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The profile of chronic skin wound microbiota in hospitalized dermatology patients

Mihaela Georgescu[1], Luminita Marutescu[2,*], Viorel Trifu[1], Viorica Marinescu[1], Ionel Toropoc[1], Doru Aurel Chirita[1], Marcela Poenaru[1], Monica Sorina Darmanescu[1], Daniel Costache[1], Mariana Carmen Chifiriuc[2, 3]

[1]Dr. Carol Davila Central Military Universitary Emergency Hospital "SUUMC Dr. Carol Davila", Romania
[2]University of Bucharest, Faculty of Biology, Microbiology Department, Romania
[3] Research Institute of the University of Bucharest, Romania

*corresponding author e-mail address: ***lumidascalu@yahoo.com***

ABSTRACT

Bacterial isolates cultured from hospitalized patients with chronic skin wounds were evaluated for virulence factors expression and resistance to commonly used antibiotics. 40 swab specimens were homogenized in thioglycolate broth (transport media) and then transferred on 5% sheep blood agar. The identification of microbial isolates was performed using Gram staining, conventional biochemical tests and API Biomerieux systems (20E, NE, 20Staph and 20Strep). The following soluble virulence factors were evaluated: haemolysins, other pore forming toxins (lecithinase, lipase), proteases, DN-ases. The antibiotic susceptibility testing was performed by Kirby-Bauer standard disk diffusion method (CLSI, 2013). *Staphylococcus sp.* constituted 47% of isolates, the frequencies of *Enterococcus spp.* and Gram negative isolates were 13%, respectively 20% of cultures. The majority of the analyzed isolates expressed proteolytic activity capable of degrading components of the extracellular matrix important for wound healing and pore forming toxins. A total of 29.16% of the *Staphylococcus spp*. isolates expressed multidrug resistance to antibiotics (beta-lactams, macrolids, aminoglycosides and lincosamides), oxacillin resistance in 14 cases. *Enterococcus spp.* isolates exhibited resistance to penicillin and tetracycline, gentamicin and vancomycin. The enterobacterial strains showed variable resistance. The majority of the non-fermentative Gram negative bacteria were susceptible to the tested antibiotics. This study demonstrates the presence of virulence factors responsible for matrix degradation and the rapid emergence of antibiotic-resistant bacteria, suggesting that treatment of chronic wounds should include appropriate management of colonizing bacteria.

1. INTRODUCTION

Chronic wounds associated to ulcers of different etiologies, like pressure, diabetic foot, venous and arterial ulcers, inflammation [1] represent an important medical problem and a public health burden [2]. There are several causes identified as being involved in the process of impairing the wound healing, such as: an abnormal inflammatory phase [3], biofilm associated infections caused by surface – attached microbial communities with increased phenotypic and genetic antibiotic resistance [4-6], loss of skin cells response to reparatory stimuli. Almost all chronic wounds are colonized with commensal bacteria from the skin microbiota. However, the presence of microorganisms does not signify an infection, therefore it is not obligatory the cause of a delayed wound healing [7]. Culture methods have been over the past 150 years an essential key in the treatment of chronic wounds, aiding in differentiating true pathogens from the commensal microorganisms [8]. Although the gold treatment of chronic wounds is that of the underlying causes, nonetheless antibiotics are frequently prescribed to patients with this kind of pathologies and recommendations differ according to the ulcer aetiology [9]. Due to important morbidity and mortality rates associated to diabetic foot ulcers most physicians prescribe antibiotics even in uninfected ulcers. On the other hand, when dealing with venous ulcers, physicians are advised to use antibiotics only in infected ulcers [9]. Nonetheless, the increased prescription of antibiotics in chronic wound patients has led to an increase in the antibiotic resistance rates of frequently isolated pathogens, such as the problematic methicillin resistant *Staphylococcus aureus* (MRSA) [10].

2. EXPERIMENTAL SECTION

2.1. Patients recruitment.

Patients were recruited from the Department of Dermatology of the Central Emergency Universitary Military Hospital "Carol Davila", Bucharest between July 2013 and April 2014. Inclusion criteria included age (> 18 years) and no systemic antibiotic therapy in the last 24 hours. The patient or the legally authorized representative had to be able to read and sign the informed consent.

