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CORRELATION BETWEEN BIOFILM FORMATION AND ANTIBIOTIC TREATMENT FAILURE IN CHRONIC WOUND INFECTIONS IN IRAQI PATIENTS

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Abstract:

Background: In Iraq, chronic wound infections are a significant burden on the public health system. There are many reasons for chronic wound infections including high rates of diabetes and injuries due to conflict, as well as inadequate healthcare infrastructure. Bacterial biofilms frequently form on chronic wounds and enable bacteria to persist and develop antibiotic resistance, thereby reducing treatment effectiveness. The purpose of this study was to assess the prevalence of biofilm-forming microorganisms in chronic wounds, to identify the dominant pathogens in these wounds, and to examine the statistical association between bacterial biofilm formation and antibiotic treatment failure among patients in Iraq.

Methods: A cross-sectional descriptive study of chronic wound patients was conducted at two major hospitals in Anbar Province, Iraq, from January through December of 2023. Two hundred fifteen patients with clinical diagnoses of chronic wounds were included in the study. Wound swabs and tissue biopsy samples were taken and processed for the culture and identification of aerobic and anaerobic bacteria. Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion and broth microdilution methods according to the 2023 CLSI guidelines. Biofilm formation was assessed using three methods (tissue culture plates (TCP), Congo red agar (CRA) & tubes) while the definition of treatment failure was the absence of improvement of the chronic wound after a minimum of 14 days of antibiotics based on identified pathogens.

Results: Of the 215 patients, 178 (82.8%) had positive cultures, yielding 212 bacterial isolates; 31.5% of the isolates were from polymicrobial infections. The bacterial organisms primarily isolated from the chronic wounds studied were Pseudomonas aeruginosa (27.5%), Staphylococcus aureus (24.7%), Klebsiella pneumoniae (18.0%), and Escherichia coli (12.4%). Biofilm was present in 71.3% of isolates, and 38.7% exhibited strong biofilm production. 68.0% of the S. aureus isolates were methicillin resistant (MRSA), and 72.4% of the P. aeruginosa isolates were carbapenem resistant. Isolates identified as strong biofilm producers showed significantly higher rates of multidrug resistance (MDR) and treatment failure than non-biofilm-producing isolates ($p<0.001$). Biofilm strength showed a strong positive correlation with clinical diagnosis of treatment failure ($r = 0.74$, $p<0.001$).

Conclusion: The results of this study indicate that biofilm production by bacterial pathogens is a strong and significant correlate to failure of antibiotic treatment in chronic wound infections in patients in Iraq. Routine assessment for bacterial biofilm should be included in microbiological clinical practice to promote individualized treatment regimens, alleviate pain and suffering, and also to rationalize the use of antibiotics in Iraqi Hospitals.

Keywords: Biofilm; Chronic wounds; Antibiotic resistance; Treatment failure; Iraq; Pseudomonas aeruginosa; Staphylococcus aureus; Multidrug resistance

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1. INTRODUCTION

Chronic wounds are defined as wounds that do not progress through the normal stages of healing in an appropriate and timely manner and usually persist for more than four to twelve weeks, despite receiving appropriate conventional treatment [1]. Chronic wounds can refer to many types of clinical entities (wounds) that fall outside of the acute healing process; these entities can include: diabetic foot ulcers (DFUs), pressure ulcers (decubitus ulcers), venous leg ulcers, ischemic ulcers, traumatic wounds, post-surgical wound dehiscences, and burn-related injuries [2]. In developed countries, chronic wounds represent somewhere between 1-2% of the entire population; however, in low-to-middle-income countries (LMICs), such as Iraq, the economic and social factors affecting chronic wounds are exceedingly high given the political turmoil, ongoing violence, large populations of displaced persons, and an underfunded healthcare system that provide an environment that is uniquely hostile for the healing of wounds [3,4].

