DOI: 10.5281/zenodo.3404090
UDC: 616.5-002.44-022.7:579.84+615.33.015.8

Antibiotic susceptibility and some persistence factors of Gram-negative bacilli isolated from trophic ulcers

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Manuscript received on August 05, 2019; revised manuscript September 03, 2019

Abstract

Background: Infections that are difficult to treat might lead to high morbidity and mortality rates. In some infections, however, despite a proper antibiotic therapy, microorganisms might persist, under certain circumstances, and produce recurrent or chronic infections. It is a well-known fact that the persistence of microorganisms might influence their viability within the macro-organism, whereas the suppression of the microbial persistence via drug preparations might greatly reduce therapeutic duration. This study is aimed at assessing the antibiotic sensitivity and some factors, contributing to persistence of Gram-negative bacilli strains isolated from trophic ulcers.

Material and methods: Data were collected and examined from 128 samples of patients with trophic ulcers. The bacteriological examinations, factors determining the persistence and the antibiotic susceptibility of the isolated strains were carried out in accordance with the current method.

Results: 211 microbial strains were isolated. The identified microorganisms revealed a high taxonomic diversity, whereas Gram-negative bacilli made up 50.2%. Isolates showed multiple resistances to antimicrobial drugs in 76.4% of cases, 43.4% strains showed hemolytic, 88.7% – anti-lysozyme and 93.4% – anti-complementary activities, whereas 70.8% strains produced a detectable biofilm. The strains isolated from mixed infections exhibited a higher percentage of pathogenicity factors compared to those isolated from monoinfections.

Conclusions: Gram-negative bacteria showed great resistance to the antimicrobial drug tests and multiple persistence factors. The results of the study proved that trophic ulcers are difficult to treat, thus being a major problem, which requires coherent monitoring and control.

Key words: trophic ulcer, Gram-negative bacilli, antibiotic resistance, persistence factors.

Introduction

Infections may cause serious complications in patients with trophic ulcers that commonly lead to inappropriate treatment, long-term hospital stay, high morbidity and mortality rates within medical units [1].

Infected trophic ulcers may result from the interaction between the macroorganism and the microorganism inoculated at this level. This interaction is influenced by the level of its contamination and the immune status of the host organism [2]. The denuded tissue is contaminated with microorganisms from the skin microbiota or spread by foreign bodies [3]. The risk of developing infections is directly proportional to the dose of microorganisms, as well as deficiencies in both general and local body's immune defenses [4]. The critical concentration of the disease-causing pathogens at which the bacterial colonization might shift to infection is also related to the accumulation of pathogenicity factors within the tissue, produced by microorganisms (enzymes, toxins, etc.) [5].

Recently, a qualitative transformation has been recorded in some species of microorganisms involved in the infectious disease pathology, which tends to increase the incidence of mixed infections, due to a simultaneous exposure of certain etiological agents. Each of these species revealed a complex of pathogenicity factors, such as adherent, hemolytic, anti-lysozyme, anti-complementary, and anti-interferon activity, etc. [6].

Long-term persistence of microorganisms in trophic ulcers is due to multiple factors that inactivate the antimicrobial activity of the immune system. Therefore, it is advisable to study the persistence properties of microorganisms in purulent infections, since these are responsible for the elimination rate at the site of inflammation, as well as for the disease prognosis. The microbial persistence determines the length of time that pathogens can persist within the macroorganism, whereas its suppression via drug preparations may potentially weaken the infectious microorganisms [7, 8].

Studies, which have been reported across different countries, revealed a range of species isolated from trophic ulcers, as well as antibiotic susceptibility cases and an increased number of patients associated with multiple resistance, thus, suggesting that administration of empirical antimicrobial therapy might increase the chances of a treatment failure [9, 10].

In other instances, some species of microorganisms may produce biofilms, which show a far greater resistance to both treatment and immune effector actions. The biofilm represents a microbial complex, wherein the cells adhere firmly to each other or to various surfaces, surrounded by the cells' exopolysaccharide matrices and exhibiting a modified, as well as a different rate of gene transcription that of planktonic cells [11]. Microbial biofilms are responsible for chronic, persistent, difficult to treat infections [12].

