# ORIGINAL RESEARCHES

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## Antibiotic susceptibility and factors involved in virulence and persistence of *Acinetobacter baumannii* strains

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### Abstract

**Background:** *Acinetobacter baumannii* has emerged as a medically important pathogen because of the increasing number of infections produced by this organism over the preceding three decades and the global spread of strains with resistance to multiple antibiotic classes. Recently, a particular attention has been drawn to the study of the microbial persistence properties and their correlation with the rate of elimination from the source of infection, as well as the prognosis of the disease progression.
**Material and methods:** There were examined 53 strains of *A. baumannii*, isolated from patients with trophic ulcers. The bacteriological examination, as well as tests on determining both the persistence factors and the antibiotic susceptibility of the isolated strains were carried out according to the current method.
**Results:** *A. baumannii* strains were highly resistant to all antibiotics tested, 38 (71.7%) showed multidrug resistance. The studies regarding the persistence factors of *A. baumannii* strains, revealed that 100% exhibited an antilysosyme activity, 78.0% – anticomplementary activity, 73.6% – produce biofilms, 58.5% – hemolytic activity, 28.3% and 13.2% – lecithinase and plasma coagulation activity, respectively.
**Conclusions:** Isolated strains showed higher level of antimicrobial resistance and multiple persistence factors. The study results proved that treatment of trophic ulcers is still a major problem, requiring rational monitoring and management strategies.
**Key words:** *Acinetobacter baumannii*, trophic ulcers, antibiotic resistance, persistence factors.

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### Introduction

The genus *Acinetobacter* includes aerobic Gram-negative and non-fermenting cocobacilli, which are widely spread in nature and might be commensal or conditionally become pathogenic to humans. The most common species to cause infections is *A. baumannii*, being the most frequently isolated from clinical specimens, followed by *A. lwoffii*, *A. haemolyticus* and *A. johnsonii*. These bacteria have been long considered as low-virulent strains with a reduced pathogenic potential, however, today they play an important role in colonization and infections of immunocompromised patients. These strains are able to colonize the skin and airways of patients, particularly of those admitted within intensive care units, where high bacterial incidence has been recorded (up to 75%) [1, 2].

These bacteria are recognized as disease-causing agents, involved in healthcare-associated infections, displaying high morbidity and mortality rates in critically ill patients. These patients are more prone to germ transmission due to invasive medical procedures, the widespread use of a variety of antimicrobial agents, particularly the broad-spectrum ones and inappropriate infection control [3].

The infections associated with different types of immunodeficiencies, virulence and antibiotic resistance of the *Acinetobacter* strains, make up 28.3% – 84.3% of mortality cases among patients. Currently, of particular concern is the selection of some *A. baumannii* strains that can express serum bactericidal resistance to various factors and biofilm-forming capacity, thus often being involved in bacteremia associated with high mortality rate (up to 75%) [3-6].

The treatment of *Acinetobacter* spp. infections is often challenging, since the bacteria show an intrinsic resistance to many antimicrobial agents and due to a variety of mechanisms, as well as have an extraordinary ability to develop resistance to all classes of antimicrobials, used in the treatment of gram-negative bacillus infections [4, 7].

It is well recognized that Acinetobacter species have a natural resistance to cephalosporins of generations 1-2 and aminopenicillins. The bacterial resistance to other classes of antibiotics is due to various mechanisms as, enzyme inactivation (penicillinases, cephalosporinases, carbapemenases, amino acid acetyltransferases), changes in the target sites (Penicillin Binding Protein), impaired membrane permeability, and activating the pumps [8].

The worldwide spread of multidrug-resistant or pan-resistant Acinetobacter baumannii strains has increased dramatically since 1990. The World Health Organization (WHO) has included these bacteria in the group of highly infectious agents that would spiral out of the antibiotic control [1].

According to the 2011-2014 European surveillance network EARS-NET (European Antimicrobial Resistance Surveillance – Net-work) data, the highest antibiotic resistance levels of gram-negative bacilli have been reported in southern and eastern Europe. The highest resistance level was recorded in species of the genus *Acinetobacter* [9].

*Acinetobacter baumannii* is one of the most serious *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter species* (ESKAPE) organisms, as declared by the WHO that can escape the effect of antibacterial drugs [1].

Although trophic ulcers are a major health problem, the current methods of treatment are sometimes not enough and often lead to amputation due to misunderstanding regarding microbiology of these infections or methods of their eradication.

Although it is well known that the associated microbial infections might significantly worsen and delay the healing processes of trophic ulcers, recent research studies suggest that microbial biofilms highly contribute to their chronicity. Over 90% of chronic wounds infections are related to biofilms, whereas only 6% occur in acute wounds [10]. The protective and hostile nature of these biofilms makes the treatment of these infections extremely difficult.

