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Biofilm-associated Multidrug Resistant Bacteria Among Burn Wound Infections: A Cross-sectional Study

Biyofilm İlişkili Çoklu İlaç Direnci Olan Bakterilerin Yanık Yarası Enfeksiyonlarındaki Yeri: Kesitsel Bir Çalışma

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Abstract

Introduction: Biofilm consists of an organized colony of bacterial cells that adhere to a self-produced polymeric matrix. These biofilms contribute to the pathophysiology and clinical manifestations of many illnesses, frequently leading to treatment failure. Microorganisms that produce biofilm exhibit increased resistance to antimicrobial agents compared to nonbiofilm-producing microbes. This study aimed to determine the proportion of biofilm-producing aerobic bacteria in burn wound microbiota and ascertain the percentage of multidrug-resistant (MDR) bacteria among burn wound infection-causing bacteria.

Materials and Methods: This cross-sectional study was conducted at the Department of Microbiology at the Maharishi Markandeshwar Institute of Medical Science and Research Mullana, Ambala, Haryana. A total of 50 burn wound swab samples were obtained from the patients along with their detailed clinical history. The swab samples were subjected to bacterial identification employing standard microbiological methods. An antimicrobial susceptibility test was conducted for all bacterial isolates using the disk diffusion method of the modified Kirby-Bauer technique. This technique was employed using Mueller-Hinton agar plates and commercially available antimicrobial disks. The tube adherence method and the modified Congo red agar method were employed to identify biofilm-forming bacteria.

Results: Of the isolates that were obtained, 56.8% were biofilm-forming bacteria. A total of 48% of the biofilm-producing bacteria were MDR. A marked resistance was noted for frequently used antibiotics like quinolones, cephalosporins, and cotrimoxazole. *Staphylococcus aureus* isolates were resistant to ofloxacin, penicillin G, and amikacin; *Klebsiella* spp. isolates were highly resistant to ampicillin, ceftazidime, trimethoprim sulfamethoxazole, tetracycline, and chloramphenicol; *Pseudomonas aeruginosa* isolates were highly resistant to trimethoprim sulfamethoxazole; *Acinetobacter* spp. isolates were resistant to cefotaxime, ceftriaxone, cefixime and trimethoprim sulfamethoxazole. For Gram-positive bacteria, *Staphylococcus aureus* exhibited 100% susceptibility to linezolid, vancomycin, and netilmycin while coagulase-negative *Staphylococci* isolates were sensitive to all antibiotics. *Klebsiella, Proteus, Pseudomonas aeruginosa*, and *Acinetobacter* spp. exhibited the highest sensitivity to carbapenem antibiotics among Gram-negative bacteria.

Conclusion: The study highlights the rising prevalence of MDR bacteria in burn wound infections and the necesssity of effective infection control measures and treatment methods to combat these biofilm-forming MDR bacteria.

Keywords: Biofilm, multidrug-resistant bacteria (MDR), antimicrobial resistance (AMR), bacterial infection

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Öz

Giriş: Biyofilm, kendi ürettiği polimerik bir matrise yapışan organize bir bakteri hücresi kolonisinden oluşur. Bu biyofilmler birçok hastalığın patofizyolojisinde ve klinik belirtilerinde rol oynar ve sıklıkla tedavi başarısızlığına yol açar. Biyofilm üreten mikroorganizmalar, biyofilm üretmeyen mikroplara kıyasla antimikrobiyal ajanlara karşı daha fazla direnç gösterir. Bu çalışma, yanık yarası mikrobiyotasında biyofilm üreten aerobik bakterilerin oranını belirlemeyi ve yanık yarası enfeksiyonuna neden olan bakteriler arasında çoklu ilaca dirençli bakterilerin yüzdesini tespit etmeyi amaçlamaktadır.

Gereç ve Yöntem: Bu kesitsel çalışma, Maharishi Markandeshwar Tıbbi Bilim ve Araştırma Enstitüsü Mullana, Ambala, Haryana'daki Mikrobiyoloji Bölümü'nde yürütülmüştür. Hastalardan ayrıntılı klinik özgeçmişleriyle birlikte toplam 50 yanık yarası sürüntü örneği alındı. Sürüntü örnekleri standart mikrobiyolojik yöntemler kullanılarak bakteri tanımlamasına tabi tutuldu. Tüm bakteri izolatları için modifiye edilmiş Kirby-Bauer tekniğinin disk difüzyon yöntemi kullanılarak antimikrobiyal duyarlılık testi yapıldı. Bu teknik Mueller-Hinton agar plakaları ve ticari olarak temin edilebilen antimikrobiyal diskler kullanılarak uygulandı. Tüp Yapışma yöntemi ve modifiye edilmiş Kongo kırmızısı agar yöntemi biyofilm oluşturan bakterileri tanımlamak için kullanıldı.

