Antibiotic Susceptibility of Monomicrobial and Polymicrobial Cultures Isolated from Chronic Wounds

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Abstract

Chronic wounds, a significant healthcare burden, often harbor complex bacterial flora that impede healing and increase the risk of developing hard-to-treat infections. This study investigates the antibiotic susceptibility of bacterial pathogens isolated from chronic wounds, focusing on monomicrobial and polymicrobial cultures. For this purpose, 110 samples from chronic wound of patients were collected together with the administration of a questionnaire for obtaining demographic information. Bacterial strains were isolated, and identified, after which the antibiotic sensitivity of monomicrobial as well as polymicrobial cultures was assessed against several antibiotics using the Kirby-Bauer disc diffusion method. The findings implied that 91% of the samples were positive for bacterial growth and the most prevalent isolates were *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. Antibiotic sensitivity testing of monomicrobial cultures revealed that Linezolid was the most effective antibiotic against Gram positive bacteria whereas, Aztreonam and Cefoxitin were the least effective. Furthermore, Colistin was the most efficacious drug against Gram negative bacteria while Ceftazidime and Ceftriaxone were the least efficacious. The polymicrobial cultures exhibited elevated resistance and showed multi-drug as well as extensive drug resistance, underscoring the need for personalized treatment strategies. The findings emphasize the importance of understanding antibiotic resistance patterns to inform effective management of chronic wound infections. The study's results indicate a significant increase in antibiotic resistance among bacteria isolated from chronic wounds, suggesting the necessity for novel drug discovery and antibiotic stewardship.

Keywords: Polymicrobial culture; *P. aeruginosa*; *S. aureus*; K. pneumonia; Linezolid; Colistin

1. Introduction

Wounds, specifically chronic wounds, have become a challenging issue in healthcare, causing substantially adverse consequences for patients and imposing a considerable strain on global healthcare systems. Wounds are the physical injuries that frequently result in the rupturing of the skin covering. This results in the exposure of skin to the external environment, potentially disrupting normal functions and anatomy (Falanga et al., 2022). Based on the pathogenesis, wounds are categorized as acute and chronic wounds. In the case of acute wounds, a series of molecular events might help in re-establishing the structural integrity in a specific process known as wound healing (Raziyeva et al., 2021). A delay in the healing process might lead to progression towards the chronic wounds, resulting in the associated complications. Chronic wounds occur when a wound fails to heal correctly and recurs. These non-healing wounds can lead to tissue necrosis and decreased angiogenesis (Bonnici et al., 2023). Diabetic foot ulcer is one of the major categories of chronic wounds that occur in elderly individuals suffering from diabetes mellitus (Oliver, 2023). Because of the rising prevalence and increased acknowledgment of the accompanying morbidity, chronic wounds have garnered significant attention as an important clinical issue. Inappropriate management of chronic wounds can lead to infections, compromising the survival of the patient (Falcone et al., 2021). Chronic wounds can harbor different microbes or pathogens, including anaerobes and facultative anaerobes. Chronic wounds feature a complicated colonizing bacterial flora that evolves with time. The most prevalent isolates are *Staphylococcus aureus* and coagulase-negative Staphylococci (Siddiqui & Bernstein, 2010).

Bacterial infections in chronic wounds hinder the normal healing process and increase the risk of systemic complications. Arsenic and mercurial compounds were used to treat such wounds before the invention of antibiotics, but they led to higher penalties than the illness itself (Strachan & Davies, 2017). The 'Plague' outbreak, caused by *Yersinia pestis*, was responsible for numerous historical pandemics, including the 'Justinian plague', which killed nearly 100 million people, illustrating the catastrophic conditions of people in the pre-antibiotic era (Uddin et al., 2021). Antimicrobial agents, or antibiotics, were then introduced to reduce infection-related mortality rates. Microbes, plants, and animals produce potent antimicrobial substances such as peptides, aminoglycosides, etc. (Stan et al., 2021). Antibiotics meant to battle bacterial infections have saved many lives and enabled previously impossible medical operations. When antibiotics are taken, enzymes in the body metabolize antibiotics and transform them into sactive chemical that limit microbial development, hence treating the infection (Chin et al., 2023). The link between antibiotic efficacy and resistance is complex, with inappropriate administration being the principle cause for increase in resistance among microbial strains. Unnecessary use of antibiotics can create selective pressure for cellular growth and survival of resistant strains (Chinemerem Nwobodo et al., 2022). Antimicrobial resistance (AMR) is a natural process where microorganisms stop responding to previously active antibiotics, causing them to no longer remain susceptible to these drugs (Mancuso et al., 2021).

