Analysis of the flora of venous and diabetic ulcerations

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Abstract The aim of the study was to analyze the microflora of leg ulcerations of different etiology. The research materials were swabs from bottom of ulcers taken from 44 patients with venous ulcerations (group I) and 44 patients with diabetic ulcerations (group II). Microorganisms were detected in 77.27% of patients with venous ulcerations compared with 86.36% of patients with diabetic ulcerations. In group I the most common bacterium was *Staphylococcus aureus*, whereas in group II they were *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. In patients with diabetic ulceration microflora consisting of several kinds of bacteria was detected more often than in patients with venous ulceration. Obtained results revealed significant differences between the microflora of venous and diabetic ulcerations.

Key words diabetic ulceration • microflora • venous ulceration

Introduction

Ulcerations of the lower leg of venous and diabetic origin are the most frequent of all chronic wounds. One of the reasons for delayed healing of such wounds is disturbance in the supply of nutrients and removal of metabolic products, caused by the pathology of blood vessels. In consequence, this leads to hypoxia of tissues resulting in necrosis. Another important reason for delayed healing of ulcerations is their high susceptibility to infections [2, 9, 17, 24].

Infection may be caused by pathogenic bacteria originating from the external environment, as well as bacteria forming physiological microflora of the skin (e.g. *Staphylococcus epidermidis, Staphylococcus aureus, and Propionibacterium acnes*) [23]. Pathogenic microflora is often transferred unconsciously by medical personnel and materials and substances used for treatment. Both chronic venous ulceration and diabetic ulceration may generally be deemed to be colonized, although such wounds do not always show clinical signs of infection in every case. The presence of infection depends mainly on the number of microorganisms residing in the wound, while the healing process depends on the type of bacterial strains and their pathogenicity. Ulcerations are prone to colonization by nearly every microorganism that can come in contact with their surface. Usually ulcerations contain mixed flora, consisting of several strains of bacteria. Most often these are aerobic bacteria, such as *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and anaerobic bacteria, e.g. *Bacteroides fragilis, Clostridium perfringens, Peptostreptococcus sp. and Prevotella oralis*. The mechanism by which bacteria delay the healing process is not completely understood [3, 6, 11, 14, 26]. It is suggested that the main role is played by the pathogenic virulence factors of bacteria, such as:

- bacterial adhesions, proteins present on the surface of bacterial cells which are responsible for their adhesion to host cells, allowing colonization of the ulceration;
- exoenzymes decomposing cell material such as collagen and fibrinogen, allowing deeper penetration to tissues and modifying bacterial resistance;
- toxins, protein substances released from bacterial cells, responsible for clinical signs of infection.

Decrease of oxygen perfusion in the ulceration and necrotic tissues provide aerobic and anaerobic bacteria with good conditions for growth [8, 12, 15]. Additional changes in microbial virulence may also be caused by the synergistic reaction between aerobic and anaerobic bacteria as well as by modification of bacterial resistance. In the resulting wound infection, the healing process is inhibited and inflammatory state is maintained. The presence of pathogenic microorganisms in the wound intensifies the production of exudate with pathological composition. Excess of cytokines, exoenzymes, bacterial toxins and proteolytic enzymes destroys growth factors, proteins, decreases collagen synthesis, increases its lysis and destroys freshly produced extracellular matrix. On top of that, these substances inhibit migration and fibroblast activity. Moreover, infection decreases the amount of oxygen that is available for the process of collagen synthesis. In extreme cases, where there is lack of proper treatment, significant colonization of the wound may take place, which leads to infection of the ulceration, its enlargement and tissue necrosis. Infected ulcerations may also be a source of generalized infections, such as inflammation of lymph vessels or sepsis, which may eventually require amputation of the limb [2, 4, 6, 11, 13, 15, 21, 26].

From the point of view of the presence of bacteria in the ulceration and clinical signs of inflammation, wounds may be divided into three groups:

1. uncolonized ulceration without clinical signs,
2. colonized ulceration without visible clinical signs,
3. infected ulceration with clinical signs of infection.

The aim of this work was to analyze bacterial flora of lower leg ulceration, depending on etiology.

Material

The prospective study included 88 patients treated for ulceration of the lower limb. Patients were treated in the out-patient setting, in the Surgical Clinic, Department and Clinical Ward of General Surgery, Silesian Medical University, Bytom, Poland, in the period from January 1998 to January 2005. The patients were divided into two groups, depending on etiology of the ulceration.