Bulgular: Elde edilen izolatların %56,8'i biyofilm oluşturan bakterilerdi. Biyofilm üreten bakterilerin toplam %48'i çoklu ilaca dirençliydi. Kinolonlar, sefalosporinler ve kotrimoksazol gibi sık kullanılan antibiyotiklere karşı belirgin bir direnç tespit edildi. *Staphylococcus aureus* izolatları ofloksasin, penisilin G ve amikasine dirençliydi; *Klebsiella* spp. izolatları ampisilin, seftazidim, trimetoprim sülfametoksazol, tetrasiklin ve kloramfenikole karşı oldukça dirençliydi; *Pseudomonas aeruginosa* izolatları trimetoprim sülfametoksazole karşı oldukça dirençliydi; *Acinetobacter* spp. izolatları sefotaksim, seftriakson, sefiksim ve trimetoprim sülfametoksazole karşı dirençliydi. Gram pozitif bakterilerden *Staphylococcus aureus* linezolid, vankomisine ve netilmisine %100 duyarlılık gösterirken koagülaz negatif *Staphylococci* izolatları tüm antibiyotiklere duyarlıydı. Gram negatif bakterilerden *Klebsiella, Proteus, Pseudomonas aeruginosa* ve *Acinetobacter* spp. karbapenem antibiyotiklerine karşı en yüksek duyarlılığı gösterdi.

Sonuç: Bu çalışma, yanık yarası enfeksiyonlarında çoklu ilaca dirençli bakterilerin artan yaygınlığını ve bu biyofilm oluşturan çoklu ilaca dirençli bakterilerle mücadele için etkili enfeksiyon kontrol önlemlerinin ve tedavi yöntemlerinin gerekliliğini vurgulamaktadır.

Anahtar Kelimeler: Biyofilm, çoklu ilaca dirençli bakteriler (MDR), antimikrobiyal direnç (AMR), bakteriyel enfeksiyon

Introduction

Biofilm is composed of an organized colony of bacterial cells that attach themselves to a self-produced polymeric matrix. Compared to microbes that do not generate biofilm, those that can produce it exhibit a high level of resistance to antimicrobial agents[1]. According to the National Institute of Health, microbial films are responsible for nosocomial infections, and approximately 65% and 80% of all microbial infections and chronic illnesses, respectively[2]. Biofilms are implicated in causing burn wound infections. One study discovered that 90% of burn wound samples exhibited positive bacterial cultures, with 46.6% of the isolates developing biofilms. The most commonly isolated bacterium related to biofilm development included *Pseudomonas aeruginosa*, *Klebsiella species, Proteus species,* and methicillin-resistant *Staphylococcus aureus* (MRSA)[3-5]. Biofilms play a crucial role in pathophysiology and clinical manifestation of several illnesses, facilitating the establishment of multidrug resistant organisms (MDRO) and treatment failure. These biofilms function as a barrier, obstructing the penetration of antimicrobial agents and host immune system defenses[3]. Burn wounds have been classified as one of the most severe types of injuries in the past decade. Annually, 11 million individuals require treatment, and 300,000 individuals succumb to burns worldwide[6]. Over 75% of burn injury fatalities are caused by bacterial infection. Burn patients experience loss of their natural barrier (skin) and protracted hospital stays and therapeutic procedures, making them susceptible to diverse infections[7]. Burn wound infections, particularly in low- and middle-income countries, result in high morbidity and mortality rates and continue to pose a challenge in most hospitals[8]. Biofilm-producing bacteria cause these infections and exihibit high resistance to antimicrobial treatments due to their biofilm-forming nature[9]. Reports suggest that these biofilms are a significant contributor to chronic inflammatory diseases[10] by enhancing the pathogen's capacity to elude both host defenses and antibiotics. The emergence of MDRO strains has further complicated treatment options, resulting in treatment failures and adverse clinical outcomes, despite the efforts being made to manage burn wound infections[11]. This study aimed to determine the proportion of biofilm-producing aerobic bacteria in burn wound microbiota and ascertain the percentage of multidrug-resistant (MDR) bacteria among burn wound infection-causing bacteria. The study findings regarding the prevalence of MDRO and their association with biofilm production may help gain insights into the challenges encountered in the clinical management of burn wounds and the significance of infection control measures.

Materials and Methods

This cross-sectional study was conducted at the Department of Microbiology at the Maharishi Markandeshwar Institute of Medical Science and Research Mullana, Ambala, Haryana. It included 50 swabs collected from burn patients admitted to the hospital along with comprehensive documentation of the patients' clinical histories.

Sample Collection

Burn wound swabs were obtained from each patient aseptically and stored in a sterile test tube containing normal saline. The samples were subsequently transported in a sterile container to the laboratory for culturing on 5% blood agar and MacConkey agar, which were then incubated overnight at 37 °C aerobically for 24 h (Figure 1).

Laboratory Analysis of Swabs

The standard microbiological protocol was followed to initially identify the various colonies by analyzing their colony morphology and culture characteristics. The bacterial colonies on the blood and MacConkey agars were subjected to Gram-stain. Subsequently, biochemical reactions and VITEK® 2 Compact Automated Systems were implemented to identify the bacteria species that were present.

