and the chi-square test. The Statistical Package for the Social Sciences (SPSS), Version 25, was used to

evaluate significant associations between variables ($p < 0.05$). The study was also completed using Microsoft Excel 2021.

3 RESULTS AND DISCUSSION

A total of 546 clinical samples were analyzed, from which 331 (60.6%) ESKAPE pathogens were isolated. Of the 331 ESKAPE isolates, 11 fully antibiotic-sensitive isolates were excluded from biofilm analyses, resulting in 320 isolates tested by the microtiter plate assay. For the sodium alginate bead assay, two additional isolates failed to grow, leaving 318 isolates for evaluation of sessile biofilm formation. Of the patients included in the study, 146 (44.1%) were female, and 185 (54.8%) were male. Wound specimens were most common (190, 34.8%), followed by urine (155, 28.4%), sputum (50, 9.2%), blood (47, 8.6%), ear swabs (36, 6.6%), nasogastric tube (NGT) aspirates (20, 3.7%), high vaginal swabs (HVS) (17, 3.1%), and both cerebrospinal fluid (CSF) and urethral discharge (10, 1.8% each). ESKAPE isolates were most frequently recovered from wounds (41.1%), catheter-associated urinary tract infections (32.6%), and sputum (8.7%). Negative cultures were observed in 18.13% of samples, particularly CSF, HVS, and blood.

Among bacterial isolates from hospitalized patients in Iraq, *Acinetobacter baumannii* (20.54%) and *Klebsiella pneumoniae* (19.9%) were most prevalent, followed by *Pseudomonas aeruginosa* (16.62%) and Staphylococcus aureus(15.1%). *Enterobacter* spp. (14.2%) and *Enterococcus* spp. (13.6%) were less frequent but still contributed significantly to the hospital-acquired infection burden.

Out of the 331 ESKAPE isolates, 170 (51.4%) were multidrug-resistant (MDR), 109 (32.9%) were extensively drug-resistant (XDR), and 31 (9.4%) were pandrug-resistant (PDR). MDR isolates were predominantly *Staphylococcus* aureus (29.4%), *Enterococcus spp.* (21.8%), *Enterobacter* spp. (17.6%), *Klebsiella pneumoniae* (17.1%), *Pseudomonas aeruginosa* (10%), and *Acinetobacter baumannii* (4.1%). XDR isolates were mainly *A. baumannii* (44.9%), followed by *P. aeruginosa* (27.5%) and *K. pneumoniae* (14.7%), while PDR strains were primarily *K. pneumoniae* (61.3%), *A. baumannii* (22.6%), *P. aeruginosa* (12.9%), and *Enterobacter* spp. (3.2%). These findings are consistent with the WHO's 2017 global priority pathogen list, highlighting *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* as critical targets for the development of novel antimicrobial therapies [20]. Antibiotic resistance is defined by increased minimum inhibitory concentration (MIC) values [21]. Among 546 clinical specimens, 331 (60.6%) yielded ESKAPE pathogens, with *Acinetobacter baumannii* (20.5%) being the most common, followed by *K. pneumoniae, P. aeruginosa, Enterobacter* spp., *S. aureus*, and *Enterococcus* spp. Variations from other studies likely reflect differences in population, setting, and diagnostic criteria, emphasizing the need for surveillance and infection control [22–26].

Among 320 ESKAPE isolates, 278 (86.9%) formed biofilms by microtiter plate assay, including 94 (33.8%) Gram-positive and 184 (66.2%) Gram-negative. Forty-two isolates (13.1%) were non-biofilm producers (1 (2.4%) Gram-positive and 41 (97.6%) Gram-negative). Based on the intensity of biofilm formation, 42 isolates (15.1%) were strong producers, 131 (47.1%) were moderate, and 105 (37.8%) were weak biofilm producers. Pearson chi-square analysis ($\chi^2 = 223.953$, $p < 0.001$) indicated a strong correlation between biofilm formation and multidrug resistance.

