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Bacterial flora of chronic venous leg ulcers: shifts over a two-decade period

Bakterijska flora hroničnih venskih ulceracija nogu: promene tokom dvadesetogodišnjeg perioda

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Abstract

Background/Aim. Venous leg ulcers (VLUs) are often colonized by various types of bacteria, which can lead to infection that requires empirical antibiotic therapy. Bacterial types and their antibiotic susceptibility vary widely over time and by geographic location. The aim of the study was to determine the change trends of causative bacteria and antibiotic susceptibility in patients with VLUs over the past two decades. **Methods.** The study was conducted at a tertiary care dermatovenereology clinic. Data on the microbiological analysis of ulcer swabs from patients with VLUs were collected over two consecutive months at three time points: 2001, 2014, and 2020. **Results.** The study included 250 patients divided into three groups. The first group (2001) had 111 participants, the second group (2014) had 64, and the third group (2020) had 75 participants. Out of the 250 swabs sent for microbiological testing, only 4 (1.6%) samples yielded negative results. In 2001, the bacteria identified were 31.83% Gram-positive (G+) and 68.13% Gram-negative (G-). In 2014, the ratio between G+ and G- bacteria was 27.18% vs. 72.82%. In 2020, the predominance of G- bacteria was even more pronounced, with 89.86% compared to 10.14% G+ ($p < 0.001$). In 2001, *Pseudomonas (P.) aeruginosa* (*P. aeruginosa*) (26.54%) was the predominant G- bacterium, and *Staphylococcus* (*S.*) *aureus* (24.78%) was the most common G+ bacterium. In 2014, *P. aeruginosa* (30.10%) and *S. aureus* (23.30%) remained prevalent. By 2020, *P. aeruginosa* (35.14%) increased further, while *S. aureus* (7.43%) decreased significantly ($p < 0.001$). Antibiotic sensitivity varied over the years, with older antibiotics showing decreased efficacy and newer classes demonstrating increased sensitivity. **Conclusion.** Throughout the study period, there was a notable shift toward G- bacterial dominance, particularly *P. aeruginosa*. Antibiotic resistance patterns also evolved. This study highlights the importance of local surveillance of bacterial flora of VLUs and antibiotic resistance profiles.

Key words:
anti-bacterial agents; bacterial infections; drug resistance, bacterial; gram-negative bacteria; gram-positive bacteria; varicose ulcer; microbiology.

Apstrakt

Uvod/Cilj. Venske ulceracije nogu (VUN) su često kolonizovane različitim vrstama bakterija, što može dovesti do infekcije koja zahteva empirijsku terapiju antibioticima. Vrste bakterija i njihova osetljivost na antibiotike znatno variraju tokom vremena i u zavisnosti od geografske lokacije. Cilj istraživanja bio je da se utvrde trendovi promene uzročnih bakterija i osetljivosti na antibiotike kod bolesnika sa VUN u poslednje dve decenije. **Metode.** Istraživanje je sprovedeno u tercijarnoj dermatovenerološkoj klinici. Podaci o mikrobiološkoj analizi briseva uzetih od bolesnika sa VUN prikupljani su tokom dva uzastopna meseca, u tri vremenska perioda: 2001, 2014. i 2020. godine. **Rezultati.** Istraživanjem je obuhvaćeno 250 bolesnika, podeljenih u tri grupe. Prva grupa (2001) imala je 111 učesnika, druga grupa (2014) imala je 64, a treća grupa (2020) 75 učesnika. Od 250 briseva poslatih na mikrobiološku analizu, rezultat je bio negativan kod samo 4 (1,6%) uzorka. U 2001. godini identifikovano je 31,83% Gram-pozitivnih (G+) i 68,13% Gram-negativnih (G-) bakterija. Tokom 2014. godine, odnos G+ i G- bakterija bio je 27,18% vs. 72,82%. U 2020. godini, dominacija G- bakterija postala je još izraženija, sa 89,86% u poređenju sa 10,14% G+ ($p < 0,001$). U 2001. godini, *Pseudomonas* (*P.) aeruginosa* (26,54%) bila je pretežna G- bakterija, dok je *Staphylococcus* (*S.) aureus* (24,78%) bila najčešća G+ bakterija. U 2014. godini, *P. aeruginosa* (30,10%) i *S. aureus* (23,30%) i dalje su preovlađivali. Do 2020. godine, učestalost javljanja *P.*