Bacterial strains can resist antibiotics by altering target regions, inactivating antimicrobial substances via efflux pumps, or modifying genetic sequences via molecular bypass (Blair et al., 2015). For instance, antibiotic resistance in *P. aeruginosa* results due to the bacteria producing alginate aggregates, which prevent antibiotics from entering the cell. The most advanced mechanism of resistance is the utilization of multidrug efflux pumps, which tend to eliminate practically all antibiotics except polymyxins (Dorniani et al., 2016). Methicillin-resistant *S. aureus* (MRSA) contains a variety of virulence factors that are either readily secreted or linked to a substrate and have the ability to modify the immune system by altering leukocyte recruitment, restricting the complement system as well as the adaptive immune system, as well as preventing antibiotic peptides from performing effectively (DeLeo et al., 2009).

Biofilm formation serves as a defense mechanism against potentially hazardous environmental impacts such as antibiotics and other antimicrobial substances and it enhances resistance to antimicrobials and sterilizing agents (Abebe, 2020). Furthermore, the coexistence of several bacterial strains in chronic wounds complicates the clinical picture, introducing additional obstacles in antibiotic therapy. The antimicrobial resistance is further enhanced by the polymicrobial environment found in chronic wounds, where horizontal gene transfer and cooperation processes among bacterial species can help disseminate resistance genes. As a result, traditional antibiotic medications may become less effective, demanding a more tailored and personalized approach to wound care (Durand et al., 2022). *S. aureus* and *P. aeruginosa* are the two most common bacteria identified from chronic wounds, and past research has looked at how they interact. For example, co-infection with these two bacteria was linked with enhanced inflammatory responses, increased antimicrobial resistance, and wound chronicity (Pouget et al., 2022).

The increasing prevalence of multidrug-resistant (MDR) bacteria points to a post-antibiotic era (Tzaneva et al., 2016). Antibiotics' mechanism of action and multidrug resistance raises concerns for new drugs' development and antibiotic stewardship. Further, the emergence of antibiotic-resistant strains necessitates a comprehensive understanding of the antibiotic sensitivity profiles of bacterial pathogens associated with chronic wounds. The knowledge about resistance against and susceptibility to antimicrobial agents determines the course of patient treatment. Therefore, this study aims to evaluate the variety of bacterial pathogens found in chronic wounds, their distribution, and probable synergy amongst species by co-culturing. The findings will help to improve knowledge of interspecies interactions and antibiotic effectiveness.

2. Materials & Methods

2.1. Sample Collection & Processing

The study was performed from September 2022 to June 2023 at The Women University Multan, Pakistan. The Institutional Ethical Review Committee approved the study and all the procedures were in accordance with the declaration of Helsinki. A total of 110 samples of pus and wound swabs were collected from the chronic wounds of patients admitted to Nishtar Hospital, Multan, Pakistan in sterile, labeled, tightly sealed containers. All the data regarding the patient's demography, type of sample, etc was carefully recorded. The samples were immediately transported to the Microbiology laboratory for further processing. All the samples were initially inoculated onto nutrient, blood and MacConkey agar, following the microbiological guidelines to avoid contamination. After 24 hours of incubation at 37^{o}C, the streak plate method was used to purify the strains for positive cultures. The purified bacterial strains were stored as glycerol stocks by adding 40% glycerol to the bacterial cultures in BHI broth in a 1:1 ratio, followed by storage at -20^{o}C.

2.2. Identification of Bacterial Isolates

The isolated strains were initially identified based on the morphological characteristics of individual colonies, followed by Gram staining to classify the isolated strains based on their cell wall characteristics. Subsequently, biochemical tests, including catalase test, oxidase, citrate, triple sugar iron, and indole motility tests were performed according to the standard protocols for accurate identification of isolated strains following the Bergey's manual of systematic bacteriology.