At present, there are evidences to suggest that the ability of bacteria living within cells (e.g. macrophages) and biofilm formation are associated with persistent infections [11].

One of the causes of bacterial long-term persistence in the host organisms is the multiple factors that inactivate the antimicrobial mechanisms of the immune system. It is also well known that the microbial persistence potential determines the length of their stay in the macro-organism, whereas its suppression by antimicrobial drugs leads to a reduced infectious potential of the microorganism [12, 13].

The study was aimed to conduct and evaluate the antibiotic susceptibility and the persistence factors of *A. baumannii* strains isolated from trophic ulcers.

## Material and methods

The study group included 53 strains of *A. baumannii*, isolated from trophic ulcers. Solid Oxoid culture media were used to isolate *A. baumannii*: Columbia Blood Agar Base with 5% sheep blood, MacConkey Agar.

The identification was assessed by classical biochemical tests and confirmed via VITEK 2 COMPACT automatic system.

The Kirby-Bauer disk diffusion method was used for antimicrobial susceptibility testing , whereas the *in vitro* antibiotic test results were interpreted according to 2019 EUCAST (European Committee on Antimicrobial Susceptibility Testing) [14].

The *A. baumannii* strains were tested for four classes of antimicrobial drugs: aminoglycosides (gentamicin, amikacin, tobramycin), carbapenems (meropenem, imipenem), fluoroquinolones (levofloxacin, ciprofloxacin) and sulfanylamides (trimethoprim-sulfamethoxazole). *A.baumannii* strains that showed resistance to three or more different classes of antimicrobials were classified as multidrug-resistant (MDR) *A. baumannii* [15].

The lecithinase activity was assayed on the egg yolk salt agar, the hemolytic activity – on a blood agar plate, the plasma coagulase activity – on an inoculated culture into a sterile citrate plasma and the anti-lysozyme and anti-complementary activity we determined according to the method described by Bukharin O. et al. [13, 16, 17].

*Detecting the anti-complimentary activity*

The microbial suspension was inoculated on a surface of 1.5% agar plate; by using the bacteriological loop (the optical density of the microbial suspension corresponded to Mac Farland turbidity standard 1.0). The petriplates were inoculated at a temperature of 37°C during 18-24 hr. in order to manifest biological properties of bacteria. Furthermore, the studied cultures were inactivated in chloroform vapors for 10 minutes, then the plates were covered with a second layer of 1.5 ml of agar and 1 µl complement, so that the final concentration of the compliment corresponded to 20; 10 and 5 UH / ml, respectively. The petriplates were incubated in the inverted position at 37 ° C for 1 hour to develop the bacterial anti-complementary action and products of their activity. Then the plates were coated with a third layer of 0.7% agar containing 0.1 ml of bacterial suspension based on the indicator culture of *Escherichia coli* ГИСК 212, which showed an increased sensitivity to the bactericidal action of complement system. The plates were incubated at 37°C for 18-24 hr. to detect bacteria-mediated complement inactivation. Anti-complementary activity was assessed by the presence of indicator culture growth areas surrounding the tested bacterial cultures, where complement inactivation occurred.

*Detecting the anti-lysozyme activity*

The tested strain was cultured on a sloping agar for 18-24 hours at 37°C, then transferred into peptone broth and cultured at 37°C for 6 hours. The optical density in peptone broth was adjusted to 0.15, which corresponded to $1x10^8$ CFU/ml.

Simultaneously, the lysozyme suspension was prepared in peptone broth with 12.5µg /ml concentration. The use of

a higher concentration of lysozyme might inhibit the bacterial growth, whereas lower lysozyme concentrations do not allow identification of this phenomenon.

100µl of lysozyme broth was added in the wells of a plate, designed for enzyme-linked immunosorbent assay, at a 12.5µg/ml concentration, to which 25µl of microbial suspension was added. 100 µl of peptone broth and 25µl of microbial suspension were added into the control wells (2 wells), which were incubated for 4 hours. The optical density was measured over 2 and 4 hours. The results were read using an ELISA reader, whereas the optical density (OD) was measured at a wavelength of 600 nm (A600).

The strains were distributed according to the extent of this phenomenon, based on the following criteria:

1. Low expression levels of antilysosyme activity: K <0.49;
2. Mean expression levels of antilysosyme activity: K in the limits of 0.5-2.49;
3. High expression levels of antilysozyme activity: K> 2.5.

