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Original Research Article

CLINICO MICROBIOLOGICAL STUDY OF SECONDARY BACTERIAL INFECTIONS IN PATIENTS WITH VENOUS LEG ULCERS AND THEIR ANTIMICROBIAL SUSCEPTIBILITY PROFILES

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Abstract

Background: Venous leg ulcers often harbor microbial colonization, and this situation can be particularly concerning when the ulcer is infected with alert pathogens highly virulent microorganisms with robust mechanisms of antibiotic resistance. The current study aimed to assess the microbiological status of venous leg ulcers, aiming to identify clinicodemographic predictors associated with culture-positive ulcers, with a specific emphasis on antibiotic resistance patterns.

Methods: Material for microbiological analysis was exclusively collected upon enrolment from patients who had not undergone any antibiotic treatment. Before swabbing, the ulcer underwent cleansing to remove necrotic tissues, exudate, and foreign bodies like dressing remnants. Subsequently, the wound was rinsed with phosphate-buffered saline (PBS). Depending on the clinical state of the wound, swabs were either collected from the surface (superficial ulcers) or the deepest point (deep ulcers) using Levine's technique.

Results: The most common microorganisms isolated from leg ulcers were *Staphylococcus aureus* (26%), *Pseudomonas aeruginosa* (10%), and *Escherichia coli* (10%). All the *S. aureus* (MSSA) isolates were susceptible to penicillin, erythromycin, amikacin, ciprofloxacin, co-trimoxazole, clindamycin, and gentamicin. 91.7% of *S. aureus* (MSSA) isolates were susceptible to tetracycline. *S. aureus* (MSSA) isolates were susceptible to vancomycin and clindamycin. None of the 6 *S. aureus* (MRSA) isolates were susceptible to penicillin, clindamycin, or vancomycin. *E. coli* 85.71% susceptible to amikacin, ciprofloxacin, piperacillin-tazobactam, and cefotaxime. *K. oxytoca* is 50% susceptible to all antibiotics tested. *K. pneumoniae* is 100% susceptible to piperacillin-tazobactam, cefotaxime, imipenem, cefoxitin, and ceftazidime. *Proteus mirabilis* is 75% susceptible to all antibiotics tested.

Conclusion: The primary pathology associated with these ulcers is perforator incompetence. Noteworthy risk factors include deep vein thrombosis (DVT), obesity, and varicose veins. The diagnosis of infection can be effectively accomplished through the quantitative culture method using tissue biopsy, revealing ulcers as either monomicrobial or polymicrobial with *Staphylococcus aureus* being the most common pathogen, followed by members of *Escherichia coli*. Methicillin-resistant *Staphylococcus aureus (MRSA)* is a significant pathogen in the etiology of venous leg ulcers

Keywords: Leg Ulcers, Microbiological Profile, Antibiotic Sensitivity, Venous Insufficiency

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Introduction

Venous ulcers, a prevalent consequence of chronic venous insufficiency (CVI), stand as the primary cause of leg ulcers. These ulcers, stemming from CVI, are painful, prone to infection, and significantly impact individuals' morale, leading to social withdrawal. Managing venous ulcers requires considerable time and financial resources, influencing both the quality of life and workplace productivity. According to the American Venous Forum (AVF), a venous ulcer is defined as a non-healing, full-thickness skin defect, typically found in the ankle region, sustained by Chronic Venous Diseases (CVD) as determined by duplex studies. [1] Venous leg ulcers (VLU) are irregular, shallow, and recurrent wounds, often persisting for extended periods. Early identification and effective management of the underlying venous issues are crucial steps to prevent the recurrence of venous ulcers. Bacteria naturally colonize all chronic wounds. [2] Research indicates that leg ulcers harbor both Gram-positive and Gram-negative bacteria. Common Gram-negative strains include *Pseudomonas aeruginosa* and *Escherichia coli*, while Gram-positive bacteria, notably *Staphylococcus aureus*, prevail. These microorganisms often exhibit resistance to one or more antibiotics. Less frequently encountered bacteria include Proteus mirabilis, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *Enterobacter cloacae*, Proteus vulgaris, *Acinetobacter baumanni*, *Morganella morganii*, *Klebsiella oxytoca*, *Citrobacter koseri*, *Citrobacter freundii*, *Coagulase-negative Staphylococcus*, and *Stenotrophomonas maltophilia*. [3, 4]