Among the 320 isolates tested, 278 (86.9%) formed biofilms and 42 (13.1%) were non-biofilm forming. Of these isolates, 15.1% were strong, 47.1% were moderate, and 37.8% were weak biofilm-forming isolates (Table 1). A high percentage of biofilm producers was detected among *Enterococcus* spp.: 44/45 isolates (97.8%) produced biofilms, and 1 (2.2%) was a non-producer. The majority of these isolates were multidrug-resistant (MDR, n = 37), then extensively drug-resistant (XDR, n = 8). Most *Enterococcus* biofilm producers were weak (55.6%), while 31.1% were moderate and 11.1% were strong producers. The statistical analysis yielded an odds ratio (OR) of 0.95 and a p-value of 0.70, indicating no significant association between biofilm formation and antimicrobial resistance in this group. The 50 *S. aureus* isolates were all biofilm producers and multidrug-resistant (MDR); 44% were strong producers and 56% were moderate producers, indicating a high biofilm-forming potential. The odds of biofilm formation were indeed higher in MDR isolates (OR = 6.04, 95% (1.01–10.19), p = 0.018), and there was a strong relation between biofilm strength and multidrug resistance.

K. *pneumoniae* exhibited relatively lower biofilm formation, with 36 out of 64 isolates (56.2%) producing biofilms and 28 (43.8%) classified as non-producers. Among the biofilm producers, most were moderate (26.6%) or weak (23.4%), with the majority being MDR (n = 29), PDR (n = 19), and XDR (n = 16). The

odds of biofilm formation in highly resistant isolates (MDR/XDR/PDR) compared with less-resistant or sensitive isolates were lower (OR = 0.77, 95% CI: 0.45–1.33, p = 0.015), suggesting an inverse relationship between resistance level and biofilm formation in this species. A. *baumannii* also exhibited a significant capacity for biofilm formation, with 60 (95.2%) out of 63 isolates producing biofilms. The majority were classified as moderate producers (65.1%), particularly among XDR (49) isolates, with 7 isolates for each PDR and MDR. They were followed by weak producers (22.2%), strong producers (7.9%), and non-producers (4.8%). However, there was no significant difference in biofilm formation between MDR/XDR/PDR and less-resistant isolates (OR = 1.08, p = 0.31).

The biofilm production pattern of *P. aeruginosa* was comparatively balanced, with 44 out of 51 isolates (86.3%) forming biofilms at all three levels: weak (35.3%), moderate (41.2%), and strong (9.8%). While 13.7% of the isolates did not generate biofilm, the majority were XDR (n = 30), MDR (n = 17), and PDR (n = 4). Statistical analysis showed no significant association between antimicrobial resistance level (MDR/XDR/PDR) and biofilm formation (OR = 1.01, p = 0.94).

Among 47 isolates of *Enterobacter* spp., 44 (93.6%) were biofilm producers, primarily classified as weak biofilm producers (70.2%), with a very small number of strong producers (2.1%) and moderate producers (21.3%). The majority exhibited MDR (31), XDR (5), and PDR (1). The proportion of non-biofilm producers was 6.4%. The association between antimicrobial resistance (MDR/XDR/PDR) and biofilm formation was not significant (OR = 0.90, p = 0.29).

This study assessed biofilm formation of ESKAPE clinical isolates using microtiter plates (categorized as strong, moderate, weak, or non-biofilm producers) and quantified CFUs in sodium alginate beads. Biofilms, linked to antimicrobial resistance, protect bacteria from antibiotics and host defenses, promoting persistence and driving chronic and hospital-acquired infections [20]. Biofilm formation was observed in 278 of 320 ESKAPE isolates (86.9%) using the microtiter plate model, contrasting with lower rates in previous studies [3, 20]. The high proportion of biofilm-forming isolates underscores the significance of this virulence factor among multidrug-resistant pathogens. In particular, *Acinetobacter baumannii* and *Klebsiella pneumoniae* exhibited strong biofilm production, consistent with their well-documented ability to persist on medical devices and hospital surfaces. The results also showed that biofilm formation was more prevalent among MDR and XDR isolates, suggesting a strong correlation between antimicrobial resistance and biofilm-forming capacity. Similar trends have been observed in studies by [14, 15], who reported that biofilm-associated resistance often complicates infection control and treatment outcomes.