Staphylococcus aureus

The beta-hemolytic microbe *Staphylococcus aureus* was isolated from blood agar after an overnight incubation. The colonies were surrounded by distinct beta-hemolysis zones. The identification was corroborated through microscopic examination, which revealed Gram-positive cocci organized in clusters when observed following Gram staining. A catalase test was performed to differentiate between *Staphylococcus*, which is catalase positive, and *Streptococcus*, which is catalase negative. A coagulase test was also conducted to distinguish *Staphylococcus aureus* (coagulase-positive) from other *Staphylococcus* species (coagulase-negative *Staphylococcus*-CoNS).

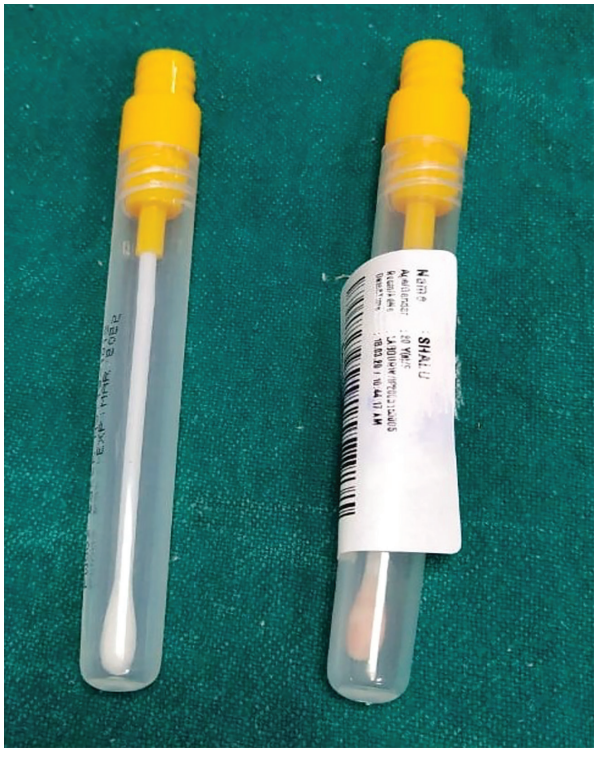

Figure 1. Swabs sticks for obtaining pus samples

Pseudomonas aeruginosa

Pseudomonas aeruginosa was isolated from MacConkey agar medium that was incubated overnight. The identification was verified through the distinctive appearance of the colonies, which were round, flat, and colorless, indicating that the organism was a lactose non-fermenter. Additionally, the organism was identified microscopically as Gram-negative bacilli after being stained with Gram-stain. Furthermore, the bacteria was identified as oxidase-positive on performing oxidase test.

Klebsiella spp.

Klebsiella spp. was isolated from MacConkey agar medium with growth appearing as mucoid and pink in color due to lactose fermentation. Gram staining showed that isolates were Gram-negative, encapsulated, and rod-shaped bacteria. A lactose fermentation test was performed, in which the bacteria was positive.

Acinetobacter spp.

Acinetobacter spp. was isolated from MacConkey agar medium, with its colonies appearing small, translucent, and shiny. The colonies were subjected to the Gram staining technique and were microscopically characterized as Gram-negative bacilli. A lactose fermentation test was conducted, yielding a negative result for the bacteria.

Proteus spp.

Proteus spp. was isolated from MacConkey agar medium, with the colonies appearing smooth and colorless (no swarming growth). Gram staining demonstrated that the colonies were composed of Gram-negative rod-shaped bacteria. The lactose fermentation test revealed that the bacteria was a negative lactose fermenter.

Antimicrobial susceptibility test: The antimicrobial susceptibility test was conducted on all bacterial isolates using Mueller-Hinton agar plates and commercially available antimicrobial disks, utilizing the disk diffusion procedure of the modified Kirby-Bauer technique. The Clinical and Laboratory Standards Institute (CLSI) M02 document was used to conduct the procedure in accordance with the Performance Standards for Antimicrobial Disk Susceptibility Tests[12]. The Mueller-Hinton agar was prepared by emulsifying the starch in a small quantity of cold water and subsequently poured into beef infusion and casein hydrolysate. This was followed by the addition of agar. The volume was increased up to one liter using distilled water. The constituents were dissolved by heating gently at 100 °C with agitation. The mixture was then filtered, and the pH was

adjusted to 7.4. The mixture was dispensed into screw-capped bottles and sterilized by autoclaving at 121 °C for 20 minutes. The Mueller-Hinton agar plates were labeled based on the different bacteria isolated. A direct broth suspension was prepared using 3-5 isolated colonies from an 18-24 hour nonselective agar plate with a turbidity equivalent to a 0.5 McFarland standard. The inoculum was applied to the agar within 15 minutes by inserting a sterile cotton swab into the suspension and streaking the entire agar surface in three overlapping streaks, rotating the plate 60 degrees each time. The antimicrobial disks were subsequently deposited aseptically on the inoculated agar, ensuring that they were evenly distributed and that there was sufficient spacing to prevent overlapping zones of inhibition. The plates were then inverted and incubated at 35 °C for 16-18 hours. The inhibition zones were measured using a ruler above a black background and interpreted in accordance with CLSI M100 breakpoint tables[12]. The CLSI M100 document provides breakpoint tables that classify bacterial isolates as susceptible, intermediate, or resistant based on the diameter of the inhibition zones. The breakpoint tables delineate the zone diameter range for each category of antimicrobial agents.