It is essential to highlight that ulcer colonization doesn't invariably lead to clinically evident infections. In most cases of colonization, innate immune mechanisms effectively curb microbial overgrowth, preventing symptomatic infections. However, if the ulcer is colonized by highly virulent pathogens, particularly those capable of biofilm formation, overt infections may ensue, potentially causing delayed wound healing. [5-7] Additionally, prolonged empirical antibiotic therapy may contribute to the emergence of drug-resistant microbial strains within the wound. [8, 9] This scenario could be particularly detrimental if the ulcer is infected with alert pathogens, characterized by high virulence and well-established antibiotic resistance mechanisms. [10] The continual presence of bacteria in venous ulcers triggers the host immune defenses, prompting the release of inflammatory mediators, cytotoxic enzymes, and free oxygen radicals as neutrophils migrate into the ulcer. Thrombosis and vasoconstrictive metabolites induce wound hypoxia, fostering bacterial proliferation and sustained tissue damage. [11] Bacterial evasion of the body's immune system complicates host defense mechanisms, leading to the development of "immune tolerance," potentially masking infections and hindering effective treatment. Chronic wounds, due to their moist environment, often foster biofilm formation, where bacteria aggregate and embed themselves in a self-secreted exopolysaccharide matrix. The presence of such biofilms hampers efficient bacterial eradication by antibiotic treatment and host defenses, [12] causing delays in wound healing and promoting the emergence of resistant bacterial strains.

Analyzing clinical observations and microbiological assessments in individuals with chronic leg ulcers, ranging from colonization to infection, provides valuable insights for clinicians in ulcer management. Quantitative wound cultures aid in assessing bacterial burden. This study aims to identify the etiological agents infecting and colonizing venous leg ulcers, along with their antimicrobial sensitivity patterns. Its utility extends to distinguishing patients with infected ulcers from those with colonization, preventing unwarranted antibiotic use, and ensuring targeted treatment for the appropriate infected population. The current study aimed to isolate and identify the bacteria infecting the patients with venous leg ulcers.

Material and methods

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This cross-sectional study was conducted in the Department of Microbiology, Prathima Institute of Medical Sciences, Naganur, Karimnagar, Telangana State. Institutional Ethical approval was obtained for the study after following the ethical clearance protocol. Written consent was obtained from all the patients of the study after explaining the nature of the study in the vernacular language during sample collection.

Inclusion criteria

1. Patients older than 18 years and above.
2. IP/OP Patients with Venous leg ulcers with one or more of the clinical signs of infections
3. Fever
4. Increased pain Discharge Malodour
5. Increased oedema

Exclusion criteria:

1. Patients with arterial ulcers, Filarial ulcers
2. Patients with neurotrophic ulcers- Diabetic ulcer, Leprotic ulcer
3. Patients with venous leg ulcers have no clinical signs of infection.

The patients' details were collected which included sociodemographic details of the participants, comorbidity information, chronic venous insufficiency history, current leg ulcerations history, and details regarding the location, depth, area, and number of ulcerations. Ulcer depth classification was based on skin involvement, designating ulcerations affecting solely the epidermis as 'superficial' and those affecting the dermis as 'deep,' which included both partial dermal involvement and penetration across the entire dermal thickness. The analysis encompassed additional clinical features of the ulceration, such as warmth, redness with a diameter exceeding 2 cm, swelling, purulence/abscess, unpleasant odor, and pain.