These findings highlight the importance of incorporating biofilm assessment into antimicrobial surveillance programs. The combined use of microtiter plate and sodium alginate bead models provided both qualitative and quantitative insights into biofilm behavior. Understanding biofilm dynamics among ESKAPE pathogens can inform more effective infection control measures and guide clinicians in selecting appropriate therapeutic strategies.

Biofilm growth in 318 ESKAPE isolates was compared using microtiter plates and sodium alginate beads. Microtiter plates measured biomass, whereas alginate beads quantified sessile cells (CFU/mL) in a 3D matrix. Overall, 278 isolates (86.9%) formed biofilms in microtiter plates, and 262 (82.4%) produced sessile cells in alginate. *Staphylococcus aureus* and *Enterococcus spp.* showed high biofilm formation (100% and 95.5–97.8%). *Enterobacter* spp. differed significantly (93.6% vs. 68%, p = 0.002), *Klebsiella pneumoniae* slightly increased (56.25% to 62.5%, p = 0.472), *Acinetobacter baumannii* remained high (95.2% vs. 96.8%, p = 0.731), and *Pseudomonas aeruginosa* decreased (86.3% to 74.5%, p = 0.135). Chi-square analysis confirmed method variability (χ^2 = 57.147, p < 0.001) and a strong planktonic–sessile correlation (χ^2 = 83.992, p < 0.001) (Table 2; Figure 1). In sodium alginate beads, 262/318 isolates (82.4%) produced sessile cells (χ^2 = 57.147, p < 0.001) [27]. Biofilm analysis showed 42 strong (13.1%), 131 moderate (40.9%), and 105 weak (32.9%) formers, with *A. baumannii* (56.4%) and *Enterococcus* spp. (32%) as main contributors. Biofilms protect bacteria from antibiotics and host defenses, increasing resistance 500-fold, and colonize skin, mucosa, and gut, forming complex, site-specific microbial ecosystems [28–30].

Planktonic and sessile cell percentages indicate the proportion of isolates forming cells in sodium alginate beads or microtiter plates, respectively. Figure 1 shows the number of isolates of each ESKAPE pathogen under different conditions. *Enterococcus* spp. ranged from 43 (sessile cells) to 47 (no ESKAPE isolates), Staphylococcus aureus from 36 (formed biofilm) to 50 (no ESKAPE isolates), *Klebsiella pneumoniae* from 50 (sessile cells)

Table 1 Distribution of biofilm formation using microtiter plates by ESKAPE pathogens according to AMR profiles