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aeruginosa (35,14%) je dodatno povišena, dok je učestalost *S. aureus* (7,43%) značajno smanjena (p < 0,001). Osetljivost na antibiotike varirala je tokom godina, pri čemu su stariji antibiotici pokazivali smanjenu efikasnost, dok su novije klase pokazale povećanu osetljivost. **Zaključak.** Tokom perioda istraživanja, došlo je do značajnog prelaska ka dominaciji G- bakterijske flore, posebno *P. aeruginosa*. Obrasci rezistencije na antibiotike su se takođe menjali. Ova studija naglašava značaj lokalnog praćenja bakterijske flore kod VUN i profila otpornosti na antibiotike.

Ključne reči:
antibiotici; infekcija, bakterijska; lekovi, rezistencija bakterija; gram-negativne bakterije; gram-pozitivne bakterije; venska ulceracija; mikrobiologija.

Introduction

Venous leg ulcers (VLUs) represent the most common type of chronic wounds. It is estimated that 70–90% of all chronic leg wounds are caused by chronic venous insufficiency (CVI) [1, 2]. VLUs are often colonized by various types of bacteria without showing signs of infection. However, in certain cases, signs of infection may develop. If left unrecognized and untreated, this can lead to prolonged healing times, enlargement of the ulcer surface, and an increased risk of complications.

Recognizing signs of infection and promptly initiating treatment is one of the most challenging tasks for doctors treating such patients. This assessment is highly subjective and relies primarily on the physician’s experience. It is recommended that VLUs with clinical evidence of infection be treated with systemic antibiotics [3]. When antibiotic therapy is necessary, it is typically initiated empirically, based on assumptions about the type of bacteria and their antibiotic sensitivity, until the results of the microbiological analysis of the swab taken from the ulcer are obtained.

Significant variations exist in the most frequently isolated bacteria depending on geographical distribution. Moreover, the commonly isolated types of bacteria and their antibiotic sensitivity can change over time.

The aim of the study was to investigate whether a change has occurred in the types of most commonly isolated bacteria and their antibiotic sensitivity over a twenty-year period.

Methods

The study was conducted at the Phlebology Department of the Clinic of Dermatovenereology Diseases, University Clinical Center of Vojvodina, Serbia, the only subspecialist department of its kind in the region, serving approximately 2 million inhabitants and specializing in the treatment of VLUs. The study was approved by the Ethics Committee of the University Clinical Center of Vojvodina (No. 00-08/332, from September 26, 2024). We anonymously collected microbiological data from ulcer swabs of patients with single or multiple VLUs. Data were gathered over two consecutive months at three time points: 2001, 2014, and 2020. All patients treated at the Phlebology Department during these periods were included, provided they met the inclusion criteria. Only outpatients with CVI confirmed by duplex scan, ankle-brachial index (ABI) values between 0.9 and 1.3, and those over 18 years were eligible. Microbiological samples were collected from patients who had not received systemic antibiotics, topical antibiotics, or topical antiseptics for at least four weeks prior to swab collection. All patients received standard local therapy for VULs (regular wound cleaning with saline solutions and maintaining a moist wound environment by applying hydrocolloids, hydrogels, alginates, and foam dressings).

The study included an initial ulcer swab for each patient. Prior to sampling, each ulcer was thoroughly cleaned with a saline solution, and any necrotic material or crust was removed. The sample was taken using the Levine technique. The swab was gently pressed into the wound bed over an area of about 1 cm². It was rotated while applying enough pressure to express fluid from the tissue. Afterward, each swab was sent for standard microbiological testing. The time between sampling and incubation was no more than 2 hrs. The swabs were tested for microbial sensitivity and resistance using a standard antibiotic set.