Material for microbiological analysis was exclusively collected upon enrolment from patients who had not undergone any antibiotic treatment. Before swabbing, the ulcer underwent cleansing to remove necrotic tissues, exudate, and foreign bodies like dressing remnants. Subsequently, the wound was rinsed with phosphate-buffered saline (PBS). Depending on the clinical state of the wound, swabs were either collected from the surface (superficial ulcers) or the deepest point (deep ulcers) using Levine's technique. Sterile swabs, pre-moistened with sterile PBS, were gently pressed over a 1 cm^2 area for at least five seconds to ensure a thorough capture of tissue fluid. A simple swab without a transport medium was employed, and clinical swabs were promptly placed back into a dry, sterile tube for immediate transportation to the laboratory. Microorganisms from the swabs were cultivated on selective media, following incubation under standard conditions. [13, 14]

Different agar media were used to isolate and identify bacteria from swabs. Total viable bacteria, Gram-negative lactose fermenters, fastidious bacteria, and *staphylococci* were isolated using nutrient agar, MacConkey agar, blood agar, and mannitol salt agar, respectively. After incubation at 37°C for 24 hours, pure bacterial colonies were obtained from mixed cultures. Morphological and biochemical tests, including Gram staining, motility, catalase, oxidase, indole, methyl-red, Voges-Proskauer, urease, citrate utilization, starch hydrolysis, nitrate reduction, and sugar fermentation, were performed to characterize the isolates. Bacterial cultures were grown on plates containing nutrient agar (Oxoid, England) and then incubated for 24 hours at 37°C. Approximately 100 µl of bacterial cells were placed in sterile normal saline to achieve a turbidity equivalent to the 0.5 McFarland standard. This standard is a

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solution of barium sulfate created by adding 0.6 ml of 1% barium chloride to 99.4 ml of sulfuric acid. [14, 15]

The antibiotic susceptibility of bacterial isolates was determined using the disc diffusion technique. Bacterial cultures were grown on nutrient agar and then spread onto Muller Hinton agar plates. Various antibiotic discs were placed on the plates and incubated overnight at 37°C. The zone of inhibition around each disc was measured to determine the susceptibility of the bacteria to the antibiotic. The antibiotics tested were amikacin, amoxicillin, ampicillin, ceftazidime, cefazolin, ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, gentamicin, imipenem, linezolid, methicillin, netilmicin, ofloxacin, oxacillin, penicillin, piperacillin, sulfamethoxazole, trimethoprim, and vancomycin.

Statistical analysis: All the available data was uploaded to an MS Excel spreadsheet and analyzed by SPSS version 21 in Windows format. Characteristics of quantitative variables were presented as mean, standard deviation, lower and upper quartiles. Qualitative variables were shown as numbers and percentages.

Results

Table 1 shows the distribution of cases of venous leg ulcers included in the study. Out of the 50 cases included in the study, the majority of cases (64%) are in the 51-70 age group. There are also a significant number of cases in the 41-50 (18%) and 61-70 (28%) age groups. The smallest number of cases is in the <30 age group (4%). This suggests that venous leg ulcers are more common in older adults and also most commonly occurring This is likely because older adults are more likely to have other risk factors for venous leg ulcers, such as varicose veins and poor circulation.

Table 1: Distribution of cases of venous leg ulcers included in the study

Age groups	*Males*	*Females*	*Total (%)*
< 30	2	0	2(4%)
31- 40	6	1	7(14%)
41- 50	8	1	9(18%)
51- 60	10	2	12(24%)
61- 70	11	3	14(28%)
> 71	5	1	6(12%)
Total	42	8	50(100%)

The most common position of the Ulcers was above the Medial malleolus (80%). Followed by Lateral malleolus (16%) and the least were located in the gaiter region (4%) depicted in Figure 1.

