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Identification and phenotypic characterization of the most frequent bacterial etiologies in chronic skin ulcers

MARA MĂDĂLINA MIHAI[1,2)], ALINA MARIA HOLBAN[3)], CĂLIN GIURCĂNEANU[1,2)], LILIANA GABRIELA POPA[2)], MARIANA BUZEA[2)], MARILENA FILIPOV[2)], VERONICA LAZĂR[3)], MARIANA CARMEN CHIFIRIUC[3)], MIRCEA IOAN POPA[1)]

[1)] *"Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*

[2)] *"Elias" University Emergency Hospital, Bucharest, Romania*

[3)] *Department of Microbiology, Faculty of Biology, University of Bucharest, Romania; Research Institute of the University of Bucharest (ICUB), Romania*

Abstract

Chronic wounds represent an important burden on the healthcare system, requiring frequent hospitalizations and expensive treatments. It is now recognized that a primary factor contributing to a non-healing trajectory and a low therapeutic response is the biofilm infection. The purpose of this study was to identify the bacterial isolates collected from chronic skin wounds of hospitalized patients and to evaluate their antimicrobial susceptibility profiles, virulence factors, as well as the ability to develop biofilms *in vitro*. A number of 44 wound samples were collected from 39 patients. The isolated strains belonged to seven different microbial species, *i.e.*: *Staphylococcus aureus* (32 strains), followed by *Pseudomonas aeruginosa* (4), *Escherichia coli* (3), *Klebsiella pneumoniae* (2), *Proteus mirabilis* (1), *Citrobacter freundii* (1), group G β-hemolytic streptococci (1). In comparison to the other isolates, *P. aeruginosa* strains exhibited the highest capacity to develop complex biofilm structures *in vitro*, followed by *S. aureus*, with insignificant differences between MRSA and non-methicillin resistant isolates. The *Enterobacteriaceae* strains expressed less virulent phenotypes, lower adherence to epithelial cells and biofilm forming capacity, but also significant resistance phenotypes with a potential of unfavorable epidemiological outcome. The isolation of MRSA, ESBL-producing microorganisms and multiple antibiotic resistant *P. aeruginosa* suggests the potential risk of nosocomial spread and the potential severe outcome in case of bacteremia and sepsis. This study represents an important step in elucidating the host-wound microbiome interaction, by describing various resistance and virulence threats of microorganisms colonizing and/or infecting the chronic wounds. However, in order to establish a statistical relevant correlation, larger studies are needed.

Keywords: chronic wound, virulence, antibiotic resistance, biofilm.

Introduction

Skin is a vital organ, acting as a physical and biochemical barrier to reduce the adherence and invasion of microorganisms. Different microbial populations colonize the cutaneous surface forming the resident microbiota, with a relatively stable composition, unique for each individual, and the transient microbiota, in a perpetual change [1]. When skin barrier is impaired, microorganisms immediately colonize the surface of the wound and, under favorable conditions, multiply, leading to acute or chronic infections [2].

Nowadays, due to the higher prevalence of various predisposing factors (sedentary lifestyle, nutritional disorders, higher survival rate of the population) the incidence of chronic wounds is increasing. Venous leg ulcers, arterial ulcers, diabetic foot ulcers, pressure sores and non-healing surgical wounds represent an important burden on the healthcare system, requiring frequent hospitalizations and expensive treatments [3]. Also, due to severe complications such as sepsis, malignancy, limb loss, they have an important impact on the individual's quality of life.

It is now recognized that a primary factor contributing to the non-healing trajectory of chronic wounds is the mono- and polymicrobial biofilm infection [4–6]. Bacterial biofilms are communities of sessile organisms attached to a surface or interface, comprising cells with modified metabolism, characterized by an increased tolerance to antimicrobial therapy and the ability to escape physiological immune response [7, 8]. A better understanding of the precise mechanisms by which microbial biofilms delay repair processes together with optimizing methods for biofilm detection and prevention may enhance opportunities for chronic wounds healing [9].

In a retrospective study carried out in 2012 on a cohort of 213 patients, Bessa *et al.* (2013) identified the most frequent microorganisms isolated from ulcerative acute and chronic wounds. From a total of 312 samples the most frequent isolated microorganism was *Staphylococcus aureus* (37%), followed by *Pseudomonas aeruginosa* (17%), *Proteus mirabilis* (10%), *Escherichia coli* (6%), *Corynebacterium* spp. (5%) and other 23 species. Polymicrobial infection was found in 59 wound samples (~27%) and was mainly constituted with two species [10].

Rhoads *et al.* went further and compared the results of traditional culturing techniques with those obtained by molecular microbial diagnostic tests applied on chronic wounds samples. While aerobic cultures revealed 17 different bacterial species, the most frequent being *Staphylococcus* spp., *Enterococcus* spp., *Serratia marcescens*, *Pseudomonas* spp., *Proteus mirabilis*, *Citrobacter*

freundii, *E. coli*, *Klebsiella pneumoniae*, molecular methods identified a significant higher number of 338 bacterial taxa. A number of 105 out of 168 samples were in complete concordance regarding the microorganisms identified by the two methods, although anaerobes represented an important population of the wound microbiota. Traditional culture revealed single bacterial species in 57% samples, two types of microorganisms in 24% and three different bacterial species in 7% samples [6].