2.3. Antibiotic Sensitivity Testing

Kirby-Bauer disk-diffusion method was used to evaluate the antibiotic sensitivity of the monomicrobial as well as polymicrobial bacterial cultures. MHA medium was prepared aseptically as per the standard protocol, followed by swabbing of the pure cultures on the plates. 0.5 McFarland standard was used for this purpose. Antibiotics utilized for testing against Gram-positive cultures included Aztreonam (ATM), Cefoxitin (CFX), Ceftriaxone (CRO), Clindamycin (CM), Fusidic acid (FA), Gentamycin (CN), Imipenem (IPM), Linezolid (LZD), and Trimethoprim/sulfomethoxazole (SXT). For Gram-negative isolates, Amikacin (AK), Aztreonam (ATM), Cefepime, (FEP) Ceftazidime (CAZ), Ceftriaxone (CRO), Ciprofloxacin (CIP), Colistin (CL), Gentamycin (CN), Imipenem (IPM), and Trimethoprim/sulfomethoxazole (SXT) were used. The antibiotic discs with specific concentrations were placed onto the plates at an appropriate distance. For polymicrobial strains, two different bacterial strains were co-cultured or swabbed onto a single MHA plate, followed by the placement of antibiotics aseptically. Antibiotics used for polymicrobial cultures included Amikacin (AK), Ampicillin (AMP), Cefepime, (FEP) Cefixime (CFM), Ceftazidime (CAZ), Ceftriaxone (CRO), Ciprofloxacin (CIP), Gentamycin (CN), Meropenem (MEM), and Trimethoprim/sulfomethoxazole (SXT). Following the incubation at 37ºC overnight, zones of inhibition were observed and recorded in accordance with the CLSI guidelines to evaluate whether a strain is sensitive or resistant to a particular antibiotic.

3. Results

3.1. Characteristics of Study Subjects

Demographic information including gender, age and place of residence as well as disease-specific information of patients, recorded prior to sampling, is shown in **Table 1**. Patients from all over South Punjab come to the Nishtar Hospital as it is one of the main hospitals in this region. Analysis of data showed that majority of the patients of chronic wound infections were males, belonged to the middle age group and resided in rural areas. Most of the patients had burn wounds while the surgical wounds were the least prevalent. Mostly, the wounds were on the upper or lower limbs, were 1-10 cm^2 in size and were present since <12 weeks.

Table 1: Demographic and clinical characteristics of the study population

Characteristic	Category	N (%)
Gender	Female	47 (42.7)
	Male	63 (57.2)
Age (yrs)	<5	12 (10.9)
	6-15	25 (22.7)
	16-30	39 (35.4)
	31-45	16 (14.5)
	46-60	10 (9)
	>60	8 (7.2)
Location	Rural	76 (69)
	Urban	34 (40)
Wound Etiology	Infection	35 (31.8)
	Burn	40 (36.3)
	Diabetic Foot Ulcer	30 (27.2)
	Surgical	5 (4.5)
Wound Site	Head/Neck	17 (15.5)
	Upper Limb	38 (34.5)
	Trunk	12 (10.9)
	Lower Limb	43 (39.1)
Wound Size (cm^2)	1-10	70 (63.6)
	10.1-20	37 (33.6)
	20.1-50	3 (2.7)
Wound Duration	<12 weeks	60 (54.5)
	12-24 weeks	48 (43.6)
	<24 weeks	2 (1.8)

3.2. Isolation & Identification of Bacterial Strains

Out of 110 specimens, 100 samples turned positive for bacterial growth. Of these positive samples, 32 were from infections, 40 from burn wounds, 24 from diabetic wounds and 4 from surgical wounds. A total of 140 bacterial strains were isolated and identified from these 100 specimens on the basis of morphological appearance, Gram staining properties and the results of biochemical tests. Of these, 42 strains were isolated from infections, 58 from burn wounds, 33 from diabetic wounds and 7 from surgical wounds. Gram-positive isolates included *S. aureus* and methicillin-resistant *S. aureus* (MRSA). Gram-negative isolates included *P. aeruginosa*, *K. pneumoniae*, *K. oxytoca*, *P. vulgaris*, *P. mirabilis*, *Enterobacter* spp. and *E. coli*. Of these, *P. aeruginosa* were the predominant ones (N=60), followed by *K. pneumoniae* (N=30), MRSA (N=15), *S. aureus* (N=10), *P. vulgaris* (N=8), *E. coli* (N=7), *Enterobacter* spp. (N=5), *P. mirabilis* (N=4) and *K. oxytoca* (N=1). Their distribution in different categories of wounds is shown in **Figure 1**.