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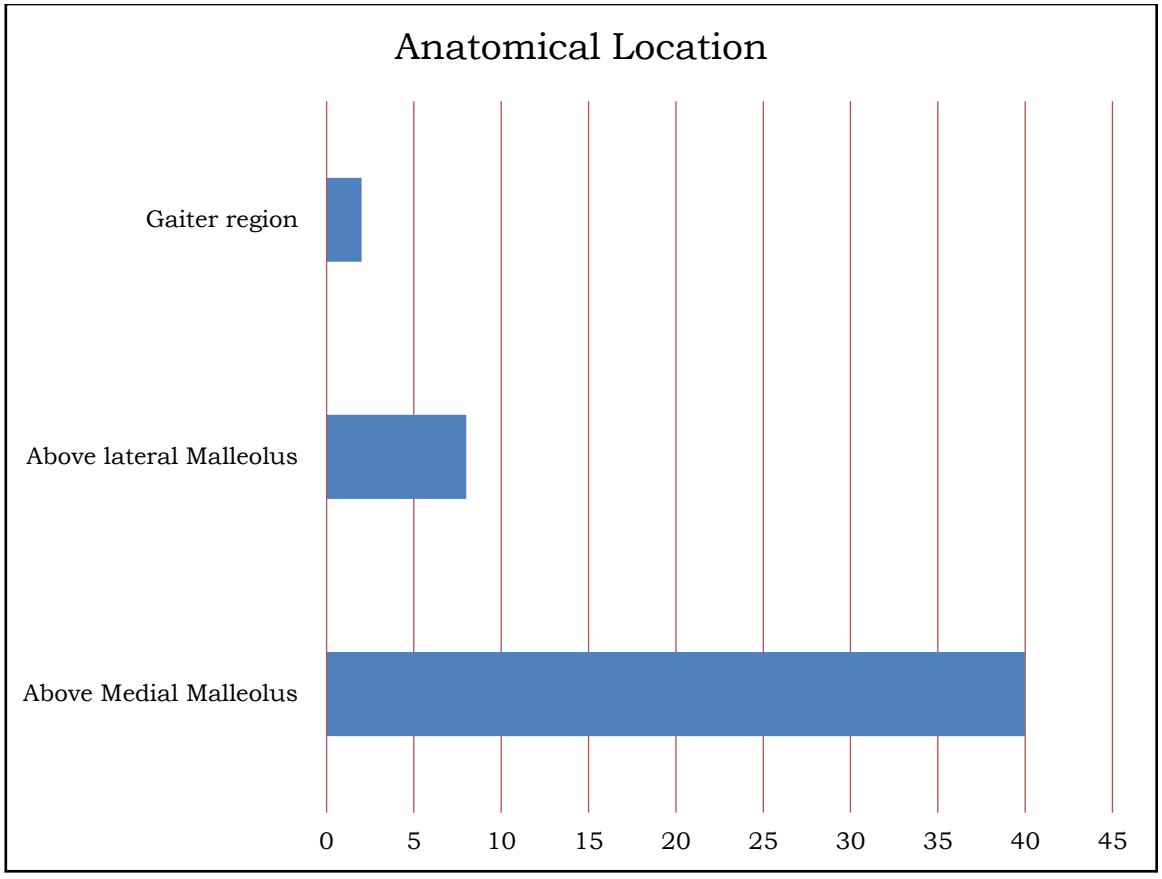

Figure 1: Showing the anatomical location of leg ulcers in the cases of the study.

Among the study cases, diabetes mellitus emerged as the most prevalent comorbid condition, with 18% of individuals indicating its presence. Hypertension ranked as the second most common comorbidity, with 4% of participants acknowledging its occurrence. The combination of diabetes and hypertension was also noted, with 4% of participants reporting both conditions. Obesity followed as the fourth most common comorbidity, with 12% of individuals disclosing its presence. Comorbidities such as cardiac diseases, chronic renal failure, and herniorrhaphy were relatively infrequent, reported by only 4%, 1%, and 3% of participants, respectively. A majority of participants (54%) reported no associated comorbid conditions.

Table 2: Association With Venous Pathology in cases of leg ulcers in the study

Venous pathology	*No of cases*	*Percentage*
DVT	5	10
Operated either for Varicose veins or SSG done	8	16
Visible Varicose Veins	33	66
IVC thrombosis operated	1	2
No pathology	3	6
Total	50	100

Table 2 shows the association between venous pathology and leg ulcers in the study. As you can see, the majority of cases (66%) have visible varicose veins. This is followed by 16% of cases who have had surgery for varicose veins or superficial venous insufficiency (SSG). Deep vein thrombosis (DVT) is less common, with only 10% of cases reporting having the condition. Iliac vein compression (IVC) thrombosis, which is a blockage of the main vein in the pelvis, is the least common venous pathology, with only 2% of cases reporting having the condition.

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This was noted that venous leg ulcers are most commonly associated in patients with visible varicose veins.