In Romania, there are very poor data regarding the epidemiology of venous leg ulcers and the implication of microbial biofilms in the delayed healing of these wounds. In a study of Georgescu *et al.* (2014), performed on patients with chronic wounds, two-thirds of infections were caused by *Enterococcus faecalis*, *S. aureus* or coagulase-negative *Staphylococcus* species [11]. A preliminary study conducted by our team on a cohort of 52 patients diagnosed with chronic venous ulcerations of the lower limbs revealed, by traditional culturing techniques, the prevalence of *S. aureus* infections (34/44), 14 being methicillin resistant (MRSA) and the prevalence of *P. aeruginosa* infections (12/44), results similar to the ones reported in the scientific literature. Observing the clinical and bacteriological outcome of the patients, we noticed the persistence of wound infections, despite systemic and topical antibiotic therapy, as well as the development of antimicrobial resistance of the isolated microorganisms.

Another retrospective study conducted in Bucharest, included 470 patients diagnosed with venous leg ulcers during the period 2009–2011. Half of the patients were younger than 68 years, being professionally and socially active, thus highlighting the negative impact of skin disease on their quality of life. Bacteriological examination was performed in about half of the patients (55.1%), with positive results in 80.3% of cases. The following microorganisms were isolated: *S. aureus* 26.3%, 17.2% *Enterobacter* spp. followed, in approximately equal proportions by *Proteus* spp. (15.8%) *E. coli* (14.8%) and *P. aeruginosa* (15.3%). There were two cases of infection with *Enterococcus* spp. and one case of infection with *Candida albicans* [12].

Although several studies, such as those mentioned above, investigated the composition of wound microbiota, none of them tested the virulence patterns together with the ability of bacteria to develop a biofilm structure.

In this study, we aim to identify the bacterial species isolated by aerobic culturing technique in samples collected from chronic wounds of a significant number of patients and to assess their antimicrobial susceptibility, their virulence pattern, as well as the ability to develop biofilms *in vitro*.

Materials and Methods

The study was performed on 44 bacterial strains isolated from 39 patients hospitalized in the "Elias" Emergency University Hospital, Bucharest, Romania, during March–May 2014.

An informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in approval by the institution's human research review committee.

Clinical data

Clinical data was collected by anamnesis, full body examination and wound assessment. The following information was gathered: the etiology, location and the presence of clinical signs of infection for each chronic wound, the patient's history of associated aggravating pathologies. Laboratory infectious markers were also assessed (C-reactive protein, erythrocyte sedimentation rate, fibrinogen, neutrophilic leukocytosis).

Microbiological examination

After moisturizing the wound bed with physiologic saline, the samples were collected using a sterile swab rotated across the surface of each lesion, from the center to the margins. The swabs were placed in a tube containing transport medium.

Samples were tested using routine aerobic culture techniques. Specimens were Gram stained. Each swab was plated onto the following media: 5% sheep blood agar, Chocolate agar, MacConkey agar (without crystal violet) and Sabouraud agar with chloramphenicol (Oxoid). All plates were incubated aerobically at 37^0C for 18–24 hours, the Sabouraud plates being incubated simultaneously at 30^0C and 37^0C for 24–72 hours. After pure bacterial colonies were obtained, we proceeded to the biochemical identification using the automated Vitek 2 system (bioMérieux) and Phoenix BD (Bekton–Dickinson). The antibiotic susceptibility pattern was assessed for each identified Gram positive/negative bacterial species using disk diffusion method according to the CLSI guideline (*Clinical and Laboratory Standards Institute*) and automated systems (Vitek2C/Phoenix BD). E-tests were also performed, following the manufacturer's recommendations.

Subsequently strains were maintained at 4^0C in the Microbial Culture Collection of Microbiology Laboratory, Faculty of Biology, Bucharest. For further experiments, bacteria were streaked in blood agar for *Streptococcus* spp. and nutrient agar for the other strains, and incubated over night at 37^0C.

Virulence assessment

Evaluation of adherence to HeLa cells

Bacterial adherence to HeLa cells was performed by the adapted Cravioto's method: HeLa cell monolayers were washed three times with phosphate buffered saline (PBS); 1 mL of fresh medium without antibiotics was added to each well [13]. Suspension of each strain from bacterial mid-logarithmic phase cultures grown in nutrient broth was adjusted at 10^7 CFU/mL and 1 mL was used for the inoculation of each well. The inoculated plates were incubated for two hours at 37^0C [14]. The adherence patterns were defined as: localized adherence (LA) when tight clusters of microorganisms were noticed on the HeLa cell surface, aggregative adherence (AA) when a microbial stacked brick pattern characterize the attachment, diffuse adherence (DA) when the bacteria adhered diffusely, covering the whole surface of the cell. For each strain, an adherence index was expressed as the ratio between the number of eukaryotic cells with adhered bacteria and 100 HeLa cells counted on the microscopic field [15].