Statistical analysis

A database was compiled with data on patient age, gender, the number and species of bacteria isolated from ulcer swabs, and their antimicrobial sensitivity. Statistical analyses were performed using SPSS for Windows, version 26 (IBM SPSS, Chicago, Illinois). Descriptive statistics, including arithmetic means and minimum and maximum values, were used to summarize quantitative variables. Qualitative variables were expressed as frequencies and percentages. The Chi-square test was applied to analyze attributive variables, allowing for comparisons across the different study groups. All statistical tests were two-tailed, with a significance level set at $p < 0.05$.

Results

The study included 250 patients divided into three groups according to the time periods. The first group (2001) comprised 111 participants, 70 females and 41 males, aged 46 to 91 years (the average age was 66.9 years). The second group (2014) included 64 respondents, 47 females and 17 males, aged 51 to 87 years (the average age was 65). The third group (2020) consisted of 75 patients, 51 females and 24 males, aged 47 to 82 years (the average age was 65.3 years).

Out of the 250 swabs sent for microbiological testing, the result was negative in only 4 (1.6%) samples. In the analyzed samples, a single bacterium was isolated in most cases, 100 (40%) out of 250, while four bacteria were found in only 18 (7.2%) samples. Detailed data on the number of isolated bacteria in individual samples are presented in Table 1.

In 2001, a total of 223 bacteria were isolated from 111 samples (mean = 2.01), identifying 33 different bacterial species. Of these, 31.83% were Gram-positive (G+) bacteria, while 68.13% were Gram-negative (G-). In 2014, the average number of bacteria *per* sample decreased to 1.61, and the number of bacterial species dropped to 23. The ratio of G+ to G- bacteria was 28 (27.18%) to 75 (72.82%). By 2020, the average number of bacteria *per* sample increased slightly to 1.97, but the number of bacterial species fell further to just 18. The predominance of G- bacteria became even more pronounced, with 133 (89.86%) compared to 15 (10.14%) G+. The difference in the frequency of isolation of G- compared to G+ bacteria through three study periods was highly significant, at $p < 0.001$ (Table 2).

In 2001, the most common G- bacterium was *Pseudomonas* (*P.) aeruginosa* (26.54% of the total number of bacteria), while the most common G+ bacterium was *Staphylococcus* (*S.) aureus* (24.78%). In 2014, the situation was somewhat different. The most common G- and G+ bacteria were still *P. aeruginosa* and *S. aureus*, but with different frequencies: 30.10% and 23.30%, respectively. In 2020, we observed a further widening in the frequency difference between the most common G- and G+ bacteria, with *P. aeruginosa* accounting for 35.14% of cases, while *S. aureus* was present in only 7.43%, and this difference in frequency was also highly significant, with $p < 0.001$ (Table 3).

Table 1

Number of isolated bacteria in individual samples of chronic venous leg ulcers across [1]three time points

Number of isolated bacteria	2001 (n = 111)	2014 (n = 64)	2020 (n = 75)
0	2 (1.80)	1 (1.56)	1 (1.33)
1	37 (33.33)	36 (56.25)	27 (36)
2	38 (34.24)	17 (26.56)	27 (36)
3	26 (23.42)	7 (10.94)	13 (17.33)
4	8 (7.21)	3 (4.69)	7 (9.34)
Total number (mean)	223 (2.01)	103 (1.61)	148 (1.97)

Results are given as numbers (percentages) except for the total number.
[1]Note: the term three time points is reffered to entire individual years, i.e., 2001, 2014, and 2020.
n – number of samples (i.e., number of respondents) during that year.

Table 2

Gram-positive and Gram-negative bacteria isolated in study samples of chronic venous leg ulcers across [1]three time points

Year	Total number of isolated bacteria in all samples	Average number of bacteria *per* sample	Number of bacterial species	Gram-positive bacteria	Gram-negative bacteria	*p*-value
2001	223	2.01	33	71 (31.83)	152 (68.13)	
2014	103	1.61	23	28 (27.18)	75 (72.82)	< 0.0001
2020	148	1.97	18	15 (10.14)	133 (89.86)	

Results are shown as numbers (percentages).
[1]Note: see explanation in Table 1.