2.2. Samples collection.

During the initial study visit, informed consent was obtained and information was collected from the patient, including age, gender, ulcers' etiology, wound's location, healing assessment, antibiotic and antiseptic administration (topical or systemic). Additionally, skin assessment and photography of the affected area were conducted.

2.3. Microbiological analysis.

Before treatment swab samples were collected for aerobic bacterial culture. The swabs were homogenized in thioglycolate broth (transport media) and then transferred on 5% sheep blood

agar. Cultures were incubated at 37°C aerobically for 24 hours. The identification of microbial isolates was performed using Gram staining, conventional biochemical tests (oxidase, catalase) and API BioMerieux systems (20E, 20NE, 20Staph and 20Strep). Bacterial isolates were evaluated for the following soluble virulence factors expression: haemolysins, other pore forming toxins (lecithinase, lipase), proteases (caseinase, gelatinase), DN-ase on specific culture media. The antibiotic susceptibility testing was performed by Kirby-Bauer standard disk diffusion method, using panels of antibiotic disks recommended by CLSI, 2013 and 2014.

3. RESULTS SECTION

Patient information is summarized in Table 1. Swab samples were collected from 40 patients, 22 males and 18 females with ages ranging from 37 to 92 (average age 62.85) years.

The etiologies of the leg chronic wounds were the following: chronic venous insufficiency, arterial insufficiency, type I&II diabetes mellitus, necrotizing vasculitis, Kaposi disease, squamous cell carcinoma, bone necrosis. 35 patients presented only one etiology of the leg ulcer(s), whereas 6 patients had an association of 2 factors: 5 patients - chronic venous insufficiency and diabetes mellitus, 1 patient - arterial insufficiency and diabetes mellitus.

Antiseptic and antimicrobial use data revealed that most patients used boric acid 2% solution before admission, followed by silver sulfadiazine cream and bacitracin zinc/neomycin sulphate powder. Table 1 summarizes the antiseptic and antimicrobial usage before admission.

Patients were monitored for at least 3 months. A total number of 48 strains were isolated, including microbiological alert pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (n =7).

In two cases the culture remained sterile, while in 26 the etiology was represented by one strain, in 11 by two strains and in three by three strains (Table 1). The Gram-positive bacteria were isolated in 32 cases, while the Gram-negative in 16. Two-thirds of infections were caused by one of the three species: *Enterococcus faecalis* (8 strains), *Staphylococcus aureus* (14 strains) coagulase-negative *Staphylococcus* species (11 strains) (*Staphylococcus xylosus*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus warnerii*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus chromogenes)*. In total, *Staphylococcus spp.* was isolated in 47 % of the analyzed cases, followed by *Enterococcus faecalis* (13%), *Serratia sp.* (4%), *Chryseomonas luteola* (4%), *Escherichia coli* (4%), *Pseudomonas aeruginosa* (4%), *Aerococccus viridians* (2%), *Streptococcus uberis* (2%), *Klebsiella pneumoniae rhinoscleromatis* (2%), *Alcaligenes faecalis* (2%) and *Burkholderia cepacia* (2%).

The most expressed virulence factor was esculin hydrolysis in 26 strains, closely followed by caseinase (n=24), lecitinase (n=16), haemolysins (n=16), gelatinase (n=12), DN-ase (n=9), amylase (n=9), lipase (n=7) (Fig. 1).

Among the isolated strains, *Staphylococcus aureus* expressed all the investigated virulent factors, followed by *Alcaligenes faecalis* (haemolysins, lecithinase, lipase, caseinase, gelatinase, DN-ase, esculin hydrolyzing enzyme), *Staphylococcus epidermidis (*haemolysins, lecithinase, caseinase, amylase, esculin hydrolyzing enzyme), *Escherichia coli* (haemolysins, gelatinase, caseinase, amylase), *Enterococococcus faecalis* (haemolysins, caseinase, lecithinase, lipase), *Enterobacter faecium* and *Aerococcus viridans* (haemolysins, caseinase, esculin hydrolyzing enzyme).