Soluble virulence factors

The bacterial virulence phenotype was assessed by performing enzymatic tests for the expression of eight soluble factors, using the following specific media: 5% sheep blood agar (for alpha and beta hemolysins), 2.5% yolk agar (lecithinase test), Tween 80 agar (lipase test), 15% casein agar (caseinase test), 1% gelatin agar (gelatinase), 10% starch agar (amylase), DNA agar (DN-ase test), 1% esculin iron salts (esculinase test).

Biofilm assessment

Overnight bacterial cultures were diluted in Brain Heart Infusion Broth for *Streptococcus* spp. and Tryptic Soy Broth for the other strains, up to a turbidity of 0.5 McFarland (approximately 1×10^8 CFU/mL) and 20 µL of the obtained suspension were seeded in 96 multi-well plates in a volume of 180 µL liquid medium in triplicate. To allow biofilm formation, the inoculated plates were incubated for 24, 48 and 72 hours at 37^0C.

After each incubation period, the biofilms were gently washed with PBS with the aim of removing planktonic cells. Washed biofilms were treated with cold methanol for 5 minutes, dried at room temperature and stained with 0.1% crystal violet solution for 15 minutes. We performed the resuspension of the adhered biomass in 33% acetic acid and spectrophotometrically read the absorbance at 492 nm. The quantity of adhered biomass is proportional to the absorbance [16].

Results

Clinical data

A number of 44 wound samples were collected from 39 patients, 29 residing in urban areas. Their age ranged from one month to 86 years, with a median of 63 years. The gender distribution revealed a predominance of women (23/39). The patients were diagnosed with the following chronic pathologies: 17 with venous ulcer of the lower limb, secondary to chronic venous insufficiency, six with arterial ulcer secondary to obliterating arteritis of the lower limbs, three with sacral pressure ulcer, two with thoracic chronic ulcer secondary to breast cancer, two with non-healing surgical wounds and nine with non-healing abscesses (Figure 1).

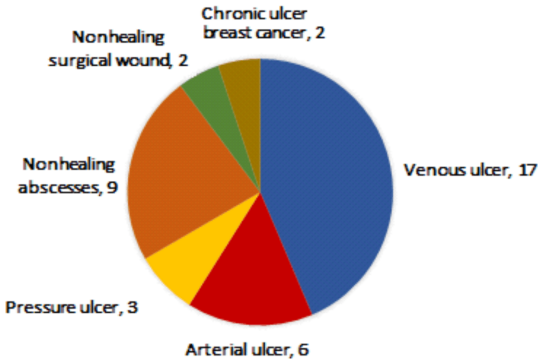

Figure 1 – ***Chronic wound diagnosis.***

From the 39 patients, 28 presented aggravating pathologies such as high blood pressure in 23 cases, diabetes mellitus type 2 in 15 cases, dyslipidemia in 12 patients. Twenty-nine individuals showed clinical signs of infection such as pain, erythema, edema, heat or purulent exudates. Laboratory blood tests pointed out an inflammatory biologic status (abnormal values of C-reactive protein, erythrocyte sedimentation rate, fibrinogen) in 24 patients, complemented with neutrophilic leukocytosis in 13 cases.

Microbiological examination

The microbial strains belonged to seven different species. The most common bacterial species was *S. aureus* (32 strains), followed by *P. aeruginosa* (4), *E. coli* (3), *K. pneumoniae* (2), *P. mirabilis* (1), *C. freundii* (1) and group G β hemolytic streptococci (1) (Table 1).

Table 1 – ***Chronic wounds isolates. For each bacterial species it is specified the total number of isolated strains and the laboratory code***

	non-MRSA	MRSA	*P. aeruginosa*	*K. pneumoniae*	*E. coli*	*P. mirabilis*	*C. freundii*	Group G β hemolytic streptococci
Venous ulcer	7 strains (5, 8, 18, 21, 28, 31, 34)	6 strains (11, 20, 27, 30, 36, 43)	3 strains (7, 16, 39)	1 strain (4)	2 strains (13, 42)	0	1 strain (1)	1 strain (9)
Arterial ulcer	2 strains (29, 44)	1 strain (22)	1 strain (37)	0	1 strain (23)	1 strain (17)	0	0
Pressure ulcer	1 strain (35)	2 strains (19, 25)	0	0	0	0	0	0
Non-healing surgical wound	1 strain (14)	1 strain (2)	0	1 strain (3)	0	0	0	0
Non-healing abscesses	8 strains (6, 10, 26, 32, 33, 38, 40, 41)	1 strain (12)	0	0	0	0	0	0
Chronic ulcer – breast cancer	2 strains (15, 24)	0	0	0	0	0	0	0

MRSA: Methicillin resistant *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. coli*: *Escherichia coli*; *P. mirabilis*: *Proteus mirabilis*; *C. freundii*: *Citrobacter freundii*.