Group I included patients with ulceration of venous origin. It consisted of 26 women and 18 men aged 52 to 68 (average 57.9 years) (Table 1). Ulceration area was 12.1 cm^2 to 20.3 cm^2 (average 15.94 cm^2), while duration of ulceration was from one year to seven years (average 3.7 years). In 19 patients ulceration was located on the left leg, and in 25 on the right leg. Full range of mobility of lower leg was recorded in 30 people, while limited mobility in 14. Ankle-brachial pressure index (API) was in the range 0.65-0.8 this is unusual for VLU(average 0.74). Body mass index (BMI) was found to be 19.8-37.8 (average 26.3).

Group II included patients with ulceration of diabetic origin. This group consisted of 30 women and 14 men aged 50 to 70 (average 59.8 years) (Table 1). The area of ulceration in these patients was 14.2 cm^2 to 18.9 cm^2 (average 16.1 cm^2), while duration of the ulceration was from 1.6 to 5 years (average 2.4 years). In 18 patients ulceration was

located on the left leg, and in 24 on the right. Full range of mobility was noted in 21 patients, and limited mobility in 23. Ankle-brachial pressure index (API) was in the range 0.68-0.85 (average 0.78). Body mass index (BMI) was 21.0 to 46.2 (average 29.1). In 23 patients of from group II, signs of diabetic microangiopathy were observed, and signs of neuropathy in 21 patients. Type II diabetes was diagnosed in all patients, and insulin was administered in the dose depending on glucose serum concentration.

Patients from both groups were previously treated in other centers. All patients were treated in accordance to the criteria published in the Helsinki Declaration of Human Rights.

Method

Before the onset of the treatment, swabs were taken from all patients onto a transport medium (Biomed). Obtained material was cultured on the medium (bioMerieux), according to routine microbiological diagnostic methods, assuring growth of aerobic and anaerobic bacteria. Identification of isolated strains of bacteria was performed with use of identification kits ID 32 and API 20 in ATB system (bioMerieux). Mechanisms of resistance of isolated strains were evaluated with anti-biograms prepared with the diffusion disk method, according to the recommendations of the National Reference Center for Drug Susceptibility of Microorganisms [22].

Table 1. Characteristics of patients with ulcerations of lower leg of venous and diabetic origin.

	Group I	Group II
Number of patients	44	44
Sex:		
men	18	14
women	26	30
Age (years)	52-68 (x=57.9)	50-70 (x=59.8)
Area of ulceration (cm^2)	12.1-20.3 (x=15.9)	14.2-18.9 (x=16.1)
Duration of ulceration (years)	1-7 (x=3.7)	1.6-5 (x=2.4)
Location of ulceration		
left lower leg	19	18
right lower leg	25	26
Mobility:		
full	30	21
limited	14	23
BMI [kg/cm^2]	19.8-37.8 (x=26.3)	21-46.2 (x=29.1)
API	0.65-0.8 (x=0.74)	0.68-0.85 (x=0.78)

Results

In the current study, absence of any bacteria was found in the case of ten venous ulcerations and six diabetic ulcerations. In 21 cases, ulcerations of patients in group I were deemed to be colonized without clinical signs, while in the remaining 13 cases signs of infection were noted. At the same time, 20 ulcerations of diabetic origin (group II) were assessed to be infected without visible clinical signs, while signs of infection were recorded in 18 cases (Table 2).

Staphylococcus aureus was found most frequently in ulcerations of patients in group I. It was found in 28 (63.63%) cases (Table 3). *Enterococcus faecalis* was cultured in 15 (34.09%) patients with venous ulceration. The next most frequently found bacterium was *Streptococcus pyogenes*, present in 12 (27.27%) cases. The most frequently isolated Gram-negative bacteria was *Proteus vulgaris*, detected in 11 (25.00%) patients, and then *Pseudomonas aeruginosa*, in 10 (22.72%) patient ulcerations from group I. At the same time *Escherichia coli* and anaerobic *Bacteroides fragilis* were found in eight (18.18%) patients with venous lower leg ulcerations. In addition, in six (13.63%) ulcerations of patients from group I *Streptococcus β-hemolitycus* was detected. *Corynebacterium sp.* was isolated from four (9%) venous ulcerations of lower leg, while *Klebsiella pneumoniae* was present in three (6.80%) cases in group I. The least frequently found bacterium was *Morganella morganii*, which was present in only two (4.50%) venous ulcerations of the lower leg.