Treatment of trophic ulcer is a challenging task for clinicians and remains a current and relevant issue [13].

As regarding to the aforementioned, this study was aimed at identifying the spectrum of microorganisms isolated from trophic ulcers, studying the antibiotic susceptibility of Gram-negative bacilli and determining the hemolytic, anti-lysozyme, and anti-complementary properties, as well as the biofilm-forming capacity.

Material and methods

The study was carried on 128 samples of trophic ulcers. The microbial strains involved in the process were isolated in pure cultures, under laboratory conditions, and subsequently identified by classical microbiological methods and Vitek2 Compact system (BioMerieux), based on the morpho-tinctorial, biological and biochemical properties.

Antibiotic susceptibility test of Gram-negative bacilli was carried out and interpreted according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing) recommendations, using phenotypic methods (Kirby Bauer disc diffusion test, synergy test) [14]. The assessed antibiotic discs included ciprofloxacin (5mg), levofloxacin (5 mg), amikacin (30 mg), gentamicin (10 mg), aztreonam (30 mg) cefepime (30 mg), ceftazidime (30 mg), amoxicillin-clavulanic acid (30mg), imipenem (10mg), meropenem (10 mg), piperacillin (30 mg) and ampicillin (10 mg).

Strains that showed resistance to three or more antibiotic groups were considered poly- resistant ones [15].

The anti-lysozyme activity was determined according to the method described by Gordina E. et al. [16]. The tested strain was cultured on an agar slant for 18-24 hours at 37°C, then subcultured in peptone water and grown for 6 hours at 37°C. The culture was adjusted to 0.15 optical density in peptone water, which corresponds to $1x10^8$CFU/ml. Simultaneously, the lysozyme suspension was prepared in peptone water with a concentration of 12.5μg/ml. The use of a higher concentration of lysozyme inhibits the growth of microorganisms, whereas lower concentrations do not allow indicating this phenomenon. Then, 100μl of lysozyme broth at a concentration of 12.5μg/ml and 25μl of microbial suspension were dispensed to the wells of the plate used for enzyme immunoassay. 100μl of peptone water and 25μl of microbial suspension were added to the control wells (n=2). The culture was thermostated for 4 hours and the optical density was measured over 2 and 4 hours. The results were read by the ELISA reader and the optical density was measured at 600 nm wavelength (A600). The distribution of the strains according to the level of expression was performed according to the following criteria: low expression levels ($K<0.49$); medium expression levels (within the limits of $0.5\leq K\geq 2.49$) and high expression levels ($K>2.5$), where K stands for the coefficient of anti-lysozyme activity of the assessed strain.

Anti-complementary activity was determined by the method described by Bukharin O. et.al. [17]. A microbial suspension (the optical density of which corresponded to the McFarland turbidity standard 1.0) was inoculated with the inoculation loop on a 1.5% agar plate surface. The inoculated plates were thermostated at 37°C for 18-24 hours, in order to reveal the biological properties of the microorganisms. Afterwards, the cultured plates were exposed to chloroform vapors for 10 minutes and then covered with a second layer of 1.5 ml of agar and 1 μl of complement on a flat surface (trated in the hemolytic system until 50 HU/ml, 25 HU/ml and 12.5 HU/ml activity), so that the final complement concentration corresponds to 20; 10 and 5 UH/ml, respectively. Plates were incubated in the inverted position at 37°C for one hour in order to perform the anti-complementary activity of bacteria and vital products. Then, the plates were covered with a third 0.7% agar layer, containing 0.1 ml of bacterial suspension from indicator culture of *Escherichia coli* ГИСК 212 (optical density of microbial suspension corresponded to McFarland turbidity standard 0.5), exhibiting an increased sensitivity to the bactericidal action of complement system. Plates were incubated at 37°C for 18-24 hours to allow inactivation of complement by bacteria. Anti-complementary action was assessed based on the growth areas of indicated culture around bacteria, where the inactivation of the complement occurred.