ESKAPE	count	AMR Profile	Total Count AMR (320) Isolates	Count of Biofilm Former 278 (86.9%)	Strong Biofilm Former 42 (15.1%)	Moderate Biofilm Former 131 (47.1%)	Weak Biofilm Former 105 (37.8%)	Non Biofilm Former 42 (13.1%)	Odds Ratio	P-value
Enterococcus spp.	45	PDR	0	0	0	0	0	0	0.95 (0.77-1.19)	0.70
		XDR	8	8	2	2	4	0		
		MDR	37	36	3	12	21	1		
		S	0	0	0	0	0	0		
		Total	45 14.1 %	44 (97.8%) (15.8%)	5 (11.1%)	14 (31.1%)	25 (55.6%)	1 (2.2%)		
Staphylococcus aureus	50	PDR	0	0	0	0	0	0	6.04 (0.01-0.19)	0.018
		XDR	0	0	0	0	0	0		
		MDR	50	50	22	28	0	0		
		S	0	0	0	0	0	0		
		Total	50 15.6 %	50 (100%) (18%)	22 (44.0%)	28 (56.0%)	0 (0.0%)	0 (0.0%)		
Gram Positive		Total	95	94 (33.8%)	27	42	25	1		
Klebsiella pneumoniae	64	PDR	19	12	4	2	6	7	0.77 (0.45-1.33)	0.015
		XDR	16	7	0	3	4	9		
		MDR	29	17	0	12	5	12		
		S	0	0	0	0	0	0		
		Total	64 20%	36 (56.25%) (13%)	4 (6.3%)	17 (26.6%)	15 (23.4%)	28 (43.8%)		
Acinetobacter baumannii	63	PDR	7	7	0	7	0	0	1.08 (0.89-1.30)	0.31
		XDR	49	46	5	28	13	3		
		MDR	7	7	0	6	1	0		
		S	0	0	0	0	0	0		
		Total	63 19.7 %	60 (95.2%) (21.6%)	5 (7.9%)	41 (65.1%)	14 (22.2%)	3 (4.8%)		
Pseudomonas aeruginosa	51	PDR	4	4	2	0	2	0	1.01 (0.75-1.34)	0.94
		XDR	30	29	2	15	12	1		
		MDR	17	11	1	6	4	6		
		S	0	0	0	0	0	0		
		Total	51 15.9 %	44 (86.3%) (15.8%)	5 (9.8%)	21 (41.2%)	18 (35.3%)	7 (13.7%)		
Enterobacter spp	47	PDR	1	1	0	0	1	0	0.90 (0.71-1.14)	0.29
		XDR	5	5	0	2	3	0		
		MDR	31	28	1	5	22	3		
		S	10	10	0	3	7	0		
		Total	47 14.7%	44 (93.6%) (15.8%)	1 (2.1%)	10 (21.3%)	33 (70.2%)	3 (6.4%)		
Gram Negative		Total	225	184 (66.2%)	15	89	80	41		
Total ESKAPE			320	278 (86.9%)	42 (13.1%)	131 (40.9%)	105 (32.9%)	42 13.1%)	**P < 0.001**	Chi-square 223.953

Statistical analysis was performed using Pearson's chi-square test to evaluate associations between antimicrobial resistance and biofilm formation. Odds ratios (OR) with 95% confidence intervals (CI) are reported. $p < 0.05$ was considered statistically significant.

to 64 (no ESKAPE isolates), *Acinetobacter baumannii* from 44 (formed biofilm) to 63 (no ESKAPE isolates),

Table 2 Detection of biofilm, microtiter plate versus sodium alginate beads

Biofilm								
ESKAPE	Total Of ESKAPE Isolates	Microtiter plates		Sodium alginate beads				
		No of ESKAPE Isolates	No of Biofilm Formers	No of ESKAPE Isolates	Sessile cells No. %	X^2	Pvalue	Planktonic cells No. %
Enterococcus spp.	45	45	44 (92,8%)	45	43 (95.5%)	0.345^2	0.557	45 (100%)
Staphylococcus aureus	50	50	50 (100%)	50	50 (100%)	NA	NA	50 (100%)
Klebsiella pneumonia	66	64	36 (56.25%)	64	40 (62.5%)	0.518^a	0.472	64 (100%)
Acinetobacter baumannii	68	63	60 (95.23%)	61	59 (96.8%)	0.118	0.731	61 (100%)
Pseudomonas aeruginosa	55	51	44 (86.3%)	51	38 (74.5 %)	2.239	0.135	51 (100%)
Enterobacter spp.	47	47	44 (93.6%)	47	32 (68.0%)	9.895	0.002**	47 (100%)
Total	331	320	278 (86.87%)	318	262 82.4%)			318 (100%)
X^2	0.027^a	83.992			57.147			
P-value	1.00^{NS}	<0.001			<0.001			

Values are presented as n (%). Statistical analyses were performed using the chi-square test to compare biofilm detection between methods. A p-value < 0.001 indicates a statistically significant overall difference between the two biofilm quantification methods.