Table 3: Results of the Doppler study among the study population with leg ulcers

Venous pathology	*Frequency*	*Percentage*
Great Saphenous Vein Pathology	11	22%
Short Saphenous Vein Pathology	17	34%
Perforator Incompetence (Above-ankle, Below Knee, Mid-calf, Above Knee)	22	44%
Total	50	100%

Table 3 shows the results of the Doppler study among the study population with leg ulcers. Perforator incompetence is the most common venous pathology, with 44% of cases reporting having the condition. This is followed by short saphenous vein pathology, which is present in 34% of cases. Great saphenous vein pathology is the least common venous pathology, with only 22% of cases reporting having the condition. The range of clinical presentations among the patients included heightened pain, with subsequent reports of pain accompanied by discharge from the wound. Swelling was observed in five patients, and three patients experienced fever.

Table 3: Gram Stain to Culture Positivity in cases of leg ulcers

	Culture Positive	*Culture Negative*	*P value*
Smear positive	26	0	P<0.012
Smear negative	19	5	

Out of the 50 cases in the study, 26 cases were positive for Gram stain, and none were negative for culture. This means that Gram stain was a perfect predictor of culture positivity in this study. Out of the 19 cases that were negative for Gram stain, 5 were positive for culture (Table 3).

Table 4: Microbial Distribution in Leg Ulcers (n=50)

No of Organisms	No of ulcers	No of isolates
Monomicrobial	23	23
Polymicrobial	22	46
No Growth	5	0
Total	50	69

This table shows the distribution of microorganisms in 50 leg ulcers. The majority of ulcers (46%) are polymicrobial, meaning that they contain two or more different types of microorganisms. This is followed by 23% of ulcers that are monomicrobial, meaning that they contain only one type of microorganism. 5% of ulcers did not grow any microorganisms (Table 4). The most common microorganisms isolated from leg ulcers were *Staphylococcus aureus* (26%), *Pseudomonas aeruginosa* (10%), and *Escherichia coli* (10%). These are all gram-negative bacteria that are commonly found in the skin.

Table 5: Total number of isolates in leg ulcers

Name of the Organism	No of isolates	Percentage (%)

Staphylococcus aureus	18	26.08
Staphylococcus epidermidis	4	5.79
Streptococcus pyogenes	2	2.90
Enterococcus faecalis	4	5.79
Enterococcus faecium	1	1.44
Micrococci	3	4.34
Diphtheroid	3	4.34
Escherichia coli	7	10.14
Klebsiella oxytoca	2	2.89
Klebsiella pneumoniae	3	4.34
Proteus mirabilis	4	5.79
Proteus vulgaris	3	4.34
Pseudomonas aeruginosa	7	10.14
Acinetobacter baumanii	1	1.44
Peptostreptococcus anaerobius	6	8.69
Bacteroides fragilis	1	1.44
Total	69	100.00

Table 5 shows the distribution of different bacterial organisms isolated from 69 leg ulcers. *Staphylococcus aureus* (18 isolates) is the most common organism, accounting for 26.08% of all isolates. *Pseudomonas aeruginosa* (7 isolates) and *Escherichia coli* (7 isolates) are the second and third most common organisms, respectively. In this study, 46% of ulcers were polymicrobial. The most common polymicrobial combination was *Staphylococcus aureus* and *Pseudomonas aeruginosa* (12.5% of ulcers). The findings of this study are consistent with previous research, which has shown that leg ulcers are often polymicrobial and that the most common microorganisms are *S. aureus*, *P. aeruginosa*, and *E. coli*. In this study out of 69 isolates, 21 aerobic isolates were found to have colony counts of 10^6 CFU/gm tissue and 7 were anaerobic isolates. In the aerobic isolates out of 21 isolates 10 were monomicrobial isolates and no monomicrobial isolates were found in anaerobic organisms. Out of the 7 anaerobic isolates, all were polymicrobial in nature.

Table 6**:** Antimicrobial susceptibility pattern of the Gram-Positive Organisms (n=32) isolates.