The most frequent result was the presence of only one species isolated from each sample. Polymicrobial infection was detected in five of the infected wounds and was constituted by three species in two samples and two bacterial species in the other three. The predominant species found in polymicrobial infections were *S. aureus* and *P. aeruginosa*.

Resistance to antibiotics

The antibiotic resistance pattern of the Gram-positive and Gram-negative microorganisms was assessed (Figure 2).

S. aureus sensitive to methicillin was present in 21 samples, being the most common isolate. Twelve strains were D-test positive, with macrolide-lincosamide-streptogramins inducible (MLSbi) phenotype (Figure 2A). Eleven

methicillin resistant *S. aureus* (MRSA) strains were isolated, all also resistant to penicillin, four strains showing resistance to gentamicin and ciprofloxacin and eight being D-test positive, with MLSbi phenotype. All 11 MRSA cultured strains were sensitive to cotrimoxazole, linezolid, teicoplanin and doxycycline.

In a 77-year-old patient diagnosed with chronic venous ulcer of the lower limb, with clinical and biological signs of infection, we isolated a multi-drug-resistant strain of *P. aeruginosa* (No. 16), sensitive only to colistin and resistant to β-lactams (cephalosporins, piperacillin, piperacillin–tazobactam, ticarcillin, ticarcillin–clavulanate), aminoglycosides (tobramycin, amikacin), second and third generation fluoroqinolones (ciprofloxacin, levofloxacin, pefloxacin) and carbapenems (imipenem, meropenem) (Figure 2B). In the same sample, *Enterococcus* spp. and *C. albicans* were also present. The patient deceased of sepsis and cardiogenic shock.

P. aeruginosa (No. 7) resistant to aminoglycosides (gentamicin, amikacin), but sensitive to all other tested antibiotics was isolated from a 77-year-old patient with chronic venous ulcer, with no clinical and biological signs of infection. In the same sample, *S. aureus* was also present (Figure 2B).

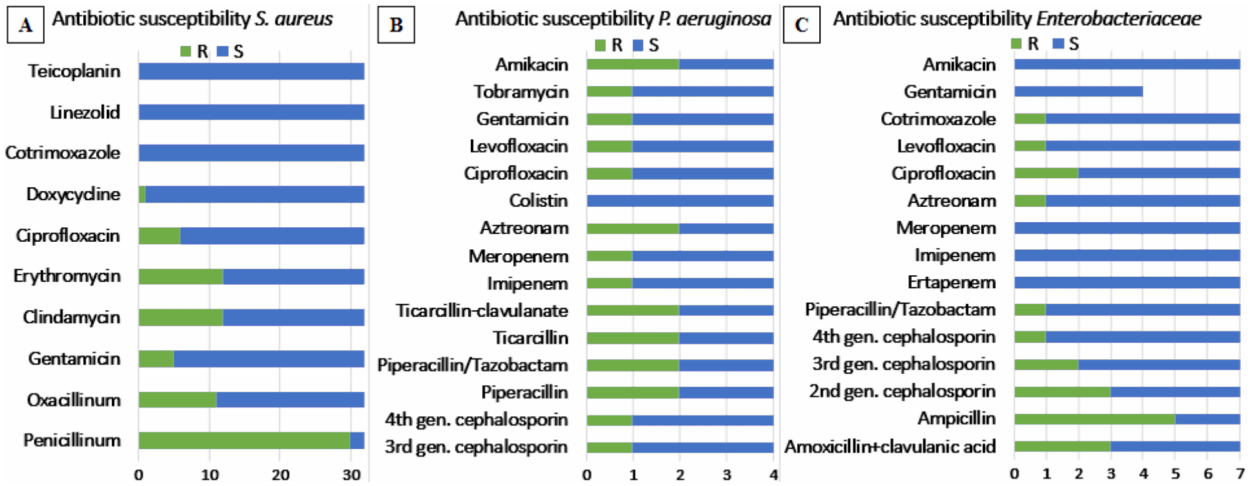

Figure 2 – ***Antibiotic susceptibility for (A) Staphylococcus aureus, (B) Pseudomonas aeruginosa, (C) Enterobacteriaceae.***

Concerning the resistance phenotype of *Enterobacteriaceae* microorganisms isolated from chronic wounds (Figure 2C), all tested strains were sensitive to carbapenem antibiotics (ertapenem, imipenem, meropenem). From the two strains of *K. pneumoniae* (No. 3) isolated from a non-healing surgical wound, produced ESBL (extended spectrum beta-lactamase), consequently with resistance to cephalosporins and aztreonam. It also proved resistant to aminopenicillins and gentamicin. From the three isolated strains of *E. coli*, only strain No. 42, isolated from the venous ulceration of a patient with previously administered fluoroquinolone antibiotic therapy, proved resistance to this class of antibiotics, the others being susceptible to all antibiotics tested. *Proteus mirabilis* showed resistance to multiple antibiotics: beta-lactams (aminopenicillins, ureidopenicillins and third generation cephalosporins), aminoglycosides (gentamicin), second generation fluoroquinolones and cotrimoxazole. *C. freundii*, isolated from venous ulceration, showed resistance to aminopenicillins and second-generation cephalosporins, being susceptible to the other tested antibiotics.