The highest antibiotic resistance rate in *Staphylococcus spp.* was registered for penicillin (16%), opposite to rifampicin resistance encountered only 4% of the isolates. All *Staphylococcus* sp. isolates were susceptible to imipenem. *Enterococcus spp.* also showed a high resistance rate to penicillin (37%), followed by tetracycline (21%), gentamicin (16%) and vancomycin (11%) (Table 2).

The two *Streptococcus spp*. isolated strains were resistant to erythromicin, vancomycin, clindamycin and cefotaxime, being susceptible to linezolid and colistin (Table 2). The *non-Enterobacteriaceae* Gram negative bacilli strains were resistant to piperacillin (30%), tobramycin, imipenem, cefoperazone, amikacin (14% each), ceftazidim and piperacillin-tazobactam (7% each). Susceptibility was observed to aztreonam.

Enterobacteriaceae spp. strains exhibited a relatively homogenous antibiotic resistance distribution, respectively 10% to cefazolin, 7% to piperacillin, cefotaxime, cefoperazone, tetracycline, amoxicillin-clavulanic acid, ampicillin, amikacin, 6% to tobramycin, gentamicin, colistin, ceftazidime, cefotaxime and 4% to ciprofloxacin and trimethoprim-sulfamethoxazole (Table 2). The antibiotic susceptibility results showed that the lowest antibiotic resistance rates (<30%) were registered for piperacillin-tazobactam, amoxicillin/clavulanate, imipenem, erythromycin, ciprofloxacin, linezolid, rifampin, aztreonam, chloramphenicol, while the highest (>70%) to penicillin and cefazolin. As mentioned by A. Cucu et al. [11], in Romania, „the national data is seldom biased by the national legislation provision that generates a conflict between monitoring the quality and safety of care and the performance of the management". Our study represents an a microbiological evaluation of the chronic wound patients.

The aetiologies of chronic wounds were similar with the ones reported in the literrature with the predominance of venous disease, which is a common problem in our country [10].

Our investigation has shown that the most frequent isolated strains were those of *Staphylococcus spp.*, similar to the studies of Gjødsbø et al. [12] and Ko¨ rber et al. [10[. The most prevalent Gram-positive bacterial species was *Staphylococcus aureus* as also reported by other studies [14, 15, 16]. The high frequency of *Enterococcus faecalis* has been reported linked to possible urinary tract infection, lack of personal hygiene, contact with poultry/poultry meat [17].

Although previous studies recorded the antimicrobial agents usage in patients with chronic wounds, no detailed study concerning the *in vitro* susceptibility assay of the isolated strains to the respective substances has been performed [13]. In our study we showed that many patients had received several systemic

antibiotic treatments without a clear record of infection. Methicillin resistance in *Staphylococcus spp.* is an important health problem. In our study methicillin resistance was both encountered in *Staphylococcus aureus* and coagulase-negative staphylococci. Demling et al. showed concern regarding the increasing of MRSA in patients from USA [18]. Although previous studies concerning MRSA have been performed in Romania [11]. however this is the first study reporting data on antibiotic resistance in strains isolated from chronic wound patients.

The isolated strains also presented the ability to produce different enzymes implicated in virulence. The most virulent strains were those of *Staphylococcus spp.*, the majority of bacterial isolates in our study. The virulence factors are responsible for infections in immuno-competent patients (10).

Table 1. Etiology, microbial colonization and treatment of the chronic leg ulcers.