Identification of multidrug resistant strain: MDR strains were identified by their resistance to at least one antibiotic agent in three or more antimicrobial categories[13].

Detection of biofilm-forming bacteria: The tube adherence method and Congo red agar method[14,15] were employed for detecting the biofilm. The modified Congo red agar method was used to inoculate isolates on a specially prepared solid medium, Blood Agar Base-2, which was supplemented with glucose and Congo red. The Congo red was prepared as a concentrated aqueous solution and autoclaved at 121 °C for 15 minutes separately from other medium constituents. The agar was subsequently added at a temperature of 55 °C. This medium was incubated aerobically at 37 °C for 24-48 hours. Isolates were then inoculated onto the modified Congo red agar.

To carry out the tube adherence method, the isolates were inoculated onto brain heart infusion broth containing 2% sucrose in a glass tube and incubated at 37 °C for 24 hours. The supernatant was decanted after 24 hours, and the sediments were washed with phosphate buffered saline (pH 7.3) and desiccated. The dried tubes were stained with 0.1% crystal violet. The excess stain was removed, and the tubes were rinsed three times with distilled water. The tubes were then dried in an inverted position, and biofilm formation was observed. Biofilm formation was verified by the observation of a visible film ascending the wall and bottom of the tube.

Statistical Analysis

The data were statistically analyzed and the results are presented in the form of tables, graphs, percentages, and tests of significance.

Results

Culture positive: Out of the 50 samples, 82% were culture positive for bacteria, while 18% exhibited no growth (Figure 2).

Gram-negative and Gram-positive bacteria isolates: Among the Gram-positive cocci, 11.4% were *Staphylococcus aureus* and 2.3% were CoNS. *Pseudomonas aeruginosa* comprised 45.5% of the Gram-negative bacteria, *Acinetobacter* spp. comprised 34.1%, *Klebsiella* spp. comprised 4.5%, and *Proteus* spp. comprised 2.3% (Figures 3, 4).

Antibiotic susceptibility of Gram-positive and Gram-negative bacteria:

S. aureus exhibited 100% susceptibility to linezolid, vancomycin, and netilmicin while demonstrating strong resistance to ofloxacin, penicillin, cefoxitin, ampicillin, and amikacin.

CoNS isolates were susceptible to all antibiotics. The most sensitive Gram-negative bacteria to carbapenems were *Klebsiella, Proteus, Pseudomonas aeruginosa,* and *Acinetobacter* spp. The *Proteus* spp. isolate was sensitive to

Figure 2. Colony morphology of the different bacteria isolated from burn wounds. (A): Growth of *Pseudomonas aeruginosa* on MacConkey agar with colonies appearing round, flat, and colorless, indicating that the organism is a lactose non-fermenter. (B): Growth of *Staphylococcus aureus* on blood agar with colonies surrounded by clear beta-hemolysis zones. (C): Growth of coagulase-negative *Staphylococcus* on blood agar. (D): Growth of *Klebsiella* spp. (right) with the colonies appearing as mucoid and pink in color due to lactose fermentation and *Acinetobacter* spp. (left) colonies appearing as small, translucent, and shiny