Virulence assessment

Evaluation of adherence to HeLa cells

The qualitative assessment of bacterial adherence to HeLa cells, revealed that 30 of the 32 tested *S. aureus* strains adhered in various patterns to the cellular substrata (Figure 3, C, E–G and I). The two strains that did not demonstrate adherence were isolated from venous ulceration (No. 35), respectively arterial ulceration (No. 38). The MRSA adherence rates ranged from 10% to 100%, with an average of 73.2% (Table 2). In seven strains out of 11, the index exceeded 80% (Table 2).

Table 2 – ***HeLa adherence pattern for each bacterial isolate***

HeLa adherence pattern	Localized adherence	Diffuse adherence	Aggregative adherence	No adherence	Adherence index
Non-MRSA	10 strains (6, 8, 10, 18, 24)	7 strains (5, 14, 15, 26, 31, 34, 44)	2 strains (21, 28)	2 strains (35, 38)	0–100% Average: 52.38%
MRSA	6 strains (2, 12, 19, 20, 22, 36)	2 strains (11, 30)	3 strains (25, 27, 43)	0	10–100% Average: 73.18%
Pseudomonas aeruginosa	1 strain (37)	3 strains (7, 16, 39)	0	0	10% (7), 80% (39), 100% (16, 37)
Citrobacter freundii	0	1 strain (1)	0	0	50% (1)
Escherichia coli	1 strain (13)	1 strain (23)	0	1 strain (42)	10% (23), 20% (13)
Klebsiella pneumoniae	0	1 strain (4)	1 strain (3)	0	30% (3), 40% (4)
Proteus mirabilis	0	1strain (17)	0	0	5% (17)

MRSA: Methicillin resistant *Staphylococcus aureus*.

The eukaryotic adherence assay showed that three *P. aeruginosa* strains (No. 16, No. 37, No. 39) possess the ability to invade epithelial HeLa cells with diffuse and localized adherence patterns and adherence rates higher

than 80%, strain No. 7 being the exception with low adherence and an index of 10% (Figure 3B; Table 2).

The adherence to epithelial HeLa cells of *Enterobacteriaceae* isolates was poorly expressed, with an adherence index ranging from 0% to 50% (Figure 3, D and H; Table 2).

Soluble virulence factors

All *S. aureus* strains produced hemolysins and amilase, but none expressed gelatinase. Esculinase and pore forming toxins such as lecithinase and lipase were produced in 10 out of 11 strains of MRSA, suggesting their increased virulence potential (Table 3).

Soluble enzymatic virulence factors patterns were assessed for all *P. aeruginosa* strains (Table 3). Pore forming toxins such as beta-hemolysins, lecithinase and lipase were produced by all strains. Gelatinase, caseinase and amilase were expressed by two strains isolated from arterial (No. 37) and venous (No. 39) ulcerations, both lacking DN-ase. The DNA degrading enzyme was produced by both strains No. 7 and No. 16, while esculinase only by the last.

The production of soluble enzymatic virulence factors was assessed for all *Enterobacteriaceae* strains (Table 3). In comparison to *S. aureus* and *P. aeruginosa* strains, *Enterobacteriaceae* isolates expressed in a lesser extent virulence factors. The majority of strains showed alpha hemolysis, while *P. mirabilis* lacked hemolytic activity. Amilase tested positive for all *Enterobacteriaceae* isolates, esculinase was produced only by *K. pneumoniae* strains and lipase only by *P. mirabilis*.