Patient no.	Etiology	Colonization	Local treatment	Sistemic treatment
1	venous disease	*Serratia marcescens*	deproteinized extract (gemoderivat) from calf blood, H_2O_2	
2	venous disease	*Chryseomonas luteola* *Serratia odorifera*	erythromycin ointment, rifampin powder, bacitracin zinc/neomycin sulphate powder	
3	venous disease	*Pseudomonas aeruginosa*	silver sulfadiazine cream, silver dressings	multiple systemic antibiotics
4	bone necrosis	*Pseudomonas aeruginosa*	boric acid 2% solution	
5	venous disease	*Alcaligenes faecalis*	bacitracin zinc/neomycin sulphate powder, gentian violet 1% solution, ciprofloxacin ointment	amoxicillin/ clavulanic acid
6	venous disease	*Chryseomonas luteola*	wound dressings	
7	venous disease, diabetes mellitus	*Alcaligenesis faecalis*	boric acid 2% solution	
8	venous disease	*Enterobacter faecium* *Aerococcus viridans*	deproteinized extract (gemoderivate) from calf blood, boric acid 2% solution, silver sulfadiazine cream	
9	venous disease	*Klebsiella pneumoniae* *Staphylococcus aureus/ Staphylococcus chromogenes*	povidone-iodine	
10	squamos cell carcinoma	*Staphylococcus warnerii* *Staphylococcus xylosus*	boric acid 2% solution	
11	venous disease	*Staphylococcus aureus*	ethacridine lactate 1 g/1000 ml, H2O2, povidone-iodine	oxacillin
12	venous disease	*Staphylococcus epidermidis* *Staphylococcus sciuri*	silver sulfadiazine cream, dipropionat betamethasone/clotrimazole/gentamicin cream	
13	venous disease, trauma	*Staphylococcus aureus*	silver sulfadiazine cream, boric acid 2% solution	
14	venous disease	*Staphylococcus hominis* *Enterococcus faecalis*	boric acid 2% solution	
15	venous and arterial disease	*Staphylococcus epidermidis*	methylene blue solution, bacitracin zinc/neomycin sulphate powder	ciprofloxacin
16	venous disease	*Staphylococcus aureus* *Staphylococcs xylosus*	boric acid 2% solution	
17	venous disease	*Staphylococcus aureus* *Staphylococcus warnerii*	boric acid 2% solution	
18	venous disease, diabetes mellitus	*Staphylococcus aureus*	boric acid 2% solution	
19	venous disease	*Staphylococcus aureus*	boric acid 2% solution	
20	arterial disease and diabetes mellitus	*Enterococcus faecalis*	boric acid 2% solution	
21	venous disease	*Enterococcus faecalis*	boric acid 2% solution	

22	venous disease	*Lactococcus lactis*	boric acid 2% solution, silver sulfadiazine cream, topical antibiotic	ceftriaxone
23	venous disease	*Lactococcus lactis/Enterococus faecalis*	boric acid 2% solution	
24	arterial and neurological disease	*Streptococcus uberis*	silver dressing	ciprofloxacin
25	venous disease	*Escherichia coli*	boric acid 2% solution	
26	venous disease	*Burkholderia cepacia*	boric acid 2% solution	
27	arterial disease, diabetes mellitus	*Staphylococcus aureus Enterobacter faecium*	boric acid 2% solution	
28	arterial and neurological disease	*Enterococcus faecalis*	boric acid 2% solution	cefotaxime
29	arterial disease	*Enterococcus faecalis*	ceftamil, povidone-iodine	cefuroxime
30	arterial disease and diabetes mellitus	*Enterobacter intermedius*	boric acid 2% solution	
31	venous disease	*Enterocobacter faecium Escherichia coli*	boric acid 2% solution	
32	venous disease	*Staphylococcus haemolyticus*	boric acid 2% solution	
33	venous disease	*Staphylococcus aureus*	boric acid 2% solution	
34	venous disease	*Staphylococcus aureus*	boric acid 2% solution	
35	venous disease	*Staphylococcus aureus*	boric acid 2% solution	
36	venous disease	*Staphylococcus aureus*	boric acid 2% solution, silver sulfadiazine cream, bacitracin zinc/neomycin sulphate powder, deproteinized extract (gemoderivat) from calf blood, zinc hyaluronate gel	cefuroxime
37	Kaposi's sarcoma	*Lactococcus lactis Enterococus faecalis*	silver sulfadiazine cream, silver dressing	
38	venous disease, diabetes mellitus	*Staphylococcus aureus*	boric acid 2% solution	
39	venous disease	sterile	Castellani solution	
40	venous disease	sterile	boric acid 2% solution	

Table 2. Susceptibility of the analyzed strains to various chemotherapeutics.