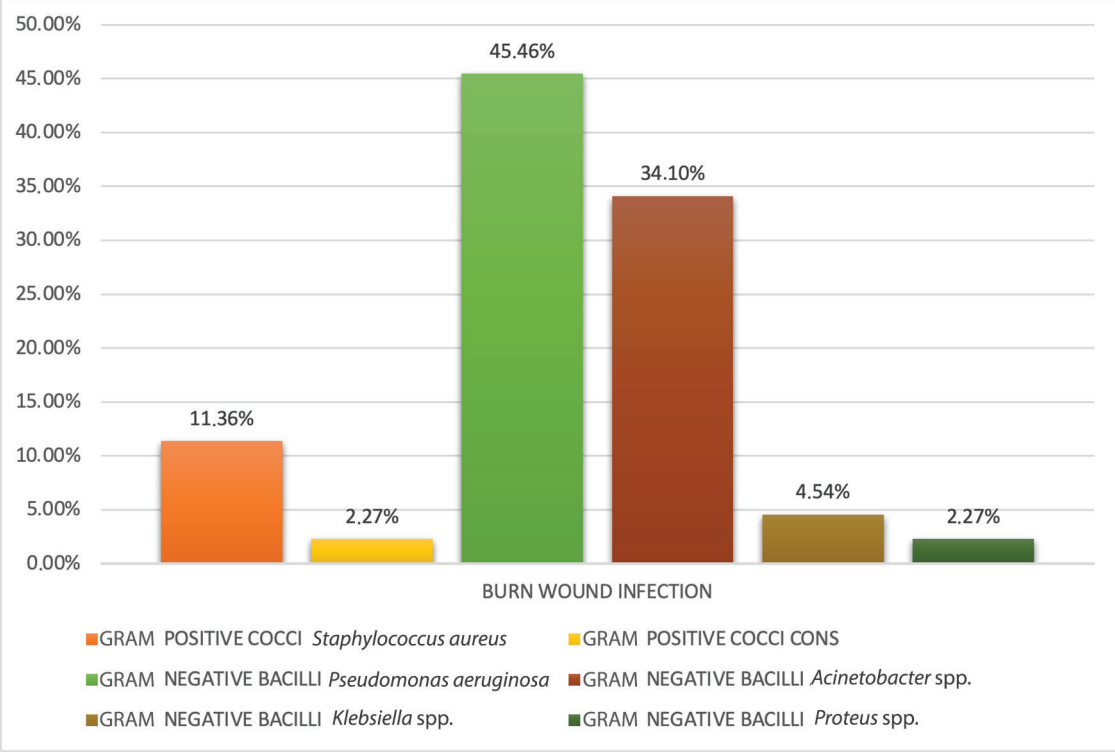

Figure 3. Distribution of Gram-positive and Gram-negative bacterial isolates in burn wound pus samples

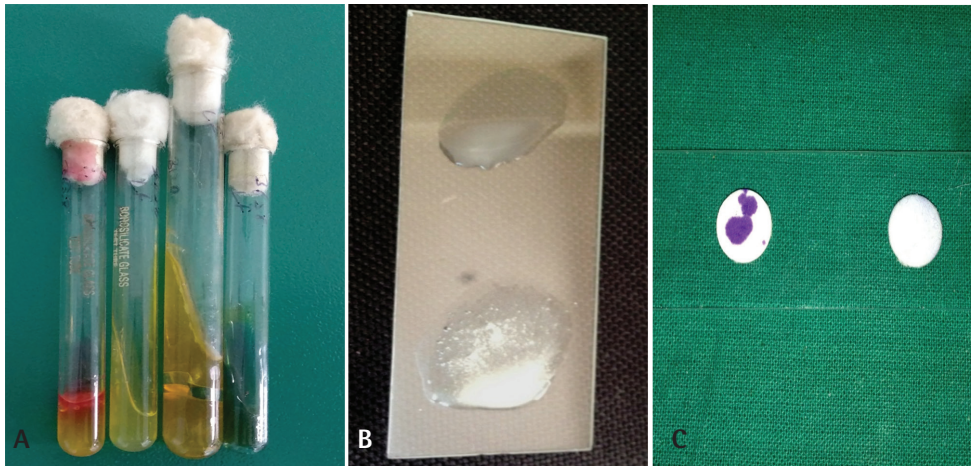

Figure 4. Biochemical testing for bacteria. (A): Biochemical test for lactose fermenter. (B): Coagulase-positive test for *Staphylococcus aureus*. (C): Oxidase-positive test for *Pseudomonas species*

all the antibiotics. *Klebsiella, Pseudomonas aeruginosa,* and *Acinetobacter* isolates were highly resistant to trimethoprim sulfamethoxazole, ampicillin, and ceftazidime. The *Klebsiella* isolates were completely resistant to ampicillin, trimethoprim sulfamethoxazole, tetracycline, chloramphenicol, and ceftazidime. The *Pseudomonas aeruginosa* isolates were highly resistant to cephalosporins, ampicillin, amoxicillin clavulanate, trimethoprim sulfamethoxazole, chloramphenicol, gentamicin, and netilmicin. *Acinetobacter* isolates were exceedingly resistant to tetracyclines, sulfonamides, cephalosporins, penicillin, and quinolones classes of antibiotics (Table 1).

The frequency of MRSA was 60%. Among the Gram-negative bacteria, 45% of *Pseudomonas aeruginosa*, 46% of *Acinetobacter* spp., and 50% of *Klebsiella* spp. were MDR.

Detection of Biofilm Producers

The biofilm detection method identifed 25 (56.81%) biofilm-producing bacteria and 19 (43.81%) non-biofilm-producing bacteria (Table 2, Figure 5).

Statistics on multidrug resistant strains: In terms of the distribution of MDR strains, three out of the five MRSA strains,

nine out of twenty *Pseudomonas aeruginosa* strains, seven out of fifteen *Acinetobacter* spp. strains, and one out of two *Klebsiella* spp. strains were MDR (Table 3).

Discussion

Gram-negative isolates comprised 86.37% of the isolates in our study, while Gram-positive isolates comprised 13.63%. Among the Gram-negative isolates, *Pseudomonas aeruginosa* (45.46%) was the most prevalent, followed by *Acinetobacter* spp. (34.10%), *Klebsiella* spp. (4.54%) and *Proteus* spp. (2.27%). For the 44 bacterial isolates, the biofilm detection rate was 56.81%. *Staphylococcus aureus* comprised the majority of Gram-positive isolates (11.36%). A similar study by Ramakrishnan et al.[16] revealed that *P. aeruginosa* (33.3%) was the most common burn wound isolate, followed by *Acinetobacter* (23.3%) and *Staphylococcus aureus* (16.6%). The frequent occurrence of *Pseudomonas aeruginosa* in our study can be attributed to its ability to thrive in moist environments[17]. It also grows in common antiseptics due to its inherent resistance to them[18]. Because of its resistance to treatment, *Acinetobacter* has emerged as a significant nosocomial pathogen, in part due to its existence as a component of the normal skin flora, ease

Table 1. Antibiotic sensitivity pattern among Gram-positive and Gram-negative bacteria in pus samples from burn wounds