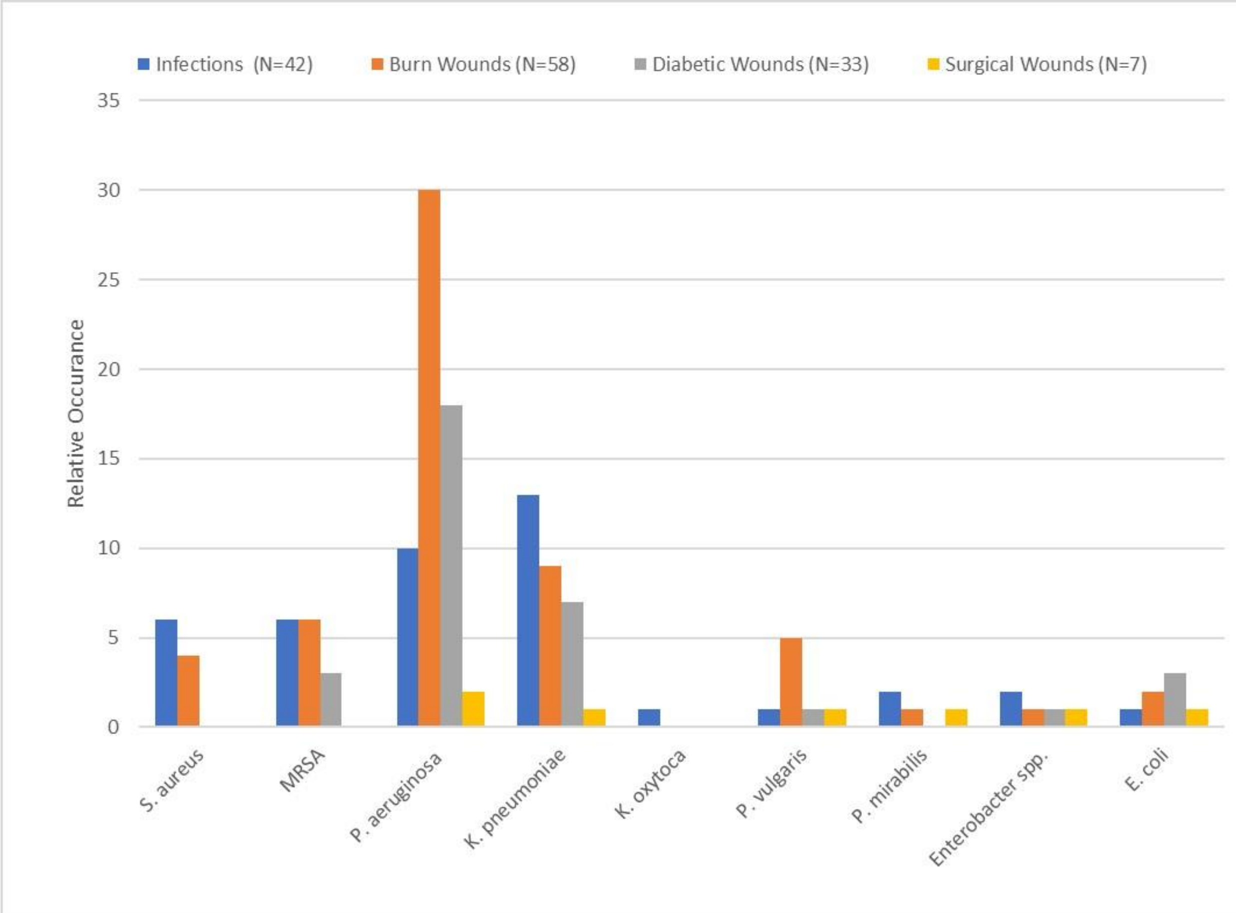

Figure 1: Distribution of bacterial isolates in different types of wounds

3.3. Antibiotic Susceptibility Testing of Monomicrobial Cultures

After comparing results of measured zones of inhibition of each antibiotic disc according to CLSI guidelines, bacterial strains were regarded as sensitive (S) or resistant (R) to specific antibiotics. Intermediate sensitive cultures were also designated as sensitive. As presented in **Figure 2**, LZD was the most effective antibiotic against Gram-positive isolates (N=25) as all the isolates were sensitive to it. Subsequently followed CM and IPM which were effective against 88% of the isolates. Majority of the isolates were sensitive to CN, CRO and FA, as well. However, SXT was effective against half of the isolates only while most isolates were resistant to ATM and CFX.