Pseudomonas aeruginosa from 38 (sessile cells) to 51 (no ESKAPE isolates), and *Enterobacter* spp. from 32 (sessile cells) to 50 (formed biofilm). Mean ± SD across pathogens were 53.3 ± 7.3 (no ESKAPE isolates), 46.3 ± 9.0 (formed biofilm), 53.3 ± 7.0 (no ESKAPE isolates), 43.7 ± 10.0 (sessile cells), and 53.0 ± 7.0 (planktonic cells). Compared with the baseline, formed biofilm and sessile cells showed 13% and 18% decreases, respectively, while planktonic cells remained similar. These results indicate lower isolate counts in biofilm-associated conditions compared with planktonic growth. Chi-square analysis comparing biofilm formation between the microtiter plate and sodium alginate bead assays showed $\chi^2 = 57.147$, p < 0.001, indicating significant method variability. There was also a significant association between sessile and planktonic isolate categories ($\chi^2 \approx 9.79, p \approx 0.002$), indicating that pathogens with higher sessile counts were more likely to have higher planktonic counts.

All bead cultures were inoculated with 10^4 CFU per bead, and 318 ESKAPE isolates formed biofilms on sodium alginate beads. Planktonic cell counts were consistently higher than sessile cell counts across all species, and significant correlations were observed in some cases. The mean $\log_{10}$ CFU/mL for all sessile isolates was 1.55E+12 ± 1.28E+11 (SE 7.8E11; 95% CI: 1.43E11-2.96E12), whereas planktonic cells showed a mean of 1.78E+17 ± 2.72E+17 (SE 1.53E+16; 95% CI: 1.48E+17-2.08E+17), indicating greater proliferation and variability in planktonic forms. Sessile biofilm formation was highest in *Enterococcus* spp. (7.501E+12 ± 3.41E+13), followed by *S. aureus* (1.472E+12 ± 1.91E+11) and *Enterobacter* spp. (1.636E+11 ± 4.65E+11). Moderate formation occurred in *Acinetobacter baumannii* (9.032E+10 ± 2.90E+11), while *P. aeruginosa* and K. *pneumoniae* had lower values. Among planktonic cells, *S. aureus* showed the highest CFU/mL (1.0316E+18 ± 6.46E+17), and *K. pneumoniae* the lowest (1.403E+13 ± 7.60E+13), reflecting acute virulence outside biofilms, Table 3.

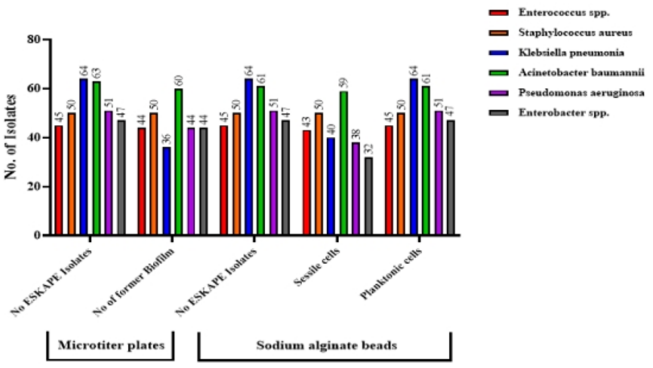

Fig. 1 Distribution of biofilm formers, in addition to planktonic and sessile cells, in the microtiter plate assay and the sodium alginate beads technique

Our findings indicate that MDR, XDR, and PDR isolates exhibit substantial biofilm formation, as reflected by CFU measurements. LSD tests at the 0.05 significance level revealed the highest counts in MDR ($2.98 \times 10^{12} \pm 1.90 \times 10^{13}$), followed by XDR ($6.43 \times 10^{10} \pm 2.43 \times 10^{10}$), PAN ($5.64 \times 10^9 \pm 2.35 \times 10^{10}$), and sensitive isolates ($1.57 \times 10^8 \pm 3.18 \times 10^8$). Although numerical differences were observed, LSD analysis showed no significant differences (p > 0.05), indicating that, although

Table 3 Comparison of bacterial concentrations (CFU/mL) in sessile versus planktonic cells of ESKAPE pathogens grown with sodium alginate beads