Antibiotics	Gram-positive bacteria		Gram-negative bacteria			
	Coagulase-negative *Staphylococci* (1 isolate)	*Staphylococcus aureus* (5 isolates)	*Klebsiella* spp. (2 isolates)	*Proteus* spp. (1 isolate)	*Pseudomonas aeruginosa* (20 isolates)	*Acinetobacter* spp. (15 isolates)
Ciprofloxacin (CIP)	1 (100)	4 (80)	1 (50)	1 (100)	12 (60)	1 (6.66)
Levofloxacin (LE)	1 (100)	3 (60)	1 (50)	1 (100)	12 (60)	2 (13.33)
Ofloxacin (OF)	1 (100)	0 (00)	-	-	-	-
Ampicillin (AMP)	1 (100)	1 (20)	0 (00)	1 (100)	5 (25)	1 (6.66)
Penicillin G (P)	1 (100)	0 (00)	-	-	-	-
Erythromycin (E)	1 (100)	3 (60)	-	-	-	-
Azithromycin (AZM)	1 (100)	3 (60)	-	-	-	-
Clindamycin (CD)	1 (100)	3 (60)	-	-	-	-
Linezolid (LZ)	1 (100)	5 (100)	-	-	-	-
Vancomycin (VA)	1 (100)	5 (100)	-	-	-	-
Piperacillin tazobactam (PIT)	-	-	2 (100)	1 (100)	11 (55)	9 (60)
Cefoxitin (CX)	1 (100)	2 (40)	-	-	-	-
Amoxycillin clavulanic acid (AMC)	1 (100)	2 (40)	1 (50)	1 (100)	9 (45)	6 (40)
Cefepime (CPM)	-	-	2 (100)	1 (100)	3 (15)	3 (20)
Cefotaxime (CTX)	-	-	1 (50)	1 (100)	7 (35)	0 (00)
Ceftazidime (CAZ)	-	-	0 (00)	1 (100)	9 (45)	1 (6.66)
Ceftriaxone (CTR)	-	-	1 (50)	1 (100)	7 (35)	0 (00)
Cefixime (CFM)	-	-	2 (100)	1 (100)	-	0 (00)
Imipenem (IMP)	-	-	2 (100)	1 (100)	19 (100)	13 (86.66)
Meropenem (MRP)	-	-	2 (100)	1 (100)	17 (85)	13 (86.66)
Trimethoprim-sulfamethoxazole (TMP-SMX)	1 (100)	3 (60)	0 (00)	1 (100)	0 (00)	0 (00)
Tetracycline (TE)	-	-	0 (00)	1 (100)	-	4 (26.66)
Doxycycline (DO)	-	-	-	-	-	6 (40)
Minocycline (MI)	-	-	-	-	-	5 (33.33)
Chloramphenicol (C)	1 (100)	3 (60)	0 (00)	1 (100)	6 (30)	10 (66.66)
Gentamicin (GEN)	1 (100)	3 (60)	1 (50)	1 (100)	9 (45)	10 (66.66)
Amikacin (AK)	1 (100)	0 (00)	2 (100)	-	16 (80)	10 (66.66)
Netilmicin (NET)	1 (100)	5 (100)	1 (50)	-	6 (30)	-
Tobramycin (TOB)	-	-	2 (100)	1 (100)	12 (60)	8 (53.33)

of transmission, and ability to survive in adverse hospital environments. *Acinetobacter* specifically inhabits aquatic environments[19].

Staphylococcus aureus was the prevalent pathogen, exhibiting a 100% sensitivity to linezolid, vancomycin, and netilmicin. It was highly resistant to ofloxacin, penicillin, cefoxitin, ampicillin, and amikacin. Similarly, Chaudhary et al.[20] and E Abou Warda et al.[21] found that *Staphylococcus aureus* isolates were susceptible to vancomycin and linezolid. El Hamzaoui et al.[22] also discovered that *S. aureus* was highly resistant to ampicillin, penicillin, cefoxitin, ofloxacin, ciprofloxacin, and erythromycin. They also observed a high degree of sensitivity to vancomycin, amikacin, gentamycin, and chloramphenicol. Among the Gram-negative bacilli, *Pseudomonas aeruginosa* displayed the highest sensitivity to imipenem accounting for 100% susceptibility, followed by meropenem and amikacin. A similar finding reported by Sabetha et al.[23] demonstrated a high susceptibility to imipenem (98-100%). Furthermore, Abdi et al.[24] reported high sensitivity to imipenem (88.9%), meropenem (77.8%), and amikacin (81.5%). Datta et al.[25] revealed 55.6% sensitivity to meropenem. *Pseudomonas aeruginosa* isolates in this study were extensively resistant to cephalosporins, ampicillin, amoxicillin clavulanate, trimethoprim sulfamethoxazole, chloramphenicol, gentamicin, and netilmicin. This is consistent with the antibiotic sensitivity pattern discoverd in a study conducted by Chaudhary et al.[20], which reported a very high rate of resistance to nearly all antibiotics, with the highest resistance (91.1%) noted for cephalosporins. Interestingly, a study by Maclean et al.[26] reported *P. aeruginosa* isolates being 100% susceptible to trimethoprim sulfamethoxazole although *P. aeruginosa* is known to use its drug efflux mechanism to gain resistance to this antibiotic.