Table 2. Frequency of appearance of bacterial flora in ulcerations with clinical signs of infection

Clinical signs of infection	Group I n (%)	Group II n (%)
Non-infected ulcerations	10 (22.73)	6 (13.64)
Ulcerations infected with bacteria, without visible clinical signs	21 (47.73)	20 (45.45)
Infected ulcerations with clinical signs	13 (29.54)	18 (40.91)
Total	44 (100.00)	44 (100.00)

The bacterial flora found in leg ulceration of diabetic origin had a different composition (Table 3). The most frequently found bacterium ,as in the case of group I, *Staphylococcus aureus*, which was found in 30 (71.43%) ulcerations. Meanwhile, *Pseudomonas aeruginosa* strain was detected in 29 (69.05%) wounds of patients from group II. The next most frequent bacteria were *Enterococcus faecalis* in 28 (66.66%) ulcerations of diabetic origin. *Proteus vulgaris* was present in 18 (42.86%) ulceration in patients from group II while *Streptococcus pyogenes* and *Escherichia coli* were found in 15 (35.71%) ulceration of diabetic origin. *Bacteroides fragilis* was obtained from seven (16.16%) ulcerations of patients from group II and *Streptococcus β-hemolitycus* and *Corynebacterium sp.* were isolated from six (14.29%) diabetic ulcerations. Meanwhile, *Klebsiella pneumoniae* and *Morganella morganii* were detected in four (9.52%) ulcerations of diabetic origin.

All infected ulcers were colonized by aerobic bacteria, and only a part of these wounds by anaerobic bacteria (18%). In the group of aerobic bacteria, *Staphylococcus aureus* was most often isolated from ulcers without clinical signs, in both group I and group II. Among ulcers with obvious signs of infection, anaerobic bacteria *Bacteroides fragilis* were found more frequently in group I than in group II.

Table 3. Frequency of individual bacteria in ulcerations of venous and diabetic origin.

Type of bacteria	Group I n (%)	Type of bacteria	Group II n (%)
Staphylococcus aureus	28 (63.63)	*Staphylococcus aureus*	30 (71.43)
Enterococcus faecalis	15 (34.09)	*Pseudomonas aeruginosa*	29 (69.05)
Streptococcus pyogenes	12 (27.27)	*Enterococcus faecalis*	28 (66.66)
Proteus vulgaris	11 (25.00)	*Proteus vulgaris*	18 (42.86)
Pseudomonas aeruginosa	10 (22.72)	*Streptococcus pyogenes*	15 (35.71)
Escherichia coli	8 (18.18)	*Escherichia coli*	15 (35.71)
Bacteroides fragilis	8 (18.18)	*Bacteroides fragilis*	7 (16.16)
Streptococcus β-hemolitycus	6 (13.63)	*Streptococcus β-hemolitycus*	6 (14.29)
Corynebacterium sp.	4 (9.00)	*Corynebacterium sp.*	6 (14.29)
Klebsiella pneumoniae	3 (6.80)	*Klebsiella pneumoniae*	4 (9.52)
Morganella morganii	2 (4.50)	*Morganella morganii*	4 (9.52)

In our study we also found that among all infected ulcers of group I, both with and without clinical symptoms, from 22 (50%) cases one bacterial species was isolated. Other ulcers were colonized by mixed microflora. In group II a single pathogenic species was found in 18 (40.91%) ulcerations. Other cases were infected with multi-species microflora. *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacteroides fragilis* were isolated very rarely both in group I and group II (Table 4, Fig. 1A-B).