Organism	*PEN 10µg*	*ERY 15µg*	*AK 30µg*	*CIP 5µg*	*COT 1.25/23.75 µg*	*CX 30µg*	*GM 10µg*	*TET10 µg*	*VAN 30µg*	*CL 2µg**
S. aureus (MSSA)(12)	100%	75%	83.33%	66.67%	75%	100%	83.3%	91.67%	NT	100%
S. aureus (MRSA)(6)	0%	33.33%	66.67%	66.67%	33.33%	0%	66.67%	33.33%	NT	100%
S.epidermidis (4)	100%	100%	75%	50%	75%	100%	50%	100%	NT	100%
Streptococcus Pyogenes (2)	100%	100%	NT	100%	NT	NT	NT	100%	100%	100%
Enterococcus faecalis (4)	100%	75%	NT	100%	NT	NT	NT	NT	100%	NT
Enterococcus faecium(1)	100%	100%	NT	0%	NT	NT	NT	NT	100%	NT
Diphtheroids(3)	100%	NT	NT	100%	NT	NT	NT	NT	100%	NT

Note: The column labeled "NT" means "not tested." The numbers in parentheses in the COT column indicate the number of isolates that were resistant to Co-trimoxazole at a higher concentration (25 µg/12.5 µg). The CL column shows the results of the cefoxitin test, which is used to screen for methicillin resistance in staphylococci. *Micrococci* were treated as normal skin commensals.

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Table 6 shows the antimicrobial susceptibility pattern of 32 Gram-positive isolates. The isolates were tested against 10 different antibiotics. The results are shown as percentages, with 100% indicating that all of the isolates were susceptible to the antibiotic, and 0% indicating that none of the isolates were susceptible to the antibiotic. All 12 *S. aureus* (MSSA) isolates were susceptible to penicillin, erythromycin, amikacin, ciprofloxacin, co-trimoxazole, clindamycin, and gentamicin. 91.7% of *S. aureus* (MSSA) isolates were susceptible to tetracycline. *S. aureus* (MSSA) isolates were susceptible to vancomycin and clindamycin. None of the 6 *S. aureus* (MRSA) isolates were susceptible to penicillin, clindamycin, or vancomycin. All 4 *S. epidermidis* isolates were susceptible to penicillin, erythromycin, ciprofloxacin, clindamycin, and gentamicin. All *S. epidermidis* isolates were susceptible to vancomycin and clindamycin. Both *Streptococcus Pyogenes* isolates were susceptible to all antibiotics tested. All 4 *Enterococcus faecalis* isolates were susceptible to penicillin, ciprofloxacin, and vancomycin. 75% of *Enterococcus faecalis* isolates were susceptible to erythromycin. The 1 *Enterococcus faecium* isolate was susceptible to penicillin, erythromycin, and vancomycin. All 3 *Diphtheroids* isolates were susceptible to penicillin, ciprofloxacin, and vancomycin. Overall, the results show that the most effective antibiotics against Gram-positive isolates are penicillin, erythromycin, ciprofloxacin, clindamycin, and vancomycin.

Table 7: Antimicrobial susceptibility pattern of the Gram-Negative Organisms

Organism	*AK 30µg*	*CIP 5µg*	*COT 1.25/23.75 µg*	*PT 100/10 µg*	*CAZ 30 µg*	*CTX 30 µg*	*GM 10µg*	*TET 10µg*	*IMP 10 µg*	*CX 30 µg*
E.Coli (7)	85.71%	85.71%	57.14%	100%	57.14%	57.14%	85.71%	85.71%	100%	100%
K.oxytoca (2)	50%	50%	50.00%	100%	100%	100%	50%	50%	100%	100%
K.pneumoniae (3)	33.3%	66.6%	100%	100%	100%	100%	33.3%	100%	100%	100%
Proteus vulgaris (3)	100%	66.6%	66.6%	100%	100%	100%	66.6%	100%	100%	100%
Proteus mirabilis (4)	75%	75%	75.00%	100%	100%	100%	75%	75%	100%	100%
Pseudomonas aeruginosa (7)	71.43%	14.28%	NT	100%	100%	NT	71.4%	NT	100%	100%
Acinetobacter baumanii (1)	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