Table 2. Number of biofilm-producing isolates in burn wound pus samples

Isolated pathogen	Biofilm producers (by any of the methods)	Non-biofilm producers (by any of the methods)
Staphylococcus aureus	3	2
Coagulase-negative *Staphylococci*	0	1
Pseudomonas aeruginosa	16	4
Acinetobacter spp.	4	11
Klebsiella spp.	1	1
Proteus spp.	1	0
Total	25 (56.81%)	19 (43.19%)

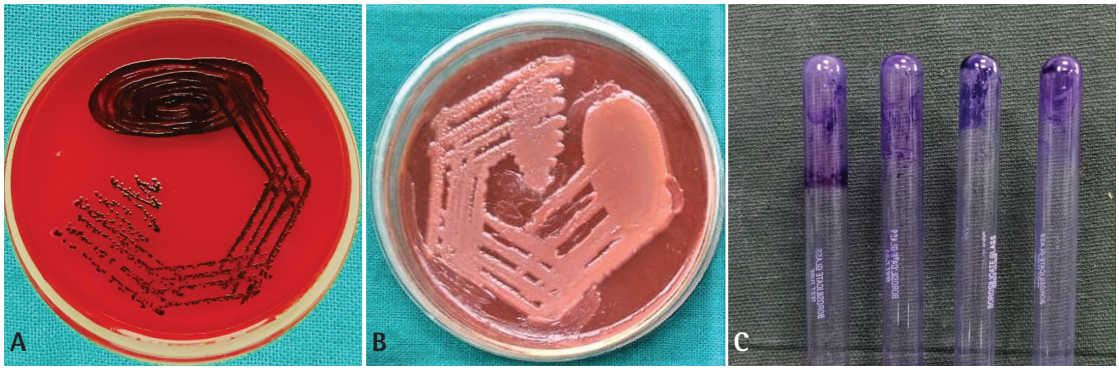

Figure 5. Techniques for identifying biofilm producers. (A): Biofilm-producing bacteria (black colonies) on modified Congo red agar method. (B): Non-biofilm-producing bacteria (pinkish-red colonies) on modified Congo red agar method. (C): Detection of biofilm formation by tube adherence method

Table 3. MDR evaluation of biofilm-forming (Bf) isolates versus nonbiofilm-forming isolates

Organisms	Count of Bf isolates	Bf multidrug resistance		Count of non Bf isolates	Non-Bf multidrug resistance		Probability value (p value)
		No.	%		No.	%	
Staphylococcus aureus	3	2	66.6	2	1	50	<0.05
Coagulase-negative *Staphylococci*	0	0	0	0	0	0	NA
P. aeruginosa	16	7	43.7	4	2	50	<0.05
Acinetobacter spp.	4	3	75	11	4	36.3	<0.05
Klebsiella spp.	1	0	0	1	0	0	NA
Proteus spp.	1	0	0	0	0	0	NA
Total	25	12	48%	18	7	38.8%	<0.05

MDR: Multidrug-resistant, NA: Not applicable

Acinetobacter species also demonstrated maximum sensitivity (86.66%) to the carbapenems, imipenem and meropenem, followed by aminoglycosides, which displayed moderate sensitivity (66.66%). This is consistent with a study performed by Asati and Chaudhary[27]. *Acinetobacter* isolates were found to be highly resistant to tetracyclines, sulfonamides, cephalosporin, penicillin, and quinolones. Comparable results were reported by Mwanamoonga et al.[28], where 21 out of 30 isolates were highly resistant to aminoglycosides, fluoroquinolones, sulphonamides, cephalosporins, carbapenems, and tetracyclines. The *Klebsiella* isolates exhibited 100% resistance to ampicillin, trimethoprim sulfamethoxazole, tetracycline, chloramphenicol, and ceftazidime. Helmy et al.[29] also reported a similar resistance profile, charactrized by complete resistance (100%) to penicillin, ampicillin, cefixime, and sulphamethoxazole/trimethoprim. Additionally, resistance rates over 65% were reported for cephalosporin, fluoroquinolones, and chloramphenicol.

The tube adherence technique and the modified Congo red agar technique were employed to detect biofilm production. Due to resource constraints, both methodologies were implemented in lieu of the gold standard tissue culture method in this investigation, which focused on resource-limited settings. Dhanalakshmi et al.[30] reported that both the Congo red agar and tube adherence methods can be considered as alternatives to detect biofilms in resource-limited conditions. The tube adherence technique was utilized to analyze the results, as it is the most dependable and prevalent method for identifying biofilm-forming organisms in laboratories, relative to the modified Congo red agar method. It is highly sensitive and specific, and it exhibits a strong correlation with the standard quantitative assay, i.e., the tissue culture plate method[31,32]. The rate of biofilm production in 44 isolates was 56.81% higher in the tube adherence method than in the modified Congo red agar method in this study. The variation was statistically insignificant (p value=0.244). This is in accordance with a study by Multani et al.[33], who reported that the rate of biofilm production was higher in the tube adherence method (55%) than in the modified Congo red agar method (46.66%). Deka[34] reported that the biofilm production rate was higher by the tube adherence method (57%) than by the modified Congo red agar method (20%). Shrestha et al.[35] reported that the tube adherence method had a higher rate of biofilm production (82.35%) and a high sensitivity and specificity (82% and 85.9%), which was comparable to the tissue culture method. Similarly, Reddy[36] and Khan et al.[37] reported that the tube method's sensitivity, as well as its specificity, were 97.3% and 100%, respectively, and 95.78% and 99.49%, respectively.