Table 3

Most common isolated bacterial species of chronic venous leg ulcer samples across [1]three time points

2001		2014		2020	
bacterial species	%	bacterial species	%	bacterial species	%
Pseudomonas aeruginosa	26.54	*Pseudomonas aeruginosa*	30.10	*Pseudomonas aeruginosa*	35.14
Staphylococcus aureus	24.78	*Staphylococcus aureus*	23.30	*Enterobacter* species	12.84
Escherichia coli	4.87	*Enterobacter* species	8.73	*Acinetobacter* species	8.78
Enterobacter species	3.98	*Proteus mirabilis*	5.82	*Staphylococcus aureus*	7.43
Proteus mirabilis	3.98	*Acinteobacter* species	5.82	*Serratia* species	6.76

[1]Note: see explanation in Table 1.

Table 4

Three most effective antibiotics against isolated Gram-positive (G+) and Gram-negative (G-) bacteria across [1]three time points

2001	2014	2020
Antibiotics against G+		
chloramphenicol (65.46)	trimethoprim + sulphamethoxazole (83.33)	cephalosporins (90.32)
gentamicin (50.91)	cephalosporins (80.28)	gentamicin (77.78)
trimethoprim + sulphamethoxazole (47.28)	tetracycline (73.91)	quinolones (76.20)
Antibiotics against G-		
ciprofloxacin (80.70)	piperacillin-tazobactam (84.48)	carbapenems (91.70)
amikacin (74.56)	carbapenems (82.03)	piperacillin + tazobactam (81.67)
ofloxacin (68.42)	cephalosporins (75.37)	cephalosporins (75.60)

Results are shown as percentages of bacteria sensitive to the specific antibiotic.

[1]Note: see explanations in Table 1.

In 2001, G+ bacteria exhibited the highest sensitivity to chloramphenicol and gentamicin, while G- bacteria were most susceptible to antibiotics from the quinolone group. According to the results of the antibiogram in 2014, G+ was most sensitive to trimethoprim combined with sulphamethoxazole (83.33%), cephalosporins (80.28%), and tetracycline (73.91), and G- was most sensitive to piperacillin-tazobactam (84.48%), carbapenems (82.03), and cephalosporins (75.37%). In 2020, G+ bacteria exhibited the highest sensitivity to cephalosporins, gentamycin, and quinolones, while G- bacteria were most susceptible to the carbapenem group, piperacillin with tazobactam, and newer-generation cephalosporins (Table 4).

Discussion

A chronic lower leg ulcer is a type of wound that fails to progress through the usual stages of healing within the expected timeframe, typically over six weeks [4]. These wounds are associated with underlying health conditions such as venous insufficiency, arterial disease, diabetes mellitus, or prolonged pressure and require specialized medical treatment to promote healing and prevent complications. VLUs are a typical example of chronic wounds. The prevalence of lower leg wounds is around 1%, and CVI is by far the most common cause [5].

Bacteria inhabit the wound bed from the onset of wound formation, with their population increasing over time. Although virtually all VLUs are colonized by bacteria, some may develop into overt infections. Chronic open wounds create an ideal environment for bacteria, which allows them to establish, survive, and evolve [6, 7].

The infection status of chronic wounds can be stratified into four stages [8–10]. The first stage is contamination – non-replicating bacteria are present in the ulcer and do not cause any damage or host response. The second stage is colonization – bacteria begin to replicate and grow on the surface of a wound, but the host's immune response is very weak. Colonization does not lead to deterioration of the ulcer's status, and healing is not compromised. The third stage is critical colonization – the number of bacteria increases significantly, which interferes with the ulcer's healing process and may cause subtle local signs of infection. However, there is still no strong activation of the patient's immune system. Finally, the fourth stage is infection – the number of bacteria is rapidly increasing. The local clinical findings of the ulcer are deteriorating. Healing is compromised. Signs of infection are prominent, and the patient complains of intense pain. There is a marked activation of the patient's immune system.