The table shows the antimicrobial susceptibility pattern of 27 Gram-Negative organisms. The organisms were tested against 10 different antibiotics. The results are shown as percentages, with 100% indicating that all of the isolates were susceptible to the antibiotic, and 0% indicating that none of the isolates were susceptible to the antibiotic. *E. coli* 85.71% susceptible to amikacin, ciprofloxacin, piperacillin-tazobactam, and cefotaxime. *K. oxytoca* is 50% susceptible to all antibiotics tested. *K. pneumoniae* is 100% susceptible to piperacillin-tazobactam, cefotaxime, imipenem, cefoxitin, and ceftazidime. *Proteus mirabilis* is 75% susceptible to all antibiotics tested. *K. oxytoca* is 50% susceptible to all antibiotics tested. Pseudomonas aeruginosa were found to be 100% susceptible to piperacillin-tazobactam, imipenem, cefoxitin and cefotaxime. *Acinetobacter baumannii* was 100% susceptible to all antibiotics tested. Overall, the results show that the most effective antibiotics against Gram-Negative organisms are amikacin, ciprofloxacin, piperacillin-tazobactam, cefotaxime, and ceftazidime.

Discussion

The study included 50 patients with venous leg ulcers who met the inclusion criteria. In terms of age distribution, 29% of the patients were in the 61-70 age group, followed by 24% in the 51-60 age group (refer to Table 1). It is noted that venous insufficiency tends to progress with

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age, with the prevalence of venous ulcers increasing by 4% beyond the age of 65 [5]. The incidence of venous ulcers is reported to rise significantly after the age of fifty as reported in other studies. [16, 17] In our study, among the 100 patients with venous ulcers, 84% were males, and 16% were females. The majority of patients in this study were engaged in occupations that required prolonged standing, leading to venous hypertension. [18] Among the study population, 20% were night security workers, followed by daily wage laborers at 18%. DJ Radak, et al. [19] in their study on risk factors for symptomatic chronic venous disorders, noted that professions involving prolonged sitting or standing were associated with an increased risk of chronic venous disease. Venous hypertension resulting from calf muscle pump dysfunction contributes to venous dysfunction and blood stasis in the lower limbs. In this study, venous ulcers were associated with incompetence in the great saphenous vein (GSV), small saphenous vein (SSV), and perforators. Specifically, 80% of venous ulcers were located above the medial malleolus, and 44% exhibited perforator incompetence. This finding aligns with the study by G. Spentzouris et al. [20] where the incidence of ulcers above the medial malleolus was reported to be 95%.

Venous stasis and inflammation stimulate peripheral nerve endings, and the addition of infection exacerbates the condition. Increased pain is considered a sign of infection. [21] In the current study, 46% of patients experienced pain, 26% had discharge, and 5% had malodorous discharge. A study by Howell et al. reported that 61% of patients presented with increased pain [30]. Direct gram stain results correlated with quantitative culture in 53% of the ulcers. This rapid indication of bacterial burden is crucial in wound assessment. P.G. Bowler et al. [22] suggested that a rapid Gram stain technique can predict a microbial load of $>10^5$ CFU/g of tissue if a single microorganism is observed on the slide preparation. Levine et al. [23] concluded that the presence of bacteria in Gram stain is associated with 10^6 bacteria or more per swab. Robson et al. [24] proposed that "quantitative bacterial counts from tissue biopsy samples of the ulcer 10^6 CFU /gram tissue indicate infection". In this study, this criterion was followed to assess bacterial burden, revealing that 48% of ulcers were infected. No growth was observed in 10% of ulcers. This contrasts with a study by Somaprakas et al. [25] where 90% of ulcers were found to be monomicrobial. Brook et al.'s [26] study on aerobic and anaerobic microbiology of chronic venous ulcers concluded that these ulcers are polymicrobial with both aerobic and anaerobic flora. Multiple bacterial species were detected by Kritine et al. [27] in their study on bacteria residing in chronic wounds.