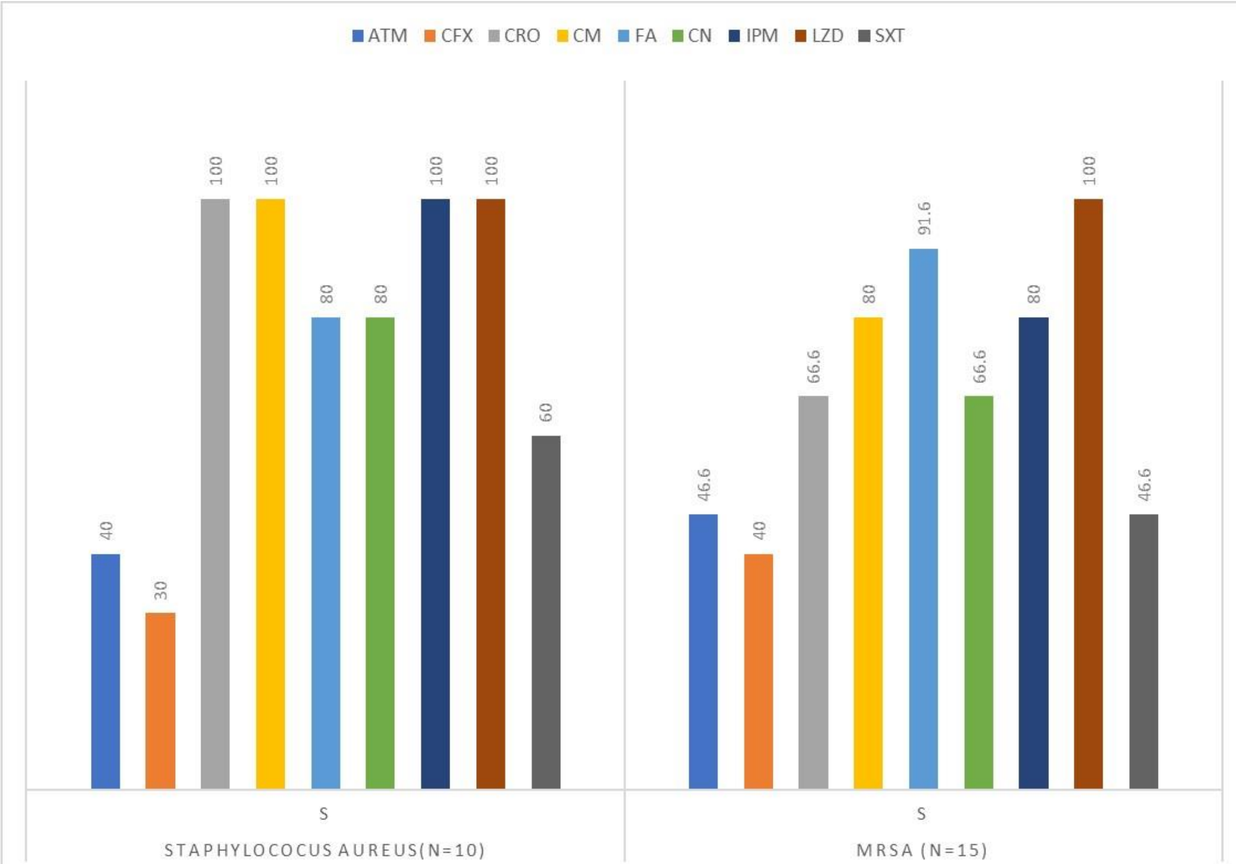

Figure 2: Antibiotic susceptibility profile of Gram-positive isolates in monoculture

For Gram-negative isolates (N=115), the results have been depicted in **Figure 3** and it was observed that CL was the most effective drug to which all isolates were susceptible. Nonetheless, it was not used against *Proteus* spp. due to their inherent resistance to CL. None of the other drugs showed very high efficacy and a vast majority of the isolates were resistant to most of the drugs, hence categorizing those as MDR pathogens. 53% of the isolates were susceptible to IPM, 48% to SXT, 45% to AK, 38% to CIP and CN, 37% to FEP and 36% to ATM. CRO and CAZ were the least effective antibiotic against which the sensitivity of isolates was 20% and 17%, respectively.