In the first two phases, the presence of microorganisms usually does not affect the healing of ulcerations. In the third and fourth phase, therapeutic intervention is necessary. The difference between colonization and infection is typically subtle and not easily determined. It primarily relies on clinical assessment rather than the microbiological status of the ulcer [9].

Various studies have recorded a large number of different bacteria isolated from VLUs. Regardless of the geographic location where the studies were conducted, the predominant G- microorganisms found in leg ulcer infections were *P. aeruginosa* and *Escherichia coli*, whereas *S. aureus* was the leading G+ microorganism [6, 9, 11, 12]. However, since bacterial colonization of VLUs tends to be polymicrobial, many other species of bacteria can be isolated from this type of chronic wound. Nevertheless, it is important to note that even extensive colonization of VLUs by various bacteria does not always result in clinically evident infections. This can likely be explained by the fact that many of the bacteria isolated from these ulcers are part of the skin's saprophytic microflora. When these microorganisms colonize the wound, they activate the body's innate immunity mechanisms, effectively preventing their overgrowth and the onset of infection [11].

It is known that there are geographic and temporal differences in the species of bacteria most commonly isolated from VLUs and their antibiotic susceptibility. Geographic differences are influenced by factors such as local healthcare practices, hygiene conditions, climate, and access to medical care. Temporal differences can be caused by changes in medical protocols, the emergence of antibiotic resistance, and the development of new treatment methods. As a result, the types of bacteria most frequently isolated from these wounds can vary considerably between different regions and over various time periods [13].

In our study, we observed significant changes in the frequency of occurrence of certain bacteria and their susceptibility to antibiotics over time. We noted that over 20 years, there was a markedly higher prevalence of G-bacteria in isolates from VLUs. *P. aeruginosa*, the most prevalent in our samples from the beginning, accounted for more than one-third of all isolated bacteria after 20 years. The most common G+ bacterium, *S. aureus*, appeared significantly less frequently in later periods. Sensitivity to antibiotics has also changed significantly. We noted resistance to older types of antibiotics and considerable sensitivity to newer classes.

There is a concerningly rapid increase in bacterial resistance to antibiotics, outpacing the development of new antibiotics entering the market [14, 15]. This trend poses a significant threat to public health, as it limits the effectiveness of current treatments. It is crucial to keep this in mind when selecting and prescribing antibiotics.

Although there are numerous guidelines for treating infections in VLUs and chronic wounds in general, there is still no consensus on the methods for diagnosing and implementing therapy. Most wound care practitioners generally evaluate and manage bacterial infections in chronic wounds by observing clinical signs and patient-reported symptoms rather than depending exclusively on objective measures [16]. After an infection is diagnosed, two types of antimicrobial treatments are available for infected VLUs: systemic antibiotics and topical preparations [17].

Most guidelines recommend systemic antibiotics as the first choice of therapy for infections, but only in cases with clear signs of the infection. No evidence suggests that the routine or preventive use of systemic antibiotics enhances healing rates for non-infected VLUs [7, 17]. Since this therapy usually needs to begin immediately after determining the infection of the ulcer and before the ulcer swab results are available, the treatment often starts empirically with later reevaluation and possible adjustment. Therefore, it is crucial to have up-to-date local data on the predominant bacterial flora and their antibiotic resistance.

The topical application of antibiotics in the treatment of VLUs is generally not recommended [3]. It leads to a temporary reduction in bacterial counts and inflammation. However, bacteria can survive in very high antibiotic concentrations, leading to the development of multi-resistant strains of microorganisms. Additionally, local antibiotic therapy often results in contact sensitization [18, 19]. Therefore, in the local treatment of an infection, we typically employ adequate debridement and the application of antiseptics and appropriate dressings.

Conclusion

Over the study period, there was a notable shift towards Gram-negative bacterial dominance, particularly *Pseudomonas aeruginosa*. Antibiotic resistance patterns also evolved, with older antibiotics showing decreased efficacy and newer classes demonstrating increased sensitivity.

This study highlights the importance of local surveillance to guide effective treatment strategies in managing venous leg ulcers, emphasizing the need for updated knowledge on bacterial flora and antibiotic resistance profiles.

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