ESKAPE Isolates	Sessile cells					Planktonic cells				
	Mean	Standard Deviation	Std. Error	95% confidence Interval		Mean	Standard Deviation	Standard Error	95% confidence Interval	
				Lower Bound	Upper Bound				Lower Bound	Upper Bound
Enterococcus spp. (45)	7.501E+12	3.41E+13	1.901E+12	3.76E+12	1.124E+13	3.3026E+16	2.29E+17	5.444E+16	7.411E+16	1.402E+17
Staphylococcus aureus (50)	1.472E+12	1.91E+11	1.822E+12	2.112E+12	5.057E+12	1.0316E+18	6.46E + 17	3.989E+16	9.531E+17	1.11E+18
Klebsiella pneumonia (64)	3.376E+8	1.78E + 09	1.594E+12	3.136E+12	3.137E+12	1.403E + 13	7.70E+13	3.6E+16	7.084E+16	7.087E+16
Acinetobacter baumannii (61)	9.032E+10	2.90E+11	1.633E+12	3.123E+12	3.303E+12	5.239E+13	3.76E+14	5.157E+16	1.014E+17	1.015E+17
Pseudomonas aeruginosa (51)	5.21E+10	**2.85E** + 11	1.786E+12	3.462E+12	3.566E+12	6.264E + 13	**2.94E + 14**	5.444E+16	1.071E+17	1.072E+17
Enterobacter spp. (47)	1.636E+11	4.65E + 11	2.096E+12	3.962E+12	4.289E+12	5.705E + 15	7.29E+16	1.02E + 71	1.95E+17	2.064E+17
Total 318	1.55E + 12	1.28 E + 13	7.18E+11	1.43E + 11	2.96E+12	1.78E + 17	2.72E + 17	1.53 E+16	1.48E+17	2.08E+17

antimicrobial resistance may be associated with biofilm formation, within-group variability prevents definitive conclusions (Figure 2).

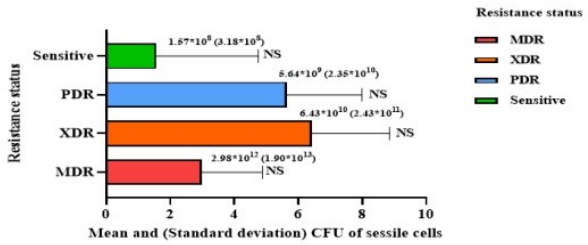

Fig. 2 Distribution of sessile cells according to multidrug, extensive and pan-drug resistant of ESKAPE isolates

Sodium alginate beads provided a reproducible in vitro model to monitor ESKAPE biofilm formation for 24–72 h. Biofilm and planktonic cell correlations were consistently observed, with differences among genera statistically assessed using the LSD test at 95% confidence. Table 4 highlights notable differences in sessile cell behavior among ESKAPE pathogens. *Enterococcus* spp. exhibited significant differences compared with Enterobacter spp. ($p = 0.004$), *Klebsiella pneumoniae* ($p = 0.003$), *Pseudomonas aeruginosa* ($p = 0.005$), and Staphylococcus aureus ($p = 0.024$), indicating a unique biofilm-forming ability. In contrast, *Acinetobacter baumannii, K. pneumoniae, P. aeruginosa,* and *S. aureus* showed no significant differences in sessile behavior ($p > 0.05$). Regarding planktonic cells, *S. aureus* differed significantly from all other genera ($p < 0.001$), whereas *A. baumannii, Enterobacter* spp., *Enterococcus spp., K. pneumoniae,* and *P. aeruginosa* displayed similar planktonic behavior ($p > 0.05$). Overall, *Enterococcus* spp. demonstrated distinct biofilm formation, while *S. aureus* was primarily distinct in its planktonic form, underscoring variability among ESKAPE pathogens with potential clinical significance.

These observations align with published evidence on biofilm formation by the *Enterococcus* genus. For example, a recent global meta-analysis reported that E. *faecalis* displays a biofilm-forming prevalence of 68.7% (95% CI: 61.3–76.0) in clinical isolates, indicating that biofilm formation is a frequent and clinically relevant trait for this genus [31]. The high biofilm potential of *Enterococcus* may be promoted by adhesins such as the Esp and EfaA proteins and by production of extracellular polymeric substances that aid surface attachment and persistence [32].