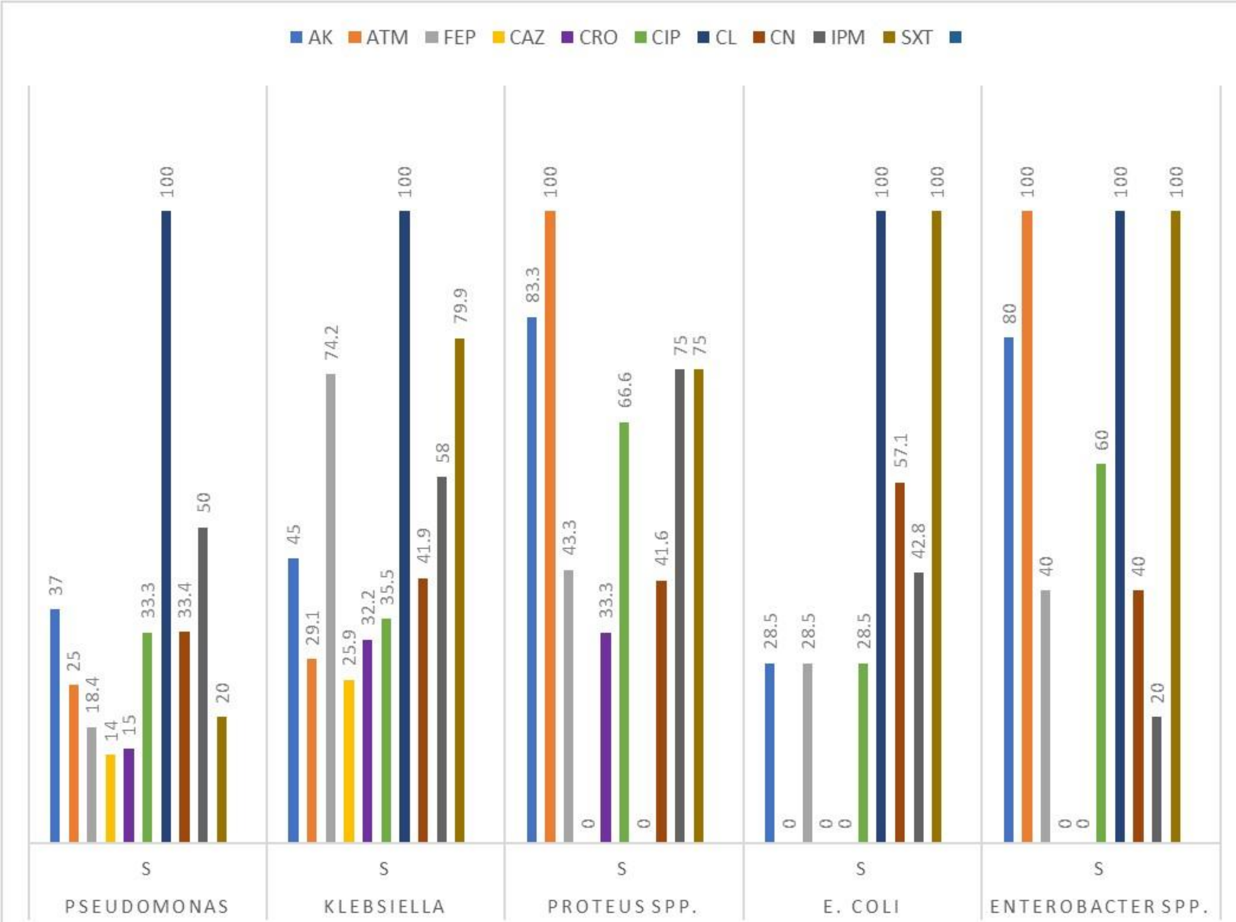

Figure 3: Antibiotic susceptibility profile of Gram-negative isolates in monoculture

With regards to specific bacteria, it was observed that CL was the most effective antibiotic against *P. aeruginosa*, followed by IPM, AK, CIP, CN, ATM, SXT, FEP, CRO and CAZ, respectively. Against *Klebsiella* spp., CL was the most effective drug followed by FEP, SXT, IPM, AK, CN, CIP, CRO, ATM and CAZ, respectively. *Proteus* spp. were completely susceptible to ATM and they were sensitive in decreasing order to AK, IPM, SXT, CIP, FEP, CN, CRO and CAZ, respectively. With regard to *E. coli*, the greatest efficacy was observed for CL and SXT, followed by CN, IPM, AK, FEP, CIP and CAZ. All isolates of *E. coli* were resistant to ATM and CRO. All isolates of *Enterobacter* spp. were susceptible to ATM, CL and SXT. Among the remaining drugs, AK was the most effective against *Enterobacter* spp., followed by CIP, FEP, CN IPM and CAZ, respectively. None of the isolates of *Enterobacter* spp. was sensitive to CRO.

3.4. Antibiotic Susceptibility Testing of Polymicrobial Cultures

Apart from monomicrobial cultures, antibiotic susceptibility testing was performed for co-cultures. For this purpose, more than one type of bacteria isolated from the same wound were co-cultured on same petri plate and selected antibiotics were applied. After 24 hours incubation, results demonstrated increased level of resistance against specific antibiotics, in general (**Table 2**). These Individual strains were sensitive to some antibiotics but in co-culture, a very high level of resistance was observed. Polymicrobial cultures demonstrated multi drug resistance as well as extensive drug resistance. All of the co-cultures were completely resistant to AMP, FEP, CFM, and CAZ. SXT was the most effective antibiotic as 46% of the co-cultures were susceptible to it. Subsequently followed MEM to which 31% of the co-cultures were sensitive. Furthermore, only 8% of the co-cultures manifested susceptibility to AK, CRO, CIP and CN. Finally, almost all of these co-cultures demonstrated multi-drug resistance or extensive drug resistance.