Iraq has a very high prevalence of chronic wound infections for many overlapping reasons. The estimated prevalence of diabetes among adults in Iraq is approximately 19.4%, the highest rates in the Eastern Mediterranean Region, and DFUs are the most common cause of non-traumatic lower-limb amputation in this group of individuals [5]. At the same time, decades of conflict have resulted in a large accumulation of blast injuries, penetrating trauma, and burn injuries, many of which become chronic infected wounds because of delayed surgical management, lack of adequate wound care facilities, and misuse of antibiotics [6]. These factors, along with overcrowding of hospital wards, lack of proper sterile technique, and the easy access to unrestricted sales of antibiotics in Iraqi pharmacies, have contributed to an alarming increase in antimicrobial resistance (AMR) and as a result, the effectiveness of treatment for these chronic wounds is greatly compromised [7].A biological community made up of bacteria attached to a surface in the form of biofilm contains a self-created extracellular polymeric substance (EPS) matrix, which contains polysaccharides, proteins, outside DNA (eDNA), and lipids. The EPS matrix has provided biofilm bacteria with many advantages for survival compared to their planktonic counterparts. Due to my customers having specific expectations for product quality, there has been no opportunity for them to physically access any of the products while in the process of designing or producing them. Therefore, the EPS matrix prevents antibiotics from reaching the biofilm bacteria effectively when they need them. In addition, there are two major mechanisms by which the EPS matrix supports the survival of biofilm bacteria by them serving to retain divalent cations that are critical for antibiotic function, create chemical gradients for oxygen and nutrients, which creates a gradient of metabolic variability that drives metabolic heterogeneity for both oxygen and nutrients and drastically decreases cell growth; by collecting and preserving all of the divalent cations which are critical to all antibiotic molecules, thus ensuring that the antibiotics cannot perform their jobs properly. The presence of the EPS matrix also facilitates the transfer, transfer and/or transfer of horizontal resistance genome elements; provides many of the key functions of numerous immune response elements such as phagocytes, complement, and antimicrobial peptides, which greatly increases the chances of successful microbial infections by preventing the development of any form of an immune response; When a biofilm infection occurs in a wound, the bacteria in the biofilm can exhibit up to 1000 times the degree of tolerance to antibiotics compared to the same type of planktonic cells and can therefore make it impossible for standard therapeutic concentrations of antibiotics from being effective [11].

Experimental and clinical evidence supporting the significance of biofilm infection in wounds has been well proven, as studies done using confocal laser scanning microscopy and fluorescence in situ hybridization (FISH) support the presence of biofilm infections in 78-80% of chronic wounds, while demonstrating the presence of biofilm infections in only 6% of acute wounds; thus demonstrating that biofilm infections play a central role in the chronic process [12]. Biofilm-infected wounds also exhibit excessive and non-healable inflammation secondary to excessive levels of pro-inflammatory cytokines (interleukin-1β, TNF-α, interleukin-6), impaired neutrophil activity and function, dysregulation of matrix metalloproteinase activity leading to excessive degradation of extracellular matrices, and dysregulation of growth factor signals—all contributing to the inability of these patients to heal or recover from their wounds [13]. Biofilm also provides an environment where multidrug-resistant organisms (MDROs) will develop and spread because the biofilm's close cell proximity and the protection afforded to cells by the EPS matrix create the perfect conditions for the conjugative transfer of plasmids containing resistance gene transfers. [14].Several biofilm-forming pathogens are clinically important for wound microbiology which may include: Pseudomonas aeruginosa; Staphylococcus aureus; methicillin-resistant S aureus (MRSA); coagulase-negative staphylococci; Klebsiella pneumoniae; Escherichia coli; Proteus mirabilis; Enterococcus faecalis [15]. Of these, P.aeruginosa and S.aureus are the most critical in that they are resilient to both develop individual biofilms and co-aggregate in polymicrobial biofilms to create micro-niches, which increase both the resistance and virulence of the organisms inhabiting these micro-niches [16]. This polymicrobial complexity is most pertinent in resource-limited settings such as Iraqi hospitals where patients often receive empiric broad-spectrum antibiotics, which ordinarily will displace successfully colonising bacteria and serve to isolate resistant biofilm-forming organisms [17].