Blood agar culture medium was used to study the hemolytic activity of the isolates [5]. The quantitative determination of biofilm-forming capacity of isolates from trophic ulcers was determined by a microtiter test [18]. Thus, 150μl of peptone water and 15μl of bacterial suspension were dispensed to a 96-well plate according to the McFarland turbidity standard 0.5 (1.5×10^8CFU/ml, respectively), previously harvested from 18- to 24-hour cultures on 5% blood agar. Duplicate laboratory tests were performed. The plates were then covered and incubated aerobically for 24-48 hours at 37°C. In order to assess the bacterial attachment to the inert substrate, the wells were rinsed five times in sterile saline solution and fixed with cold methanol for 5 minutes. Methanol was then removed; the dry plates were stained for 30 minutes with 0.1% crystal violet solution. The slide was then washed with tap water to remove the excess stain and the stained biofilm was resuspended with a 33% glacial acetic acid solution. Thus, the obtained suspensions were used to determine the optical density (OD), based on the absorbance spectrophotometer readings of stained suspensions at 490 nm (A490).

The cut-off optical density (ODc) is defined as the average OD of negative control + 3x standard deviation (SD) of negative control. The strains were tested for biofilm production and classified based on the adsorption of the Crystal Violet dye. The isolates were classified into four categories: non-adherent, the optical density lower than 0.056; poor adherent ($0.056<OD\leq 0.112$), moderately adherent ($0.112<OD\leq 0.222$) and strongly adherent, the optical density greater than 0.222.

Escherichia coli (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603) and *Acinetobacter baumannii* (ATCC 11778) reference strains were used for quality control. Statistical data analysis was carried out via EpiInfo 2000.

Ethical issues

The studied strains were obtained from routine analysis of clinical specimens. Sample collection did not involve direct contact with the patient, thus no consent was required. Permission to conduct the study was obtained from the Head of the Microbiology Laboratory. The study was conducted and approved by the ethics committee No 65/12.04.2017 of Nicolae Testemitsanu State University of Medicine and Pharmacy of the Republic of Moldova.

Results

Bacteriological examination was carried out on 128 samples collected from patients with trophic ulcers. A single species of microorganisms was isolated in 35.9% of cases, two and more species in 53.1% and no microorganisms were isolated in 10.9% of cases. A total of 211 microbial strains were isolated and identified. The most common strains isolated from trophic ulcers were the *Staphylococcus* (predominantly *S. aureus*), then enterobacteria (*Proteus* spp., *Klebsiella* spp., *Escherichia* spp.), non-fermenting bacilli *Pseudomonas* spp., *Acinetobacter* spp. and yeast-like fungi of the genus *Candida.*

Among the infections caused by a single microbial species, the most often involved was *Staphylococcus aureus* (41.3%), as well as other isolated species like *Proteus* (15.2%), *Staphylococcus haemolyticus* (10.9%), *Pseudomonas aeruginosa* (8.7%), *Acinetobacter baumannii* (8.7%), *Klebsiella pneumoniae* (8.7%) and *Escherichia coli* (6.5%). Mixed infections were caused by associations of strains like *S. aureus* and *P.aeruginosa* (23.1%), followed by associations of *S.aureus* and *A.baumannii* (20.5%). Association between two species was registered in 57.4% of mixed infections and three species associations were found in 42.6%.

In this study, 106 (50.2%) strains of Gram-negative bacilli were isolated, of which 61.3% were glucose-fermenting and 38.7% – glucose-non-fermenting.

The antibiotic susceptibility tests of Gram-negative bacilli strains, isolated from trophic ulcers, showed a high level of resistance to these drug preparations. *Enterobacteriaceae* strains exhibited a marked resistance to penicillins (100%), cephalosporins (87.7%), fluorquinolones (84.6%) and aminoglycosides (70.8%). Carbapenems proved to be the most effective antibacterial drugs (83.1% strains were sensitive to meropenem) against infections caused by enterobacteria (fig. 1).

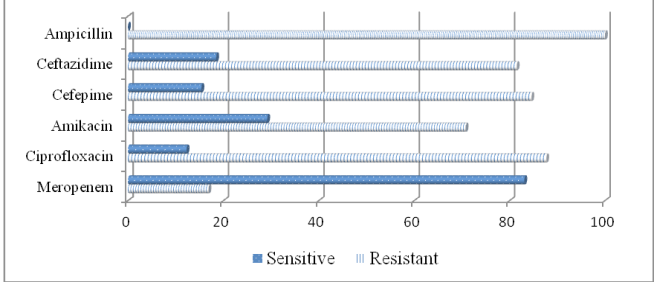

Fig. 1. Antibiotic susceptibility of *Enterobacteriaceae* strains (%).