Discussion

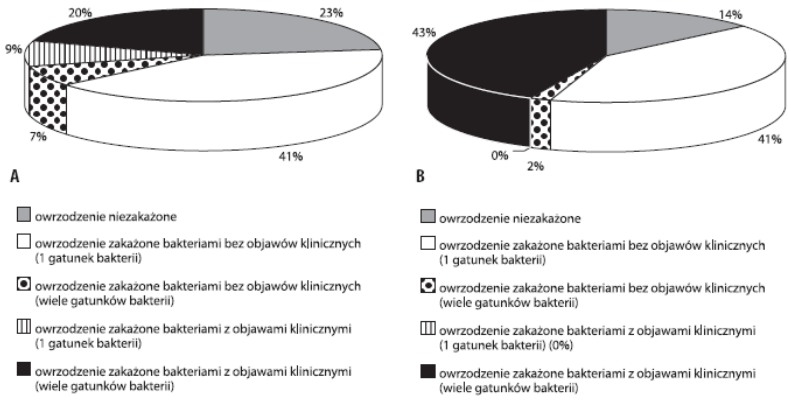
Local factors that influence long-term healing of ulceration include disturbed circulation, ischemia, hypoxia, oedema of tissues and colonization of the wound by bacteria. It is assumed that chronic ulceration of lower extremities are never septic. Sometimes venous ulcerations heal without complications even with obvious bacterial colonization. The area of ulceration also plays an important role in determining healing . Usually ulcers with the diameter of about 5cm with proper treatment have the biggest chance for healing, while, ischaemic or diabetic ulcers show prolonged healing process in the case of bacteria [1, 2, 9, 14-16, 18, 24, 26]. Schmit and colleagues evaluated bacterial flora in patients with ulceration of the lower extremities of venous, arterial and diabetic origin [25]. They found bacteria in 95% of arterial ulcerations and 71% of diabetic ulcerations. Their results showed venous ulcerations usually contained *Staphylococcus aureus*, while diabetic ulcerations most

frequently contained *Enterobacter cloacae* and *Pseudomonas aeruginosa*. Seneczko et al. assessed bacterial flora in 33 patients with ulceration of the lower leg caused by chronic venous insufficiency and flora originating from the skin of 30 healthy people (control group) [24]. The authors demonstrated that in 97% of evaluated cases from both groups pathogenic bacteria were isolated. The bacteria most frequently isolated from venous ulcerations were *Staphylococcus aureus* (30% of cases), *Pseudomonas aeruginosa* (22.50%) and *Escherichia coli* (7.50%). Mazur et al. evaluated bacterial flora from 30 venous ulcerations of lower leg [20]. *Staphylococcus aureus* was most frequently isolated bacterium.

In this study we found varying frequency of bacterial infections. Venous ulceration contained bacteria in 77.2% cases, while diabetic ulceration in 86.36% cases. *Staphylococcus aureus* was most often isolated from venous ulceration (63.63%), while *Staphylococcus aureus* (71.43%), *Pseudomonas aeruginosa* (69.05%) and *Enterococcus faecalis* (66.66%) were most frequent in diabetic ulceration. On the basis of obtained results we found that *Pseudomonas aeruginosa* was three times more often isolated from diabetic ulceration (69.05%) than from venous ulceration (22.72%). *Escherichia coli* were also twice as often present in ulceration in patients from group II (35.71%) than in wounds of patients from group I (18.18%). At the same time, *Streptococcus β-hemolitycus* was less and similarly frequent in ulceration in patients from group I (13.63%) and group II (14.29%). No significant differences of frequency of aerobic *Bacteroides fragilis* were found in venous (18.18%) and diabetic ulceration (16.16%).

In this study bacteria were not isolated in the case of ten (22.73%) ulcers of patients from group I and six (13.64%) wounds of patients from group II. As mentioned before, it is difficult to keep a chronic wound completely septic. The fact of lack of isolates from as many as 16 ulcerations does not signify absence of microorganisms in these ulcers. In such cases anaerobic bacteria may be present, which usually reside deep at the base of the wound or under necrotic tissue, and are very difficult to access for adequate collection and culture. Moreover, lack of most frequent aerobic bacteria in these ulcers confirms a possibility of the presence of anaerobic bacteria. The aerobic bacteria mentioned above were not present in ulcers where anaerobic bacteria were found. Similar results were published by Bowler and Davis, who evaluated bacterial flora appearing in acute and chronic wounds [5]. In the case of chronic wounds from which anaerobic bacteria were isolated, such as *Peptostreptococcus sp*., *Prevotella sp*. or *Clostridium perfringens*, no colonization by aerobic bacteria such as *Staphylococcus aureus* or *Pseudomonas aeruginosa* (two species of bacteria most usually connected with wound infection) was demonstrated.

Fig. 1. Content of isolates (without consideration of types of bacteria) from venous and diabetic ulcerations of lower leg.



owrzodzenie niezakażone = non-infected ulceration
owrzodzenie zakażone... bez objawów (1 gatunek...) = infected ulceration, without clinical signs (1 species of bacteria)
owrzodzenie zakażone... bez objawów (wiele gatunków...) = infected ulceration, without clinical signs (many species of bacteria)
owrzodzenie zakażone... z objawami (1 gatunek...) = infected ulceration, with clinical signs (1 species of bacteria)
owrzodzenie zakażone... z objawami (wiele gatunków...) = infected ulceration, with clinical signs (many species of bacteria)

Table 4. Frequency of bacteria in ulcerations of venous and diabetic origin, with or without clinical signs of infection.