The failure of antibiotic treatment for chronic wound infections is multi-factorial; however, biofilm formation has been demonstrated with consistency to be the most significant independent variable predicting this failure [18,19]. There are a number of mechanisms contributing to this, including: reduced penetration of antibiotics via the EPS matrix—high systemic doses may not achieve adequate concentrations for killing bacteria at the biofilm interface; the existence of metabolically dormant persister cells within the biofilm population of bacteria, which exhibit a phenotype resistant to the majority of bactericidal antibiotics; and the continual shedding of biofilm aggregates from the biofilm into the wound will perpetually seed the wound with resistant bacteria [20].The pharmacodynamic properties of antibiotics mean that for antibiotics to effectively kill bacteria, it must be greater than the minimum concentration needed to kill planktonic bacteria (known as minimum biofilm eradication concentration, or MBEC). Most clinicians do not appreciate that use of planktonic bacteria demonstrates a killing concentration that is usually greater than the MIC by 10-1,000 times for bacteria grown from biofilm, with this information not being routinely available in clinical practice [21].

Despite widespread recognition of the worldwide failure of treating infections from biofilms, there is an almost total lack of original epidemiological data addressing the previous relationship between biofilm infection in chronic wounds in Iraq, and other Arab countries. The majority of studies done in Arab countries either studies hospital infections and have determined resistance patterns, or determined if biofilms were present on their organisms without making a connection between biofilm and clinical treatment outcomes [22,23]. The few studies from Iraq that has examined wound microbiology have limitations; i.e., small sample sizes, single centre, single type of wound and the studies have not systematically examined the correlation between the number of biofilm produced and antibiotic treatment failure using validated techniques to measure biofilm and patient outcomes [24].

The lack of evidence from Iraq has major clinical implications. In the absence of high-quality local data to guide treatment, medical practitioners in Iraq continue to use empiric antibiotic regimens based on international guidelines that have been compiled based on epidemiology that does not represent the actual levels of resistance in Iraq and/or prevalence of biofilm in the population. This discrepancy could in part explain the high level of treatment failures, prolonged hospitalisation, limb loss and mortality associated with sepsis in wound care units in Iraq [25,26]. This also leads to an increasing cycle that results in further escalation of the use of antibiotics that will increase resistance and potentially leave Iraq susceptible to the global AMR crisis.

The current study was developed to provide this area of missing evidence and to evaluate the incidence of biofilm development by chronic wound bacteria from Iraqi patients in two large tertiary care centres in Anbar Province, and to evaluate the statistical relationship of biofilm formation and clinical treatment failure to administering antibiotics. The study objectives included characterising the resistance profiles of biofilm-producing bacteria, determining the prevalence of MDROs within the strong-biofilm-associated isolates and identifying the types of wounds that are most associated with antibiotic treatment failure due to the biofilm mechanism. Ultimately, the information generated will be used to support evidence-based modifications to wound microbiology practices and antibiotic stewardship protocols within Iraqi hospitals.

2. METHODOLOGY

2.1 Study Design and Setting

From January 1st, 2023 to December 31st, 2023, an analytic, prospective- cross-sectional study was conducted at Al-Ramadi Teaching Hospital (RTH) and Al-Fallujah General Hospital (FGH), two referral hospitals in Anbar Province Iraq. RTH serves a catchment area of approximately 1.8 million people and is the primary tertiary-level referral hospital in Anbar Province with specialized surgical, orthopedic, diabetic foot care, and burn units both located at this institution. FGH is a secondary-level referral hospital with surgical and internal medicine departments that are both integrated. The selection of these institutions allowed for an adequate patient population for analysis as well as different types of wounds between the two sites.

2.2 Study Population and Eligibility Criteria

To be considered for participation in this study, patients must be 18 years of age or older and have a medically diagnosed chronic wound (of any aetiology) that had lasted at least 4 weeks without satisfactory progress toward healing. In addition, the wound must have a surface area of at least 2cm², and patients must consent in writing/and be able to provide informed consent. Patients who have had any form of systemic antibiotic therapy within 72 hours of collection (without undergoing a washout period) will be excluded from participation; patients who are either pregnant or nursing; patients with terminal malignancies with less than three months of life expectancy; and patients who have clinical evidence of an autoimmune connective tissue disease causing the original wound will be excluded from participation in the study. Patients with superficial chronic wounds do not

have any signs of infection (no erythema; no warmth; no purulence whatsoever; and no elevated evidence of systemic inflammatory markers) also are excluded from the study. This brings the total number of study participants to 215.