Out of the 25 isolates that showed biofilm formation, 12 (48%) were found to be MDR, while out of the 18 isolates that did not develop any biofilm, 7 (38.8%) were found to be MDR, which is consistent with the pattern observed by Asati and Chaudhary[27]. *Staphylococcus aureus* has the potential to adhere to a broad range of matrix components to initiate colonization. Microbial surface components recognizing adhesive matrix molecules are the family of protein adhesions that frequently facilitate this attachment. Proteins that exhibit an affinity for collagen and bind to fibronectin belong to this family[38].

Pseudomonas aeruginosa was the most prevalent pathogen, with 9 (45%) of the isolates being identified as MDR strains. Asati and Chaudhary[27] also observed 70.49% MDR *Pseudomonas aeruginosa* in their study. To reduce incidence of infections caused by these antibiotic-resistant organisms, strict measures to prevent infections (such as isolation in a dedicated room, wearing gowns and gloves when interacting with the patient, and practicing hand hygiene before and after each patient encounter) and suitable initial antimicrobial treatment are crucial.

Out of the five isolates of the Gram-positive *Staphylococcus aureus*, two (40%) were reported to be methicillin-sensitive *Staphylococcus aureus* (MSSA), while three (60%) isolates were identified to be MRSA. This correlates with a study conducted by Datta et al.[25] where among the *Staphylococcus aureus* isolates, 44.44% (16/36) were MSSA and 55.56% (20/36) were MRSA. MRSA was identified as the most prevalent Gram-positive organism in burn wounds. The MDR rate of *Staphylococcus aureus* isolates was reported to be 60% in this investigation, a high rate that is consistent with a study conducted by Asati and Chaudhary[27].

The number of patients in hospitals who have infections caused by MDR strains of *Pseudomonas aeruginosa* and *Acinetobacter* spp. is concerning. Twenty years ago, *Acinetobacter* spp. was considered nonpathogenic; however, it has since emerged as a significant and challenging human pathogen, causing a variety of infections. It is the second most prevalent nosocomial, aerobic, non-fermentative, Gram-negative bacilli, followed closely behind *Pseudomonas aeruginosa*. These two microorganisms demonstrate a significant potential for biofilm formation, which accounts for their high antibiotic resistance, survival capabilities, and enhanced virulence. Isolates that produced the most biofilms in this study were *Pseudomonas aeruginosa*. Similarly, Kunwar et al.[39] discovered that *P. aeruginosa* is the most prevalent organism forming biofilm in burn wounds.

Study Limitations

The current investigation included a reduced number of samples due to a restricted number of patients with burn wound infection in a single year. Larger sample sizes would yield better results. The genes of biofilm-producing bacteria were not investigated due to a lack of resources; nonetheless, the identification of

genes in biofilm producers would enhance biofilm detection. Our study focused solely on resource-limited settings to better comprehend biofilm formation, diagnostic methods, and antibiotic sensitivity.

Conclusion

The biofilm production rate in the isolates obtained from the burn samples was 56.81%. MDR bacteria comprised 48% of these biofilm-producing bacteria. A higher incidence of MDR was observed in biofilm-producing isolates in the study. Biofilm impedes antibiotic uptake and further worsens the prognosis. Imipenem exhibited the maximum level of sensitivity, followed by meropenem and amikacin. Marked resistance was observed for commonly used antibiotics like quinolones, cephalosporins, and cotrimoxazole.

Recommendation

Biofilm production may be detected in regular laboratories employing the tube adherence method, as it is more effective in detecting biofilm-producing organisms. This is both simple to interpret and cost-effective. The study therefore recommends that every burn center should consistently identify and monitor the precise patterns of burn wound infection and the antimicrobial sensitivity levels of microbes involved. Furthermore, the timely detection of infection caused by biofilm-producing strains can aid modification of treatment strategies and enhance outcomes in burn patients.

Ethics

Ethics Committee Approval: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics and Protocol Review Committee with Protocol Identification Number: IEC/MMIMSR/1904/15-03-2019.

Informed Consent: Written informed consent and assent were obtained from all study participants before sampling.

Authorship Contributions

Surgical and Medical Practices: B.S., S.M., S.C., Concept: B.S., S.M., R.D.A., S.C., Design: B.S., S.M., R.D.A., S.C., Data Collection or Processing: B.S., S.M., S.C., Analysis or Interpretation: B.S., S.M., R.D.A., P.O.A., J.A-A., Literature Search: B.S., R.D.A., P.O.A., J.A-A., Writing: B.S., R.D.A., P.O.A., J.A-A.

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