No	Chemotherapeutic	Sensitive strains (%)	Resistant strains (%)	Intermediate strains (%)
Penicillins	Piperacillin/tazobactam	85.72%	14.28%	0%
	Amoxicillin/clavulanate	75.87%	24.13%	0%
	Ampicillin	37.94%	62.06%	0%
	Penicillin	21.87%	78.12%	0%
	Piperacillin	38.47%	61.53%	
Oxacillin		47.83%	52.17%	
	Ceftazidime	60%	40%	0%
	Cefotaxime	50%	46.6%	3.3%
	Cefoxitin	40%	60%	0%
	Cefoperazone	53.85%	46.15%	0%

	Cefazolin	0%	100%	0%
Carbapenems				
	Imipenem	100%	0%	0%
Macrolides				
	Erythromycin	90%	0%	10%
Lincosamides				
	Clindamycin	52%	48%	0%
Aminoglycosides				
	Gentamycin	59.37%	37.5%	3.2%
	Amikacin	66.6%	30.5%	2.9%
	Tobramycin	69.24%	30.76%	0%
Tetracyclines				
	Tetracycline	46.7%	53.3%	0%
Glycopeptides				
	Vancomycin	56.52%	39.13%	4.4%
Fluoroquinolones				
	Ciprofloxacin	92.3%	0%	7.7%
Sulphonamides				
	Trimethoprim/sulfamethoxazole	66.66%	33.33%	0%
Ansamycin				
	Rifampicin	82.6%	13.04%	4.4%
Oxazolidinone				
	Linezolid	100%	0%	0%
Phenicols				
	Chloramphenicol	76.48%	23.52%	0%
Monobactam				
	Aztreonam	71.43%	28.57%	0%

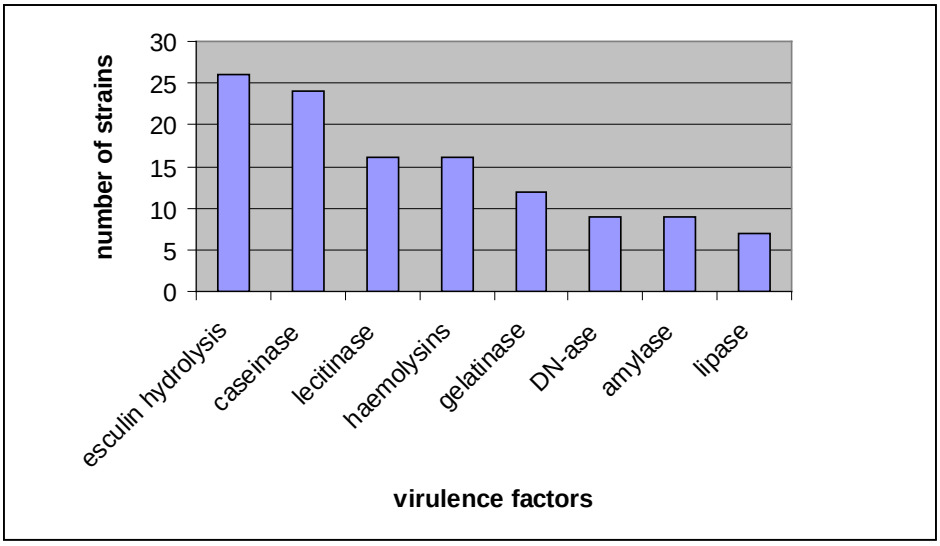

Figure 1. Graphic representation of the frequency of the investigated virulence factors in the analyzed strains.

4. CONCLUSIONS

Our study constitutes a preliminary background for the elucidation of the microbiome role in the infectious pathology of the chronic wound and the antibiotic resistance markers of the microbial strains colonizing the hospitalized patients in Romanian Dermatology Departments. However, more data are needed for statistically relevant and epidemiological studies.

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