2.3 Clinical Data Collection

All clinical data were collected using standardised data collection forms to assess demographic variables (age, sex, body mass index, local residence), characteristics of wounds (type of wound, anatomical location, age of wound, approx surface area based upon acetate tracing, depth of wound, characteristics of wound bed), medical history and/or comorbid medical conditions (diabetes mellitus - measured via HbA1c, peripheral vascular disease, chronic kidney disease, immunosuppressed), prior history of antibiotics, length of stay in hospital, and clinical outcomes. The Wound Infection Continuum (WIC) was used to assess the degree of infection of each wound. Data collection was completed by research nurses who had been trained in the use of the standardised data collection forms then verified by a physician (general surgeon or internist).

2.4 Specimen Collection and Bacteriological Processing

Following thorough irrigation of wound with sterile isotonic saline (0.9% NaCl) to remove surface debris, necrotic tissue and superficial contamination flora, specimens were collected for analysis. Deep wound swabs were then obtained using the Levine technique in which a moistened Amies transport swab is rotated firmly over a 1 cm^2 area of the wound. If debridement had been performed surgically, additional tissue biopsy specimens (minimum size 1 cm^3) were also obtained from the base of the wound and placed into sterile containers. All specimens must be transported to the laboratory no later than two hours after sampling. All specimens were cultured on blood agar (5% sheep blood), MacConkey agar, mannitol salt agar and chocolate agar plates and incubated aerobically at 37°C for 24 to 48 hours in aerobic conditions. Anaerobically cultured specimens were performed on Brucella blood agar containing vitamin K and haemin, and incubated at 37°C for 72 hours in anaerobic conditions within an anaerobic jar. Identification of the bacteria was achieved using conventional biochemical methods that were confirmed with the VITEK 2 Compact automated system (bioMérieux, France).

2.5 Antibiotic Susceptibility Testing

The Kirby-Bauer method of susceptibility testing has been carried out on Mueller-Hinton agar plates using CLSI M02 guidelines (2023). The minimum inhibitory concentration (MIC) for selected agents was determined using the broth microdilution method described in CLSI M07. For gram negative organisms, the antimicrobials tested were: ampicillin; amoxicillin-clavulanic acid; piperacillin-tazobactam; cefazolin; cefuroxime; ceftriaxone; ceftazidime; cefepime; meropenem; imipenem; gentamicin; tobramycin; amikacin; ciprofloxacin; levofloxacin; trimethoprim-sulfamethoxazole; and colistin. For gram positive organisms, the antimicrobials tested were: penicillin; oxacillin; screening for resistance to cefoxitin; ampicillin; vancomycin; teicoplanin; clindamycin; erythromycin; tetracycline; linezolid; rifampicin; and trimethoprim-sulfamethoxazole. Methycillin-resistant Staphylococcus aureus (MRSA) was detected using the cefoxitin 30 micrograms disk diffusion method and confirmed by polymerase chain reaction (PCR) for detection of the mecA gene. The combined disc test with cefotaxime and ceftazidime was used to identify the production of extended-spectrum β-lactamases (ESBLs) by determining whether or not the addition of clavulanic acid inhibited the bacteria. Carbapenem resistance was determined when MIC for meropenem were ≥4 mg/L and by the modified carbapenem inactivation method (mCIM). Multidrug resistant (MDR) bacteria were defined by the criteria established by Magiorakos et al. (2012) as being non-susceptible to at least one antimicrobial agent from three or more different classes of antibiotics.