*Determining biofilm production*

Biofilm production by isolated strains was quantitatively determined using the microtiter plate method [17]. For the purpose of study, 150µl of peptone broth and 15 µl of bacterial suspension were added to a 96-well plate and adjusted to the 0.5 McFarland turbidity standard (respectively 1.5 x $10^8$ CFU/ml), which were previously prepared from 18-24 hour bacterial culture and grown on 5% blood agar. The plates were coated and incubated for 24 hours at 37°C. Subsequently, the level of adhesion of the tested strains to inert substrate was determined by removing the content from each well and then rinsing five times with sterile saline and fixing with cold methanol for 5 minutes. After removing of the methanol, the dried plates were stained with 0.1% violet crystal solution for 30 minutes. The excess stain was removed by washing and the stained biofilm was resuspended in a 33% glacial acetic acid solution. Thus the obtained suspensions were used to determine the optical density (OD), based on the spectrophotometric absorbance readings at 570 nm colored suspension (A570). The tests were performed in duplicate.

The optical density cut-off value (ODc) is defined as the average OD of negative control + 3x the standard deviation (SD) of negative control. Biofilm formation by the tested strains was assayed and classified according to the adsorption of the violet crystal dye. The isolates were classified into four categories: non-adherent (OD ≤ ODc), poor adherent (ODc <OD ≤ 2xODc), moderately adherent (2xODc <OD ≤4xODc) and strongly adherent (4xODc <OD).

The reference strains *A. baumannii* (BAA-747) were used for quality control. EpiInfo 2000 was used in statistical data analysis.

## Results and discussion

The present study assessed the antimicrobial susceptibility profiles of *A. baumannii* strains isolated from trophic ulcers (fig. 1-4).

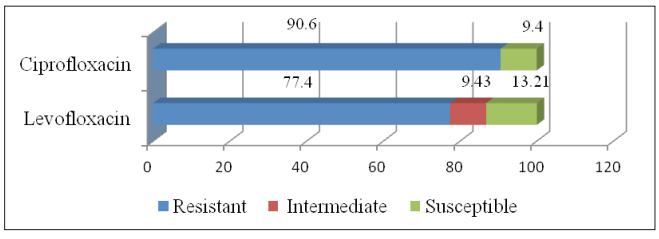


**Fig. 1. Antibiotic susceptibility testing of *A. baumannii* strains to fluoroquinolones**

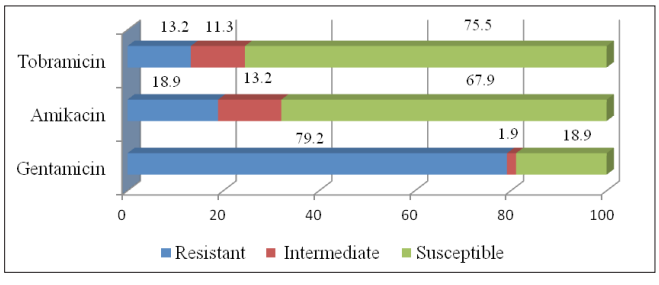


**Fig. 2. Antibiotic susceptibility testing of *A. baumannii* strains to aminoglycosides**

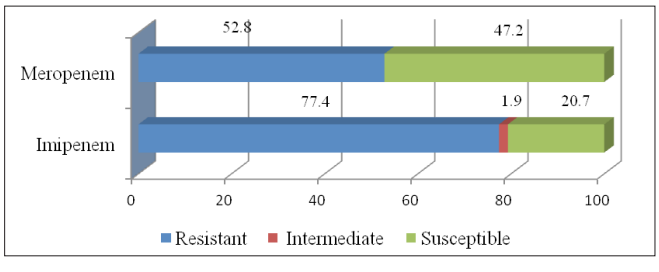


**Fig. 3. Antibiotic susceptibility testing of *A. baumannii* strains to carbapenems**

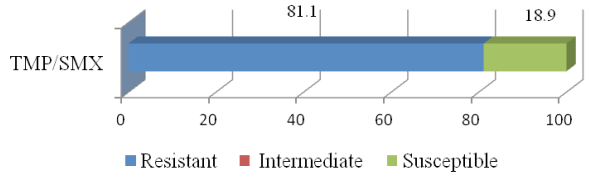


**Fig. 4. Antibiotic susceptibility testing of *A. baumannii* strains to sulfanylamides, TMP/SMX – trimethoprim/sulfamethoxazole**

The data analysis of the previous figures revealed that *A. baumanii* strains, isolated from trophic ulcers are highly resistant to most antibiotics. Of 53 strains, 38 (71.7%) were multidrug-resistant. Higher antibiotic susceptibility was registered to tobramycin 40 (75.5%) and amikacin 36 (67.9%).

The resistance of *A. baumannii* strains to carbapenems (imipenem – 77.4% and meropenem – 52.8%) is of major concern. The World Health Organization stated that carbapenem-resistant *Acinetobacter baumannii* infections have become a major public health challenge and a serious health threat in future [18].