Table 2: Antibiotic susceptibility profiling of polymicrobial cultures

Co-cultured strains	AK	AMP	FEP	CFM	CAZ	CRO	CIP	CN	MEM	SXT
P. aeruginosa - 1 *K. pneumoniae* - 1	11mm R	5mm R	12mm R	5mm R	5mm R	5mm R	14mm R	12mm R	16mm R	20mm S
P. aeruginosa - 2 *K. pneumoniae* - 2	10mm R	5mm R	11mm R	5mm R	5mm R	5mm R	12mm R	10mm R	17mm R	18mm R
P. aeruginosa - 3 *K. pneumoniae* - 3	11mm R	5mm R	5mm R	5mm R	5mm R	5mm R	18mm R	20mm S	15mm R	24mm S
P. aeruginosa - 4 *K. pneumoniae* - 4	9mm R	5mm R	8mm R	6mm R	5mm R	5mm R	14mm R	5mm R	13mm R	20mm S
P. aeruginosa - 5 *K. pneumoniae* - 5	12mm R	5mm R	10mm R	5mm R	5mm R	8mm R	12mm R	10mm R	16mm R	15mm R
P. aeruginosa - 6 *K. pneumoniae* - 6	11mm R	5mm R	13mm R	5mm R	5mm R	10mm R	10mm R	12mm R	12mm R	25mm S
P. aeruginosa - 7	5mm	5mm	5mm	16mm	5mm	26mm	5mm	5mm	22mm	10mm

K. pneumoniae - 7	R	R	R	R	R	S	R	R	S	R
P. aeruginosa - 8 *K. pneumoniae* - 8	5mm R	5mm R	5mm R	5mm R	5mm R	5mm R	5mm R	5mm R	24mm S	12mm R
P. aeruginosa - 9 *K. pneumoniae* - 9	5mm R	5mm R	5mm R	5mm R	5mm R	5mm R	20mm IS	5mm R	20mm IS	22mm IS
P. aeruginosa - 10 *K. pneumoniae* - 10	5mm R	5mm R	5mm R	5mm R	5mm R	5mm R	18mm R	14mm R	18mm R	17mm R
P. aeruginosa - 11 *K. pneumoniae* - 11	7mm R	5mm R	5mm R	5mm R	5mm R	5mm R	15mm R	5mm R	15mm R	17mm R
P. aeruginosa - 12 *P. mirablis* - 1	28mm S	5mm R	8mm R	5mm R	5mm R	15mm R	12mm R	12mm R	26mm S	25mm S
P. aeruginosa - 13 *P. mirablis* - 2	13mm R	5mm R	5mm R	5mm R	5mm R	7mm R	10mm R	10mm R	20mm R	15mm R

Discussion

Chronic wounds and their propensity for infection represent a significant healthcare concern with far-reaching consequences. As the global population continues to increase and the prevalence of chronic diseases rises, the impact of chronic wound infections on healthcare systems will likely intensify. In the current study, samples were collected from the patients suffering from chronic wound infections, and 91% of the samples were found to be positive for bacterial growth. It was observed that *P. aeruginosa* (42.8%) and *K. pneumoniae* (22%) were the predominant species in wounds that lasted over 3 months, making the condition difficult to treat. A similar study was conducted in Sierra Leone indicating 95.7% positive samples. The predominant bacterial species were reported to be *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* (Schaumburg et al., 2021). A six-month study in Italy analyzed bacterial isolates from diabetic foot and pressure ulcer wounds. In their study, 44.2% Gram positive and 55.8% Gram negative strains were found, with *S. aureus* being the most common, followed by *P. aeruginosa* (Bessa et al., 2015). The prevalence of *Pseudomonas* species might be a major contributing factor in the slow healing of wounds, as documented in the present study. A similar fact was reported in a study conducted on diabetic mice as a model (Zhao et al., 2010). It is documented that *S. aureus* and *P. aeruginosa* release destructive virulence factors, causing prolonged infections and contributing to delayed wound healing (Dissemond, 2009).