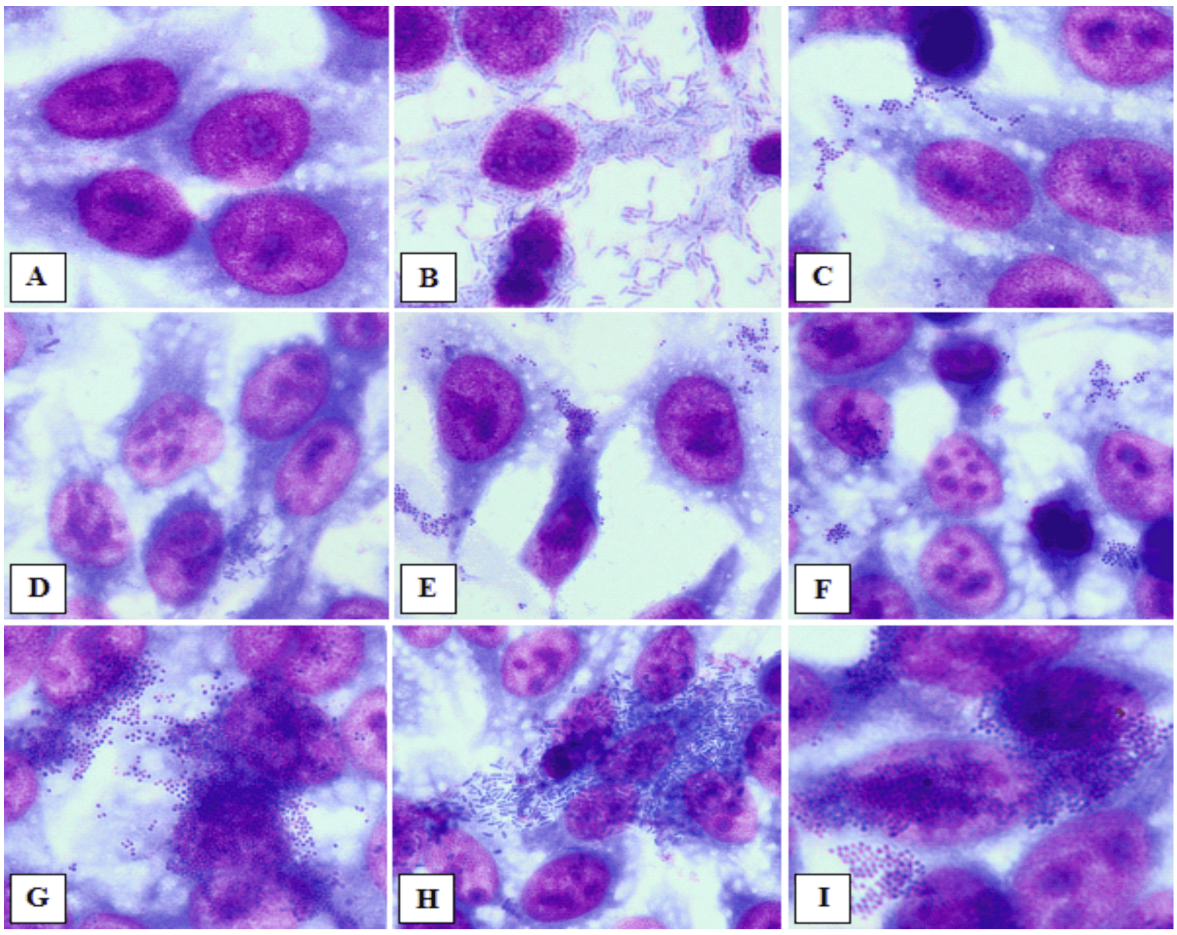

Figure 3 – ***Adherence to HeLa cells: (A) No adherence; (B) Diffuse adherence Pseudomonas aeruginosa (No. 16); (C) Diffuse adherence Staphylococcus aureus (No. 14); (D) Localized adherence Escherichia coli (No. 13); (E) Localized adherence Staphylococcus aureus (No. 32); (F) Localized adherence MRSA (No. 36); (G) Aggregated adherence MRSA (No. 25); (H) Aggregated adherence Klebsiella pneumoniae (No. 3); (I) Aggregated adherence MRSA (No. 43). MRSA: Methicillin resistant Staphylococcus aureus.***

Table 3 – ***Production of soluble virulence factors***

	non-MRSA	MRSA	*P. aeruginosa*	*C. freundii*	*E. coli*	*K. pneumoniae*	*P. mirabilis*
No. of isolates	21	11	4	1	3	2	1
Alpha-hemolysin	2	4	0	1	3	2	0
Beta-hemolysin	19	7	4	0	0	0	0
Esculinase	13	10	1	0	0	2	0
DN-ase	17	8	2	0	0	0	0
Lipase	12	10	4	0	0	0	1
Caseinase	7	1	2	0	1	1	0
Lecitinase	18	10	4	0	0	0	0

	non-MRSA	MRSA	*P. aeruginosa*	*C. freundii*	*E. coli*	*K. pneumoniae*	*P. mirabilis*
Gelatinase	0	0	2	0	2	0	1
Amilase	21	11	2	1	3	2	1

MRSA: Methicillin resistant *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. coli*: *Escherichia coli*; *P. mirabilis*: *Proteus mirabilis*; *C. freundii*: *Citrobacter freundii*.

Biofilm assessment

Biofilm results demonstrated low differences between non-methicillin resistant *S. aureus* and MRSA isolates, regarding their ability to produce biofilms (Figure 4, A and B). Usually, the biofilms of the analyzed strains followed a normal pattern and architecture (Figure 5). Biofilms development was maximum at 24 up to 48 hours of incubation and after this period, cells detach and the biofilm architecture becomes thinner (Figure 5).

All *P. aeruginosa* strains intensely formed biofilms, with a maximal value in strain No. 7 (Figures 4C and 5). In comparison to other bacterial species isolated from chronic wounds, *P. aeruginosa* isolates had the highest capacity to develop complex biofilm structures. Whether the different clinical outcomes of the patients were influenced by the ability of each strain to form biofilms remains to be elucidated.

Biofilm assessment showed that all *Enterobacteriaceae* strains had a low capacity to develop biofilms, without significant differences between isolates (Figures 5 and 6).