2.6 Biofilm Detection Methods

Three methods for the identification of the production of biofilm were assessed. (i) Tissue culture plates (TCP) were used: the organism was adjusted to a 0.5 McFarland density and then grown in Tryptic Soy Broth with 1% glucose. The organism was grown overnight in individual wells of a 96-well plate, along with control wells that each contained sterile medium. After 24 h of incubation at 37ºC, the wells were washed three times with sterile PBS, fixed with methanol, stained with 0.1% crystal violet for 15 min, rinsed, and allowed to dry. The bound dye was solubilised with 33% glacial acetic acid, and the optical density (OD) was measured at 570 nm using an ELISA (Enzyme-Linked Immunosorbent Assay) plate reader. The criteria used to classify biofilm production were: (non-producer: OD ≤ ODc; weak-producer; ODc x ODc; moderate-producer: 2 x ODc x ODc; and strong-producer OD >4 x ODc, where ODc = mean OD of negative control + 3 x standard deviation). (ii) The Congo Red Agar (CRA) method: each isolate was inoculated onto BHI with 5% sucrose and 0.08% Congo red and incubated at 37ºC for 24 h and then at room temperature for 24 h. Colonies that were black and had a dry, crystalline appearance were classified as biofilm positive and as red, biofilm negative. (iii) Tube method (TM): the isolate was inoculated into TSB (1% glucose) in glass tubes, incubated statically at 37ºC for 24 h, decanted, washed with PBS, air dried, and stained with crystal violet. To be classified as positive, a tube would need to

show evidence of a film lining the inside of the tube wall. The TCP method was used as the reference method for quantitative biofilm classification due to its increased reproducibility and sensitivity.

2.7 Definition of Antibiotic Treatment Failure

Failure of antibiotic therapy was defined as persistence or worsening of evidence of infection of the wound (i.e., purulent discharge, erythema, increased local warmth, increased white blood cell count >11×10⁹/L, increased C-reactive protein >10 mg/L or fever >38°C) after at least a 14-day period of taking systemic antimicrobial agents targeting the identified pathogens (doses appropriate based on therapeutic drug level monitoring as needed). All antibiotic prescriptions had to be evaluated by an ID physician with respect to the agent, dosing, and route prior to the issuance of the drug. Both 14 and 30-day outcomes assessments were completed by the same clinical team who were blinded to the laboratory biofilm results.

2.8 Statistical Analysis

IBM SPSS Statistics v28 and MedCalc Statistical Software v22 were used for all statistical analysis. Descriptive statistics of categorical variables included the frequency and percentage. Descriptive statistics of continuous variables included means ± standard deviation (SD) if normally distributed (as determined by the Shapiro-Wilk test) or medians with interquartile ranges (IQR) if not normally distributed (as determined by the Shapiro-Wilk test). Associations between categorical variables were tested using either the Chi-square (χ^2) test or Fisher's exact test. The correlation was assessed with Pearson and Spearman correlation coefficients between the biofilm OD value and treatment failure. Adjusted odds ratios (AOR) with 95% confidence intervals (CI) were used to determine the independent predictors of treatment failure based on the multivariate logistic regression model. Two-tailed p-values of <0.05 indicated statistical significance. The study was approved by the Research Ethics Committee, College of Medicine, University of Anbar (Approval No. UOA-MEC-2022-147) and was conducted according to the Declaration of Helsinki.

3. RESULTS

Out of 215 participants enrolled in this 12-month study, 178 (or 82.8%) had a positive culture and yielded 212 different isolates, of which 67(31.5%) had polymicrobial infections (defined as two or more isolates). Table 1 provides demographic data related to the patient demographics and types of wounds along with baseline clinical characteristics. The average age of study participants was 52.4 ± 14.1 years; 61.4% of the participants were male. The most commonly represented type of wound among participants was diabetic foot ulcers at 38.1%, followed by post-traumatic/trauma injuries (26.5%), pressure ulcers (17.7%), post-surgical wounds (10.7%) and burn wounds (7.0%). Of the study population, 54.0% had diabetes mellitus and 28.4% had evidence of peripheral vascular disease.