The present study also assessed one of the persistence factors of *A. baumannii* strains viz. the anti-lysozyme activ-

ity. The anti-lysozyme activity (the ability of microorganisms to deactivate muramidase, which is an essential link in non-specific resistance) is very common among various isolates [19]. Of the 53 *A. baumannii* strains isolated from trophic ulcers, 53 (100%) showed an anti-lysozyme activity. A high expression level of anti-lysozyme activity (K> 2.5) was recorded in 16 (30.2%) strains, a mean expression level (K0.5-2.49) – 24 (45.3%) strains and a low expression level (K <0.49) was revealed in 13 (24.5%) of strains.

Hemolysin was another persistence factor studied, which acts as an exotoxin that may lead to chronicity of various infectious process [20]. Hemolytic activity was recorded in 31 (58.5%) *A. baumannii* strains isolated from trophic ulcers.

Lecithinase was present in 15 (28.3%) *A. baumanii* strains and plasmocoagulase – in only 7 (13.2%) strains. Lecithinase is an exoenzyme that acts upon phospholipids (lecithin) within the muscle fiber membrane, erythrocytes and other cells. Plasmocoagulase is a proteolytic enzyme that leads to septic thrombi formation and exerts an antiphagocytic effect by spreading within the body. These enzymes are aggressive factors for the microorganisms [20].

The ability to deactivate the complement system of the macroorganism leads to a microbial persistence within the infectious focus, which develops into chronic processes [19]. Lipid A and serine protease of *A. baumannii* exhibit an anticomplementary activity, thus providing a long-term bacterial survival inside the host organism [21, 22].

Of the 53 strains studied, 50 (94.3%) showed anti-complementary activity, of which 39 (78.0%) strains inactivated the complement at a concentration greater than 15 CH50 / ml, 8 (16.0%) – at a concentration of 5-15 CH50 / ml and 6 (12.0%) – at a concentration of 5 CH50 / ml. Only 3 strains (5.7%) did not inactivate the complement.

Biofilm-forming ability of *A. baumannii* clinical strains is crucial for their survival inside the macroorganism (even in antibiotic treatment) [23].

Of the 53 *A. baumannii* strains tested – 39 (73.6%) produced detectable biofilms. As regarding the biofilm status, 15 (38.5%) of the isolates were determined as strong biofilm forming, 6 (15.4%) – moderate biofilm forming and 18 (46.1%) – produced weak biofilms, out of the 39 strains. Similar data have been reported by a series of studies, which reported that over 74% of *A. baumannii* clinical strains produced biofilms [24, 25].

The correlation between the biofilm formation ability and MDR strains was also studied (tab. 1). Of the 38 MDR *A. baumannii* strains – 14 (36.8%) produced strong biofilms, 4 (10.5%) – moderate biofilms, 17 (44.7%) – weak biofilms and 3 (7.9%) – produced no biofilms.

**Table 1**

**Correlation between MDR and Non-MDR strains of *A. baumannii* and biofilm formation ability by using the phenotypic method**

| Types of biofilm | MDR | Non-MDR | Total |
|---|---|---|---|
| Strong biofilm producer | 14 (26.4%) | 1 (1.9) | 15 (28.3%) |
| Moderate biofilm producer | 4 (7.5%) | 2 (3.8%) | 6 (11.3%) |
| Weak biofilm producer | 17 (32.1%) | 1 (1.9%) | 18 (34.0%) |
| No biofilm producer | 3 (5.7%) | 11 (20.7%) | 14 (26.4%) |
| Total | 38 (71.7%) | 15 (28.3%) | 53 (100%) |

A significant association between MDR and biofilm formation ability was reported (P <0.001).

## Conclusions

The research findings underline the importance of an effective surveillance of antimicrobial resistance of *A. baumannii* strains, thus suggesting the appropriate use of antimicrobials in order to prevent the emergence of bacterial resistance to these specific drugs.

The present study proved that the most expressed pathogenicity factors in *A. baumannii* strains, isolated from trophic ulcers, are lysozyme inactivation, complement and biofilm formation. The assessment of virulence and persistence factors will help the practitioners to effectively manage these infections, providing a more effective control of appropriate antimicrobials and thus resulting in a reduced mortality and morbidity rates in patients.

The study results justify the development of new strategies for the prevention and treatment of *A. baumannii* infections.

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**Author's contributions**
GB conceptualized the idea, conducted literature review, wrote the manuscript, revised and approved the final text.

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**Ethics approval and consent to participate**
The research project was approved by Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 65, 12.04.2017).

**Conflict of Interests**
No competing interests were disclosed.