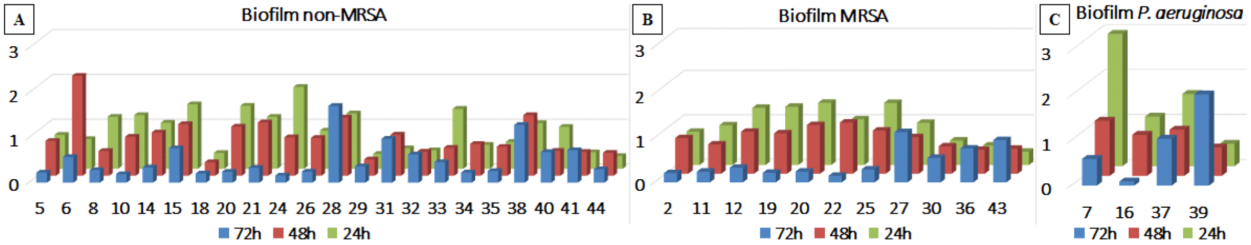

Figure 4 – ***Graphic representation of Staphylococcus aureus (A – non-MRSA, B – MRSA) and (C) Pseudomonas aeruginosa biofilm formation at 24, 48 and 72 hours. On the X-axis is represented the given strain numbers, while on the Y-axis is plotted the OD at 495 nm. MRSA: Methicillin resistant Staphylococcus aureus, h: Hours.***

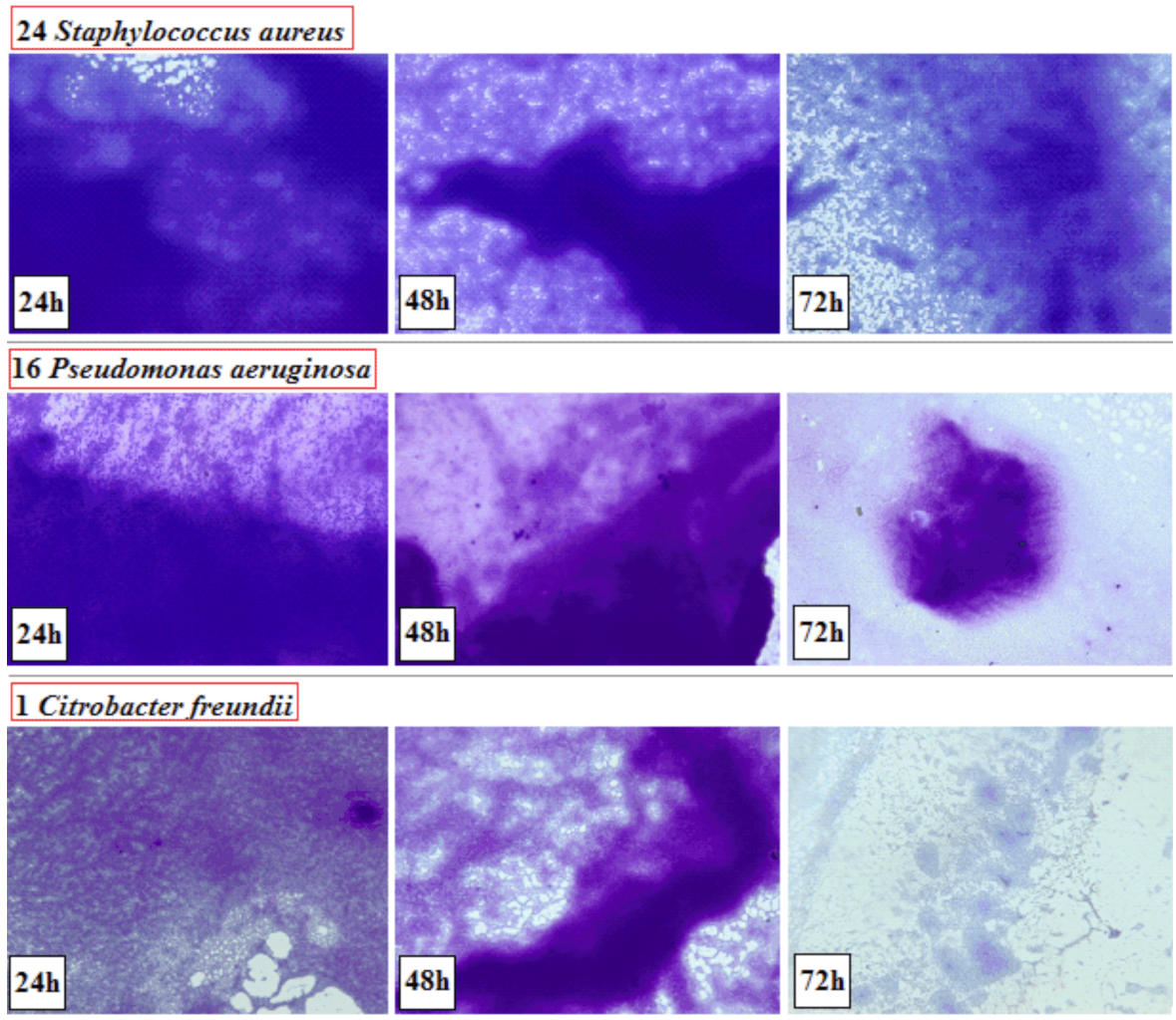

Figure 5 – ***Microscopy analysis of Staphylococcus aureus (No. 24), Pseudomonas aeruginosa (No. 16), Citrobacter freundii (No. 1) biofilms developed after 24, 48 and 72 hours. h: Hours.***