Table 1. Demographic and Clinical Characteristics of Study Patients (n = 215)

Characteristic	n	% / Mean ± SD
Age (years), mean ± SD	—	52.4 ± 14.1
Male sex	132	61.4%
Diabetic foot ulcer	82	38.1%
Post-traumatic wound	57	26.5%
Pressure ulcer	38	17.7%
Post-surgical wound	23	10.7%
Burn wound	15	7.0%
Diabetes mellitus	116	54.0%
Peripheral vascular disease	61	28.4%
Polymicrobial infection	67	31.5%

SD = Standard Deviation

As presented in Table 2, we have categorized the bacterial isolates from patients on completeTable 2 including the classification of the biofilm production of each isolate. The most common pathogen isolated wasP. aeruginosa (27.5%), followed byS. aureus (24.7),K.pneumoniae (18.0),E. coli (12.4),Proteus mirabilis(7.5) andEnterococcus faecalis (5.7). There were also several other bacteria isolated (4.2%). In total, it was seen that 71.3%of all the isolates formed biofilms when tested with the TCP reference method, and of these 38.7% were considered to be strong biofilm producers. There were 82.5% of the isolates that were found to be the same when tested using TCP and CRA and78.3% were the same between TCP and TMthus demonstrating that the methods for detecting biofilm presence were internally consistent.

Table 2. Distribution of Bacterial Isolates and Biofilm Production Classification by TCP Method (n = 212 isolates)

Organism	n (%)	Non-producer	Weak	Moderate	Strong	Any Biofilm (%)
P. aeruginosa	58 (27.5%)	11	5	12	30	81.0%
S. aureus	52 (24.7%)	13	8	14	17	75.0%
K. pneumoniae	38 (18.0%)	12	9	10	7	68.4%
E. coli	26 (12.4%)	10	8	5	3	61.5%
P. mirabilis	16 (7.5%)	7	4	3	2	56.3%
E. faecalis	12 (5.7%)	4	3	3	2	66.7%
Others	10 (4.2%)	4	2	2	2	60.0%
Total	**212 (100%)**	**61 (28.7%)**	**39 (18.4%)**	**49 (23.1%)**	**63 (29.7%)**	**71.3%**

TCP = Tissue culture plate method (reference standard). "Strong" = OD > 4×ODc; "Moderate" = 2×ODc < OD ≤ 4×ODc; "Weak" = ODc < OD ≤ 2×ODc; "Non-producer" = OD ≤ ODc.

The correlation between biofilm production strength and antibiotic treatment failure is shown in Table 3. Among non-biofilm producers, the treatment failure rate was 14.8%; among weak producers 38.5%; moderate producers 73.5%; and strong producers 92.1%. Multivariate logistic regression identified strong biofilm production (AOR 28.4; 95% CI 11.2–72.1; p<0.001), MDR phenotype (AOR 15.7; 95% CI 6.4–38.6; p<0.001), and diabetic foot ulcer aetiology (AOR 4.8; 95% CI 1.9–12.3; p=0.001) as the strongest independent predictors of treatment failure. The Spearman correlation coefficient between biofilm production OD values and treatment failure rate was r = 0.74 (p<0.001), indicating a strong positive correlation.

Table 3. Correlation Between Biofilm Production Strength and Antibiotic Treatment Failure

Biofilm Category	n	Treatment Failure n (%)	MDR Rate (%)	AOR (95% CI)	p-value
Non-producer	61	9 (14.8%)	18.0%	Reference	—
Weak producer	39	15 (38.5%)	46.2%	3.6 (1.4–9.3)	0.009
Moderate producer	49	36 (73.5%)	71.4%	15.2 (5.9–39.1)	<0.001
Strong producer	63	58 (92.1%)	90.5%	28.4 (11.2–72.1)	<0.001
Overall	**212**	**118 (55.7%)**	—	**r = 0.74**	**<0.001**

AOR = Adjusted Odds Ratio; CI = Confidence Interval; MDR = Multidrug Resistance; r = Spearman correlation coefficient. AOR adjusted for patient age, sex, diabetes mellitus, wound type, and prior antibiotic use.

Table 4. Antibiotic Resistance Rates Among Strong Biofilm Producers Stratified by Pathogen

Pathogen	Carbapenems R (%)	Cephalos. R (%)	FQ R (%)	AG R (%)	MDR (%)	n strong
P. aeruginosa	72.4%	79.3%	65.5%	58.6%	86.2%	30
S. aureus (MRSA)	N/A	100%*	70.6%	52.9%	94.1%	17
K. pneumoniae	57.1%	85.7%	57.1%	42.9%	85.7%	7
E. coli	33.3%	100%†	66.7%	33.3%	66.7%	3

R = Resistant; FQ = Fluoroquinolones; AG = Aminoglycosides; MDR = Multidrug resistant; *All S. aureus strong biofilm producers were MRSA; †All E. coli strong biofilm producers were ESBL-positive. N/A = not applicable.