Moreover, 21 (32.3%) extended-spectrum beta-lactamase producing strains (ESBL) have been identified during this study, which were sensitive to meropenem (76.2%), followed by amickacin (28.6%) and ciprofloxacin (19.0%).

Antibiotic susceptibility assessment of *P. aeruginosa* strains revealed a large number of multiple antibiotic resistant strains and only three strains (13.0%) were resistant to a single drug preparation. Of 23 strains, 20 (87.0%) were multidrug-resistant. Aminoglycoside was the most active agent tested against *P. aeruginosa* (47.8%). A high resistance level was observed in the following drug groups: penicillins (100%), cephalosporins (86.9%), monobactam (82.6%), carbapenems (78.3%) and fluorquinolones (73.9%) (fig. 2).

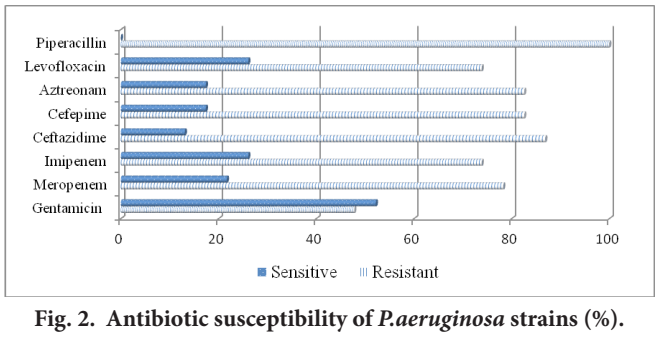

Fig. 2. Antibiotic susceptibility of *P.aeruginosa* strains (%).

Acinetobacter baumannii strains showed high resistance to most antibiotic groups. Carbapenems exhibited a higher level of sensitivity, including imipenem (72.2%) and meropenem (66.7%). Over 50% of the strains were resistant to aminoglycosides, fluorquinolones, third- and fourth-generation cephalosporins. Multiple antibiotic resistance was detected in 77.8% of strains and only 4 strains (22.2%) were sensitive to all antibiotics that were chosen for testing (fig. 3).

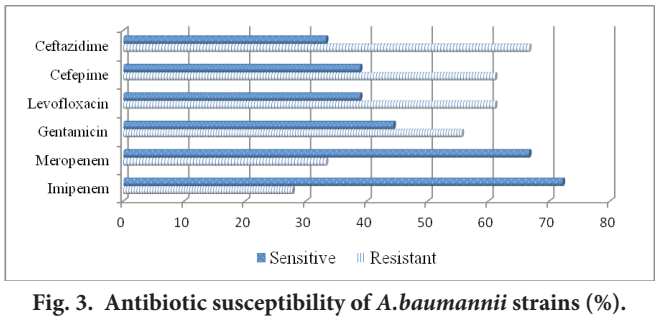

Fig. 3. Antibiotic susceptibility of *A.baumannii* strains (%).

The next stage of the study determined the level of expression of some persistence factors of Gram-negative bacilli isolated from trophic ulcers (tab. 1).

Hemolysin, which is an exotoxin, appeared to be one of the persistence factors leading to chronic infectious process [5]. Hemolytic activity was recorded in 46 (44.3%) strains of Gram-negative bacilli isolated from trophic ulcers.