Within the aerobes, Gram-positive cocci were the predominant pathogens in infected venous ulcers. Among these Gram-positive cocci, Staphylococcus aureus emerged as the most common pathogen, isolated from 17% of the ulcers, followed by *Streptococcus pyogenes* in 3% of the ulcers. Infections with *Streptococcus pyogenes* typically manifest with inflammation and have the potential to spread along draining lymphatics to focal lymph nodes, rapidly advancing through subcutaneous tissue and fascia, resulting in swift tissue destruction. [28] Madsen et al. [29] in their study on bacterial colonization and healing of venous ulcers, observed that ulcers infected with *Staphylococcus* and beta-hemolytic *Streptococcus* tended to heal slowly. *Enterococcus faecalis*, a normal flora of the skin implicated in wound infections, was isolated from 6% of the ulcers in our study, and *Staphylococcus epidermidis* was found in 6% of the venous ulcers. Mustafa Fazli et al. [30] reported *Staphylococcus aureus* in 50% of cases. Bowler et al. [22] in their study, concluded that *Staphylococcus aureus* was the most frequently isolated organism. Among the Gram-negative bacilli, *Escherichia coli* emerged as the most prevalent pathogen at 10%, followed by *Pseudomonas aeruginosa* at 10%. Brook et al. [15] reported an isolation rate of 12% for *Escherichia coli.*

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Out of the 18 *Staphylococcus aureus* isolates, 33.33% were identified as Methicillin-resistant *Staphylococcus aureus* (MRSA). In a study conducted in Brazil, the frequency of MRSA was reported to be 28%. [31] Eradicating MRSA from chronic wounds is considered almost impossible. Howell–Jones noted that with MRSA infection, challenges include cross-contamination of wounds from patients themselves, fomites, and healthcare personnel. [32] In a study by Frankel et al. [33] the incidence of MRSA was 45% among patients with chronic wounds. Despite appropriate treatment, chronic wounds often fail to heal due to the presence of biofilm. In this study, 33.33% of MRSA isolates demonstrated moderate biofilm production. The biofilm in MRSA isolates confers antibiotic resistance and is attributed to the presence of polysaccharide intracellular antigen. [34] None of the 6 isolates of *S. aureus* (MRSA) isolates were susceptible to penicillin, clindamycin, or vancomycin. Isolated Gram-negative bacilli included *Pseudomonas aeruginosa* and Escherichia coli at 10.14% each followed by *P mirabilus* at 5.79% *Acinetobacter baumanii* at 1.44%. Halbert et al. [35] reported no significant delay in wounds colonized by *Pseudomonas aeruginosa*. These organisms were found to be susceptible to all tested antibiotics. This contrasts with a study conducted in Eastern India, where metallo-beta-lactamase (MBL) producing strains of *Acinetobacter baumanii* and *Acinetobacter lwoffii* were isolated from venous ulcers. [34] Ulcers with prolonged duration and larger surfaces tend to show increased microbiological diversity. However, it's crucial to note that ulcer colonization doesn't always lead to clinically apparent infection. Innate immune mechanisms often prevent microbial overgrowth in colonized cases, averting symptomatic infections. Nevertheless, infections may arise when ulcers host highly virulent pathogens, particularly those capable of biofilm production, potentially delaying wound healing. Prolonged empirical antibiotic therapy may further contribute to the selection of drug-resistant microbial strains, especially concerning alert pathogens with robust antibiotic resistance mechanisms.

Conclusion

Within the limitation of the current study, we found venous leg ulcers are predominantly observed in the older age groups. The primary pathology associated with these ulcers is perforator incompetence. Noteworthy risk factors include deep vein thrombosis (DVT), obesity, and varicose veins. The diagnosis of infection can be effectively accomplished through the quantitative culture method using tissue biopsy, revealing ulcers as either monomicrobial or polymicrobial with *Staphylococcus aureus* being the most common pathogen, followed by members of *Escherichia coli*. Methicillin-resistant *Staphylococcus aureus (MRSA)* is a significant pathogen in the etiology of venous leg ulcers, with the majority also identified as moderate biofilm producers. Within the *Enterobacteriaceae* family, extended-spectrum beta-lactamase (ESBL) production contributes to antimicrobial resistance, although no AmpC or metallo-beta-lactamase (MBL) producers were detected in this study. Anaerobes constitute a notable proportion of the etiological agents in patients with venous leg ulcers. To guide treatment initiation for venous leg ulcers, it is essential to determine the presence of infection or non-infection in the wounds. Given the ongoing debate on whether to treat colonized wounds, direct microscopy, and quantitative microbiological culture, detecting the presence of bacteria exceeding 10^5 CFU/gram tissue, can serve as a useful guide.

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