Ulcerations infected with bacteria	Group I	Group II
Ulceration without clinical signs, caused by:		
one species of bacteria	18	18
many species of bacteria	3	1
Ulceration with accompanying clinical signs, caused by:		
one species of bacteria	4	0
many species of bacteria	9	19
Total	34	38

In the18 ulcers from group I and II colonized by one species of bacteria, no clear-cut clinical signs were noted. Only four wounds of patients in group I showing clinical signs of infection were infected by a single species of bacteria, and in ulceration belonging to group II no

single isolate caused clinical signs. Meanwhile, ulcers infected by mixed bacterial microflora were accompanied by clinical signs, both in group I and II.

Mazur et al. in their research demonstrated the appearance of one microorganism in 51.8% ulcers of venous origin [20]. Mixed infection caused by two species of bacteria was demonstrated in 33.3% of patients, and by three species in 14.8% of wounds.

Soneczko et al. demonstrated appearance of single isolates in 81.82% of patients with ulcers of the lower limb caused by chronic venous insufficiency [24]. At the same time, the presence of two or more species of bacteria was shown in 6.06% of patients.

Is it generally assumed that bacteria present in ulceration above some critical value show higher virulence, which is usually the reason for the appearance of clinical signs. The phenomenon of increased virulence is called 'quorum sensing'. In the past, it had been hypothesized that quorum sensing comes from the transmission of chemical signals between bacteria of the same species. Currently, it is assumed that a chemical exchange may take place between several species of bacteria. On the basis of this phenomenon it is possible to explain why bacteria devoid of pathogenic features and appearing with other types of bacteria cause intensified clinical signs. This theory would also confirm assumption that pathogenic bacteria present in ulcerations in the form of mixed microflora are even more virulent and resistant to treatment. This is why it is suggested that synergistic interactions between bacteria are more important than the number of bacteria in the wound [7, 19].

Appearance of mixed bacterial flora in ulceration of the lower leg, both in group I and II, allows us to hypothesize that possible synergistic interactions between bacteria significantly contribute to the pathogenesis of chronic wound infection, although these mechanisms have not been completely understood. The complexity of bacterial flora within a wound means that it is difficult to define the role of individual species in infections, or point to a specific species of bacteria responsible for intensification of clinical signs of infection manifesting in ulcers. The higher number of ulcers infected with mixed bacterial flora was found in patients from group II.

Increased level of blood glucose favors faster proliferation of bacteria in the ulceration. This is why infection poses a significant danger for diabetic foot. Infection is found in most cases that end up in amputation. The most frequent pathogens are *staphylococci* and *streptococci*, although Gram-negative bacteria are found in about 50% of patients, and the infection often contains many bacterial species [1, 10]. In the current study bacteria were isolated from 86.36% of ulcerations of diabetic origin. Analysis of microflora demonstrated that the occurrence of *staphylococci* and *streptococci* was 71.43% and 69.05%, respectively.

It has been recently suggested that synergistic bacterial interactions are more important than the exact number of bacteria, because higher variability of species in the bacterial flora is associated with impaired wound healing. It is assumed that both anaerobic and aerobic bacteria unfavorably affect the wound healing process. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are most usually isolated from infected ulcers, but they are also found in ulcers not showing any clinical signs of infection. Haemolytic *streptococci* are not

often found in of lower extremity ulcers, but if they are diagnosed and effective treatment is not started promptly, they can be a cause of serious problems which may lead to massive tissue destruction [11, 13, 14, 21].

Chronic trophic ulcerations sometimes lasting many years are infected with multi-resistant bacterial species, which is an additional difficulty in application of topical treatment. Thus, targeted antibiotic therapy connected with other treatment modalities, may contribute to limitation of infection in the chronic wound and hasten wound healing process.

Conclusions

1. There are significant differences in the complex bacterial flora between ulcerations of venous and diabetic origin.
2. *Staphylococcus aureus* is most frequently isolated from ulcerations of venous origin.
3. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are most frequently isolated from ulcerations of diabetic origin.
4. Complexity of bacterial flora occurring in lower limb ulceration allows us to hypothesize that synergistic interactions of aerobic and anaerobic bacteria have higher significance in the pathogenesis of chronic ulceration than the activity of single pathogen.

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