Lysozyme was also determined as being a universal resistance factor of the macro-organism. It is a peptidoglycan-degrading enzyme, which commonly works in Gram-positive bacteria; however, Gram-negative bacteria might be also affected by increasing the permeability of the outer membrane and lipopolysaccharides [6]. Therefore, micro-

Table 1

Hemolytic, anti-lysozyme and anti-complementary activity of Gram-negative bacilli isolated from trophic ulcers

Species	Hemolytic activity		Anti-lysozyme activity		Anti-complementary activity		Total strain number
	Abs.	%	Abs.	%	Abs.	%	
P. mirabilis	12	42.8	25	89.3	26	92.9	28
P. vulgaris	0	0	2	100	2	100	2
K. pneumoniae	12	54.5	20	90.9	21	95.5	22
E. coli	4	30.7	7	53.8	12	92.3	13
P. aeruginosa	8	34.8	22	95.7	21	91.3	23
A. baumannii	10	55.5	8	100	17	94.4	18
Total	45	43.4	94	88.7	99	93.4	106

Table 2

***In vitro* biofilm-forming ability of Gram-negative bacillus strains isolated from trophic ulcers**

Biofilm-forming ability	*Proteus* spp.		*Klebsiella pneumoniae*		*Escherichia coli*		*Pseudomonas aeruginosa*		*Acinetobacter baumannii*		Total	
	Abs.	%	Abs.	%	Abs.	%	Abs.	%	Abs.	%	Abs.	%
Non-producing	4	13.3	3	13.6	4	30.8	6	26.1	5	27.2	31	29.2
Producing	26	86.7	19	86.4	9	69.2	17	73.9	13	72.2	75	70.8
– strong	6	23.1	13	68.4	3	33.3	7	41.2	4	30.8	33	39.3
– moderate	18	69.2	5	26.3	5	55.6	9	52.9	2	15.4	39	46.4
– weak	2	7.7	1	5.3	1	1.1	1	5.9	7	53.8	12	14.3

organisms tend to protect themselves against this enzyme in order to survive longer within the host organism. Anti-lysozyme activity was recorded in 94 (88.6%) out of 106 strains, whereas 12 (11.3%) were inactive. 24 (25.5%) strains showed a high level of expression of anti-lysozyme activity, 32 (34.1%) – a medium level and 38 (40.4%) strains – a low level of expression.

Another important factor responsible for microbial persistence within the infection site is the ability of bacterial cells to inactivate the complement system of the macro-organism [6]. Of the 106 Gram-negative bacilli strains involved within the study, 99 strains (93.4%) exhibited anti-complementary activity, of which 81 (81.8%) strains inactivated the complement at a concentration greater than 15 CH50/ml, 16 (16.2%) strains – at a concentration ranging between 5 to 15 CH50/ml and 2 (2.0%) strains – 5 CH50/ml. Only 7 strains (6.6%) did not inactivate the complement.

The data study of the anti-complementary activities in monocultures compared to isolated cultures in associations showed that the latter strains are often related to medium and high anti-complementary activity ($P < 0.05$).

Studies on the persistence factors of the isolated microorganisms showed that the level of expression is higher in isolates of mixed infections (1.0-1.5 times) compared to those in monoinfections ($P < 0.05$).

Of the 106 strains of Gram-negative bacilli isolated from trophic ulcers, 75 (70.8%) strains produced detectable biofilms (OD>0.112). As regarding the biofilm status, 33 (39.3%) isolates produced strong biofilms (OD>0.220), 39 isolates (46.4%) – moderate biofilms (OD 0.112-0.220) and 12 isolates (14.3%) – weak biofilms (tab. 2).

All Gram-negative bacilli strains isolated from trophic ulcers exhibited a high ability of biofilm formation (>70%).

The antibiotic resistance of biofilm-forming compared to non-biofilm-forming strains showed that biofilm-forming strains had a higher resistance to all groups of drugs tested.

Conclusions

1. The study of the spectrum of microorganisms isolated from the major trophic ulcers has shown the important roles of the genus *Staphylococcus*, followed by Gram-negative bacilli, yeast-like fungi of the genus *Candida* and *streptococci*.

2. Gram-negative bacilli strains isolated from trophic ulcers showed a marked resistance to the antimicrobial drugs tested.

3. The study of the persistence factors of gram-negative bacilli showed that the isolated strains have a range of abilities to inactivate the natural resistance mechanisms of the macroorganisms.

4. Understanding the bacterial persistence factors might allow selecting effective targeted therapies for controlling the microbial growth in trophic ulcers.

5. The study results show that treatment of trophic ulcers is both a challenging task and a major issue requiring current management strategies.

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