The finding that *S. aureus* shows a distinct planktonic profile in our data while lacking significantly different sessile behavior compared with other ESKAPE genera may reflect its dual lifestyle. *S. aureus* is well known as both a robust biofilm former and an efficient planktonic pathogen. The literature emphasizes that biofilm formation by the ESKAPE group is associated with increased antimicrobial resistance, community stability, and increased risk of device-associated infections [33]. In particular, biofilms confer protective advantages through their extracellular polymeric substance (EPS) matrix and are often associated with multidrug resistance mechanisms, efflux pump activation, and persister cell formation.

From a clinical relevance viewpoint, the distinct biofilm behavior of *Enterococcus* suggests that this genus may pose a particular risk in persistent, device-associated infections where sessile communities dominate. The prominent planktonic difference exhibited by *S. aureus* may indicate greater potential for systemic dissemination or acute infection phases compared with the other tested genera. Thus, in environments where planktonic growth is favored (e.g., bloodstream, soft tissue), *S. aureus* may have a relative advantage, whereas in surface-colonizing scenarios (e.g., catheter surfaces, implants), *Enterococcus* may be more troublesome for long-term infection establishment.

Table 4 Comparative statistical analysis of sessile and planktonic ESKAPE isolated from clinical samples and their biofilm formation using sodium alginate beads

Least Significant Difference (LSD)					95% Confidence Interval	
	Genus 1	**Genus 2**	**Std. Error**	**Sig.**	**Lower Bound**	**Upper Bound**
Planktonic cells (I)	*Acinetobacter* spp.	*Enterobacter* spp.	2.6827E+12	0.978$^{N.S}$	-5.35312E+12	5.20665E+12
		Enterococcus spp.	2.52992E+12	0.004***	-1.23898E+13	-2.43146E+12
		K. pneumonia	2.3037E+12	0.969$^{N.S}$	-4.44397E+12	4.62394E+12
		P. aeruginosa	2.44278E+12	0.988$^{N.S}$	-4.76947E+12	4.84591E+12
		S. aureus	2.46978E+12	0.576$^{N.S}$	-6.24296E+12	3.47871E+12
	Enterobacter spp.	*Enterococcus* spp.	2.8571E+12	0.011**	-1.29605E+13	-1.7143E+12
		K. pneumonia	2.65886E+12	0.951$^{N.S}$	-5.06973E+12	5.39618E+12
		P. aeruginosa	2.78023E+12	0.968$^{N.S}$	-5.36037E+12	5.58329E+12
		S. aureus	2.80399E+12	0.641$^{N.S}$	-6.82747E+12	4.2097E+12
	Enterococcus spp.	*K. pneumonia*	2.50462E+12	0.003***	2.57123E+12	1.243E+13
		P. aeruginosa	2.63311E+12	0.005***	2.26659E+12	1.26312E+13
		S. aureus	2.65818E+12	0.024**	7.96895E+11	1.12602E+13
	K. pneumonia	*P. aeruginosa*	2.41657E+12	0.983$^{N.S}$	-4.80786E+12	4.70434E+12
		S. aureus	2.44386E+12	0.547$^{N.S}$	-6.28193E+12	3.3377E+12
	P. aeruginosa	*S. aureus*	2.57539E+12	0.582$^{N.S}$	-6.48902E+12	3.64832E+12
Planktonic cells (II)	*Acinetobacter* spp.	*Enterobacter* spp.	5.71578E+16	0.810$^{N.S}$	-1.26239E+17	9.87173E+16
		Enterococcus spp.	5.38619E+16	0.341$^{N.S}$	-1.57376E+17	5.46085E+16
		K. pneumonia	4.89765E+16	0.998$^{N.S}$	-9.62755E+16	9.64815E+16
		P. aeruginosa	5.19809E+16	0.999$^{N.S}$	-1.02238E+17	1.02344E+17
		S. aureus	5.22673E+16	0.000***	-1.14374E+18	-9.3803E+17
	Enterobacter spp.	*Enterococcus* spp.	6.12416E+16	0.539$^{N.S}$	-1.58137E+17	8.28912E+16
		K. pneumonia	5.69923E+16	0.808$^{N.S}$	-9.82887E+16	1.26016E+17
		P. aeruginosa	5.95939E+16	0.817$^{N.S}$	-1.03458E+17	1.31086E+17
		S. aureus	5.98439E+16	0.000***	-1.14489E+18	-9.0936E+17
	Enterococcus spp.	*K. pneumonia*	5.36863E+16	0.338$^{N.S}$	-5.41599E+16	1.57133E+17
		P. aeruginosa	5.64405E+16	0.363$^{N.S}$	-5.96297E+16	1.62503E+17
		S. aureus	5.67044E+16	0.000***	-1.10109E+18	-8.77915E+17
	K. pneumonia	*P. aeruginosa*	5.17989E+16	0.999$^{N.S}$	-1.01982E+17	1.01882E+17
		S. aureus	5.20863E+16	0.000***	-1.14349E+18	-9.38489E+17
	P. aeruginosa	*S. aureus*	5.49208E+16	0.000***	-1.14901E+18	-9.32862E+17