In this study, Linezolid was reported to be the most effective drug against *S. aureus*, with 100% of isolates showing sensitivity to it, followed by Clindamycin and Imipenem (88% sensitivity). On the other hand, Aztreonam and Cefoxitin were recognized to be the least effective drugs against *S. aureus*. In a previous research study evaluating the antibiotic susceptibility pattern of wound isolates, it was reported that Linezolid and Vancomycin were the most effective drugs against Gram-positive isolates, including *S. aureus* (Bessa et al., 2013). Retrospective research done in Poland found that *S. aureus* isolated from chronic wounds was resistant to Benzylpenicillin (85%), Tobramycin (23%), and Cefoxitin (22%) whereas Ceftaroline, Vancomycin, Teicoplanin, Linezolid, and Rifampicin were effective against all of the tested strains (Budzyńska et al., 2021). A similar study on *S. aureus* isolated from chronic wounds demonstrated that the resistance against Ciprofloxacin, Methicillin, Oxacillin and Vancomycin was 49%, 44%, 39% and 50%, respectively (Karthy et al., 2009).

For Gram-negative isolates, it was observed that *P. aeruginosa* was sensitive to Colistin while showing a particular pattern of resistance to other antibiotics. Ceftriaxone and ceftazidime were reported to be the least effective drugs, as the majority of isolates were resistant to them. A similar antibiotic susceptibility pattern was observed for *K. pneumoniae*, with Colistin as the most effective drug, as evidenced by 100% sensitivity to it. In an earlier research study based on burn wounds, it was observed that *P. aeruginosa* isolates showed the highest resistance to Ceftazidime (Asati & Chaudhary, 2017). A similar study revealed that Gram-negative bacterial strains exhibited high level of resistance against commonly used antibiotics i.e., penicillins, tetracyclines, cephalosporins, and aminoglycosides. These strains were recorded to be sensitive to only

Vancomycin and Carbapenems (Mehta et al., 2007). Bessa et al in 2015 manifested that Amikacin was categorized as the most efficient class of antibiotics against most isolates, although 28.3% of *P. aeruginosa* strains resisted it. Guan et al in 2021 conducted a retrospective study in China to estimate the prevalence of antibiotic-resistant strains in chronic wounds. Their results showed predominant resistance in *S. aureus* to Penicillin (92%), Erythromycin (58.3%), and Clindamycin (50.9%). Vancomycin was 100% efficient against Gram positive strains. Among Gram negative strains, *E. coli* manifested 68.1% insensitivity to Ampicillin while posed 3.9% and 3.6 % resistance to Tigecycline and Amikacin, respectively.

Apart from the monomicrobial culture, the co-culturing of two different bacterial strains against specific classes of antibiotics manifested an escalation of the resistance. In the current study, polymicrobial cultures of *P. aeruginosa* and *K. pneumoniae* mostly presented multi-drug resistance and sometimes, even extensive drug resistance. Furthermore, the polymicrobial cultures of *P. aeruginosa* along with *P. mirablis* showed extensive drug resistance as well as multi-drug resistance. This implies that microbes become more resistant when they co-exist in synergistic relationships, making it even harder to treat the wounds. Bessa et al in 2015 evaluated that *S. aureus and P. aeruginosa* strains were prominent among polymicrobial isolates and their synergism resulted in an upward surge in the resistance against antibiotics. Seth et al in 2012 conducted a study on the combined effect of polymicrobial cultures on biofilm production in a rat model. Their results approved the claim that increased virulence of polymicrobial biofilm is a combined effect of pathogenicity presented by each single strain thus enhancing resistance to the antibiotics. It is evaluated that resistant genes are transferred among bacterial species and therefore bacteria are rapidly adapting themselves not only against single bioactive group but also two or more combined bioactive groups. Hence, we have to prevent misuse and overuse of antibiotics in order to minimize antibacterial resistance.

3. Conclusion

A considerably high level of antibiotic resistance was observed among burn wounds and diabetic foot ulcers. *P. aeruginosa* and *K. pneumoniae* were the predominant strains from causing infections in wounds and presented multidrug resistance due to biofilm formation. Linezolid was reported to be the most effective drug against Gram positive strains while Colistin, Tigecycline and Polymyxin B were nominated the most efficacious antibiotics against Gram negative isolates. The polymicrobial environment of wounds poses a significant challenge as it compounds the situation by enhancing resistance among pathogens. Overall, the results imply that there is a dire need of discovery of new and alternative antimicrobial drugs as well as implementation of antibiotic stewardship programs to combat drug resistance.

STATEMENTS & DECLARATIONS

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Competing Interests

The authors have no conflict of interests to declare.

Availability of Data and Materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Ethical Approval

All the procedures used in this study complied with the declaration of Helsinki and were approved by the Ethical Review Committee of The Women University, Multan, Pakistan.

Consent to Participate & Publish

All the study participants provided written informed consent or assent with consent provided by a family member for participating in the study as well as publication of results without disclosure of identity.

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