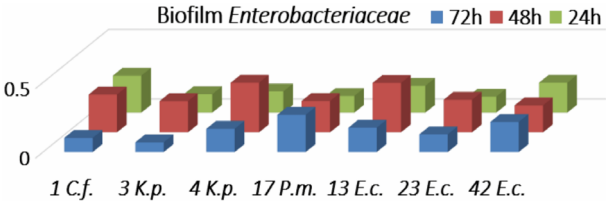

Figure 6 – ***Graphic representation of Enterobacteriaceae biofilm formation at 24, 48 and 72 hours. On the X-axis is represented the given strain numbers, while on the Y-axis is plotted the OD at 495 nm. C.f.: Citrobacter freundii, K.p.: Klebsiella pneumoniae, P.m.: Proteus mirabilis, E.c.: Escherichia coli.***

Discussion

The etiologies of chronic wounds and the prevalence of bacterial wound isolates were similar with the ones reported in the international scientific literature. Our results demonstrated that the most frequent species isolated from chronic ulcers were *S. aureus* and *P. aeruginosa*, similar with other reported studies.

We assessed the susceptibility patterns of the wound isolates to the most commonly recommended classes of antibiotics. The isolation of MRSA, ESBL producing microorganisms and multiple antibiotic resistant *P. aeruginosa* from the chronic wounds of hospitalized patients, suggests the potential risk of nosocomial spread infections and the potential severe outcome in case of bacteremia and sepsis. Furthermore, the biofilm phenotype predisposes to an increased antimicrobial tolerance and to the failure of standard therapeutic methods [17].

Bacterial adherence is an essential step in the development of biofilms *in vitro* and *in vivo*. In our study, the adherence of the isolates to eukaryotic cells varied in pattern and intensity. Although many studies conducted so far characterize the molecular mechanisms of bacterial adherence to the wound bed [18–20], we could not find correlations between the microorganism adherence patterns and the clinical severity and progression of chronic wounds.

Both *S. aureus* and *P. aeruginosa* produce various virulence factors, involved in the persistence of the infection and in delayed wound healing [10]. Moreover, microorganisms are able to modulate their virulence for a better survival and persistence in different clinical outcomes or in hostile environments [15, 21].

All *P. aeruginosa* strains intensely formed biofilms and, in comparison to the other bacterial species isolated from chronic wounds, the isolates had the highest capacity to develop complex biofilm structures. Interestingly, it has been stated that in polymicrobial biofilm infections, *P. aeruginosa* increases the virulence potential of the biofilm by sustaining the development of other microorganisms [2, 22]. *S. aureus* strains also proved a high ability to produce biofilms, with low differences between MRSA and non-methicillin resistant isolates.

The *P. aeruginosa* strain isolated from the patient with the worst clinical outcome (No. 16) had multiple resistance to antibiotics, produced various virulence factors (pore forming toxins, DN-ase) and showed 100% adherence in a diffuse pattern and possessed the capacity to form biofilm (Figure 4C).

The *P. aeruginosa* strain isolated from the patient with the best clinical outcome (No. 7) was sensitive to the antibiotics tested, with the exception of aminoglycosides, produced the same virulence factors as strain No. 16, except for esculinase, adhered poorly to cellular substrate, with an index of 10%, in a diffuse pattern and possessed the highest capacity to form biofilms (Figure 4C).

In order to establish a statistical relevant correlation between the clinical outcomes of chronic wound infections and the bacterial resistance and virulence phenotypes, larger studies are needed.

The *Enterobacteriaceae* strains expressed less virulent phenotypes, a lower capacity to adhere to epithelial cells and to develop biofilms. While these characteristics might suggest a good patient outcome, the high antimicrobial resistance rates of the isolated strains underline the risk of hospital and community dissemination of resistant strains.

Conclusions

The fact that all isolated strains prove the ability to organize in biofilms leads to the conclusions that the development of a personalized treatment approach, by combining biofilm targeting therapeutic agents with traditional treatments, might contribute to faster healing and improved patient outcomes. Further studies need to be conducted to confirm the hypothesis whether the presence of biofilm forming bacteria might represent a worsening sign, leading to severe patient outcome. This study represents an important step in elucidating the host-wound microbiome interaction, by describing various resistance and virulence phenotypes of the microorganisms isolated from hospitalized patients chronic wounds. A correlation assessment of the clinical outcomes of chronic wound infections and the bacterial resistance and virulence phenotypes would be useful in order to understand the mechanisms of delayed wound healing and, further on, in the development optimal preventive and therapeutic algorithms.

Acknowledgments

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Corresponding author
Mariana Carmen Chifiriuc, Professor, PhD, Department of Microbiology, Faculty of Biology, University of Bucharest, 1–3 Portocalelor Lane, 060101 Bucharest, Romania; Phone +40766–728 315, e-mail: carmen_balotescu@yahoo.com

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