Most ESKAPE species showed similar biofilm formation; however, *Enterobacter* spp. decreased from 93.6% in microtiter plates to 68.0% in the alginate model, reflecting assay-dependent differences, with 3D beads better representing live sessile cells. *Klebsiella pneumoniae* formed biofilms in 56.3% of microtiter and 62.5% of alginate isolates ($p = 0.472$), indicating a slight, non-significant increase under host-like conditions. *Acinetobacter baumannii* exhibited high biofilm rates: 95.2% by microtiter and 96.8% by alginate ($p = 0.731$), aligning with previous studies while contrasting others [34–38]. *A. baumannii* strongly forms biofilms on abiotic and tissue-like surfaces, causing persistent infections [39].

For *Pseudomonas aeruginosa*, biofilm formation was 86.3% via microtiter plate and 74.5% with the sodium alginate model ($p = 0.135$). The lower alginate rate reflects better in vivo simulation, consistent with Sonderholm et al., but lower than reports of 100% or 94.2% with microtiter plates [40, 41]. The alginate-based model effectively simulates the heterogeneous nutrient and oxygen gradients encountered in natural biofilms, demonstrating growth under hypoxic conditions, enhanced antimicrobial resistance, and the critical involvement of flagella in biofilm development and structural integrity [42–44]. Biofilm formation was independent of MDR, XDR, PDR, or sensitive profiles, highlighting the multifactorial nature of resistance and the need for targeted antibiofilm strategies [45–47]. This study demonstrated that ESKAPE pathogens generally form more robust sessile biofilms than their planktonic counterparts, except for *Enterococcus* spp., whereas S. aureus showed higher antimicrobial resistance. These findings underscore the heterogeneity of ESKAPE pathogens in their response to treatment. *Enterococcus* spp. showed distinct biofilm formation, mediated by adhesins, exopolysaccharides, and quorum-sensing mechanisms, consistent with observations reported by Lazăr et al. [48, 49].

Enterococcus spp. exhibited a distinct biofilm formation pattern compared with *Enterobacter* spp., *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*, driven by Esp/Ace proteins, exopolysaccharide-mediated ribosome inactivation, and Fsr quorum-sensing signaling, whereas the other ESKAPE species displayed more similar phenotypes. The study used in vitro models but was limited by assay constraints and the absence of comprehensive molecular and clinical data.

4 CONCLUSION

The present research highlights the high prevalence of ESKAPE pathogens, particularly *A. baumannii* and *K. pneumoniae*. Effective management requires combining antimicrobial resistance profiling with biofilm evaluation, as biofilm formation differs significantly among MDR, XDR, and PDR strains.

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Data availability

N/A

DECLARATIONS

Conflict of interest

Authors declare no conflict of interest.

Consent to publish

N/A

Ethical approval

Written informed consent was obtained, and the study received ethical approval from the University of Anbar (No. 61, April 17, 2024) in accordance with the Helsinki Declaration.

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