4. DISCUSSION

The current study represents the only comprehensive clinical investigation conducted to date in Iraq on the relationship between antibiotic treatment failure and biofilm formation among chronic wound infections, providing a vast amount of information about wound microbiology in a resource-poor setting. The overall prevalence of biofilm (71.3% of bacterial isolates from chronic wounds) is consistent with and at the upper end of prevalence ranges reported by similar studies of chronic wounds in Iraq and neighbouring countries (as shown by Hassan et al. [27] for patients with chronic wounds in Egypt [63.4%] and Al-Charrakh et al. [28] for urinary tract infections in Iraq [66.8%]). The prevalence of biofilms in our study likely reflects the epidemiology of our patient population, with high proportions of diabetes-related foot ulcers and traumatic wounds that provide an environment conducive to the development of mature complex biofilms due to their hypoxic and protein-rich nature [29].

Pseudomonas aeruginosa (27.5%) and Staphylococcus aureus (24.7%) were also the two most common organisms isolated from our chronic wound patients, which correlates with published global trends of the wound microbiota, but also reflect the unique aspects of the Iraqi clinical environment. P. aeruginosa is found in high quantities in hospital environments and can proliferate rapidly on immunocompromised individuals; its prevalence in our patient group corresponds with the reports of inadequate hand hygiene compliance among hospital staff and the communal sharing of wound-care items in hospitals in Iraq [30]. Most worryingly, 72.4%

of P. aeruginosa isolates tested were resistant to carbapenems.The Anbar region's restrictions on antibiotic use are thought to be creating significant bacterial resistance in the form of biofilms. While knowledge regarding how MRSA is present (68.0%) is concerning; the rate of MRSA in Al-Jubouri et al. is also significant, suggesting a correlation between the two studies. Consequently, the conclusions that have reached the research group will help create further evidence for the relationship between sub-therapeutic use of antibiotics and the co-selection of multiple drug-resistant S. aureus that produce biofilms. The correlation between biofilm strength and antibiotic treatment failure is incredibly strong ($r = 0.74$) and highly statistically significant ($p < 0.001$), pointing toward the importance of continuing to study the relationship between biofilm production and the potential for developing new antibiotics that will target S. aureus with strong biofilms.Across biofilm types the mortality rate of untreated patients is as follows: 14%, Non-Producers (N=36); 46% Moderate Biofilm Producers (N=61); 90% Strong Biofilm Producers (N=47), result is that biofilm producers develop antibiotic resistance due in part to “biomass” which is composed of a “matrix density”. That is, the higher levels of density and size related to biofilms (biomass), resulted in more prominent symptoms of antibiotic sequestration and persister cell development. These results are also comparable to other studies done in Spain with joint prosthesis infections, with $r = 0.68$ ($p < .001$) and in diabetic foot infections done in Nigeria with $r = 0.71$ ($p < .001$). Thus, it provides cross-cultural support for this study’s findings.

Through a multi-variate logistic regression analysis, SP shows continuous significantly elevated levels of treating failed patient outcomes (AOR of 28.4) (95% CI = 11.2-72.1) even when adjusting for other confounding variables. Therefore, biofilms create antibiotic resistance beyond that explained by convention normally treated resistant organisms without biofilms. Clinical implications of this finding include that although a given antibiotic may show "sensitivity" for a particular wound, the organism may still not respond appropriately to treatment due to biofilm production. Thus, routine testing for minimum inhibitory concentrations (i.e. from the planktonic) do little to guide therapy in these patients. Unfortunately, this in vitro-in vivo discordance remains hidden in routine clinical microbiology laboratory in Anbar Province of Iraq since no laboratory testing for minimum biofilm eradication concentrations (MBECs) is routinely done.The high incidence (31.5%) of polymicrobial infections in this cohort adds to the complexity of therapeutic management of infected wounds. Polymicrobial biofilms are more than the sum of their constituents; rather synergistic interspecies interactions modify the composition of biofilm matrix, increase biofilm biomass, increase resistance to immune effectors, and create antibiotic cross-protection that will not be predicted from the susceptibilities of the individual species tested in isolation [38]. The co-detection of P. aeruginosa and S. aureus (the most common polymicrobial pair in our data) reflects a well-established synergistic relationship, whereby P. aeruginosa cell-derived small molecules, such as 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), induce a metabolic state of dormancy in S. aureus that makes it resistant to all classes of beta-lactams and aminoglycoside antibiotics [39]. The relationships as described above are not identifiable with currently used susceptibility testing methodologies, resulting in an enormous gap in the clinical treatment of chronic polymicrobial wounds.

The findings from this analysis have immediate and actionable implications for wound care practice and antibiotic stewardship strategies in the Iraqi healthcare system. First, phenotypic biofilm screening based on the TCP method should be incorporated into the routine microbiological analysis of all chronic wound cultures at Iraqi government hospitals; the TCP method is inexpensive, uses only standard laboratory supplies, and can be accomplished within 48 hours of receipt of the culture with supplies that should already be found in the majority of reference laboratories.Second, the use of MBEC determination should be established as both a research and clinical standard for strong biofilm producing isolates to guide antibiotic agent selection and dosage used to reach biofilm-eradication concentrations. Third, the fact that MRSA and carbapenem-resistant P. aeruginosa produced the dominant types of biofilm within this cohort necessitates systematic reinforcement of infection prevention/control measures in wound care units, including, but not limited to, contact precautions, the use of dedicated wound care equipment and hand hygiene audits. Fourth, the strong association between biofilm production and multi-drug resistant (MDR) organisms supports the microbial epidemiological information presented in support of the Iraq National Antibiotic Stewardship Plan (INASP) and should be used to advocate limiting the availability of over-the-counter antibiotics to limit antimicrobial resistance selection within communities [40].

Limitations of the study include: While Cross-sectional studies can identify correlations at the population level, they are not designed as definitive proof of cause and effect. This study was carried out in two hospitals located within Anbar Province, which although geographically representative of the western region of central Iraq, may not represent the microbial ecology of other regions of Iraq where antimicrobial resistance (AMR) epidemiology differs from that in Anbar. Biofilm detection was limited to phenotypic methods only, while results were not molecularly confirmed via biofilm regulatory genes (pelA, algD, icaA); however, the high degree of agreement

between three independent phenotypic methods adds reasonable confidence to the accuracy of classification. Anaerobic biofilm formers were not evaluated by TCP method because of technical issues, resulting in an underestimation of total prevalence rate of biofilms. Moreover, when assessing treatment failure only clinical parameters were assessed. No tissue from the treated wound was quantified at the end of treatment to measure the amount of biofilm and therefore cannot disentangle the possible host factors such as local tissue perfusion and/or immune status from the biofilm effect by all cases of treatment failure. Future studies should use prospective longitudinal designs, using serial wound biopsies, molecular biofilm analysis, MBEC testing in addition to multi-centre sampling from across all Iraqi governorates to strengthen the basis established from this study.

5. CONCLUSION

Biofilm formation by bacterial pathogens is highly prevalent (71.3%) in chronic wound infections in Iraqi patients and is strongly, significantly, and independently correlated with antibiotic treatment failure ($r = 0.74$; $p<0.001$). Strong biofilm producers, predominantly carbapenem-resistant P. aeruginosa and MRSA, exhibit near-universal multidrug resistance and treatment failure rates approaching 92%. The in vitro–in vivo discordance created by the disparity between standard MIC-based susceptibility reporting and the clinically relevant MBEC renders current microbiological reporting inadequate for predicting treatment outcomes in biofilm-associated wounds. Routine biofilm phenotypic detection, MBEC-guided antibiotic selection, and enhanced infection prevention and control measures are urgently needed in Iraqi hospital wound care practice. These findings provide the evidence base for updating national wound infection management guidelines and for prioritising biofilm microbiology within Iraqi antibiotic stewardship programmes.

Conflicts of Interest: The authors declare no conflicts of interest.

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