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Antimicrobial susceptibility and biofilm production among *Staphylococcus* and *Candida* species

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Abstract

Background: Biofilms are surface-attached groups of microbial cells that are embedded in an extracellular matrix. One of the main features of biofilms is their resistance to antimicrobial drugs; therefore, the biofilm-based infections are extremely difficult to treat. This study aimed to investigate the biofilm-forming capacity of *Staphylococcus* spp. and *Candida* spp. strains isolated from collected clinical samples, as well as to assess their antibiotic susceptibility.
Material and methods: The study was conducted on 134 strains of *Staphylococcus* spp. and 147 strains of *Candida* spp. isolated from various clinical specimens. Both biofilm formation and antibiotic susceptibility of the isolated strains were studied using contemporary standardized microbiological methods.
Results: The results of the study showed a high biofilm-forming capacity among the clinical strains of *Staphylococcus* spp. and *Candida* spp., as well as a higher level of antibiotic resistance in biofilm-producing strains compared to biofilm non-producing ones.
Conclusions: The high rates of antibiotic resistance and biofilm-forming capacity of strains represent a major public health challenge. The study showed a strong correlation between biofilm formation and antimicrobial resistance patterns.
Key words: *Staphylococcus* spp., *Candida* spp., biofilm formation, antimicrobial resistance.

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Introduction

The advancement of biomedical science has enabled to study the microorganisms in their natural environment, whereas over 95% of microorganisms existing in nature are in biofilms [1]. Biofilm formation is an important strategy by which microorganisms survive and adapt in natural environments [2, 3].

A biofilm is defined as an aggregate of microorganisms in which the cells adhere to each other on a surface, enclosed in a synthetized extracellular polymeric substance matrix. Biofilms can occur on living or non-living surfaces, being widely spread in nature. The vast majority of bacterial infections may also involve microbial biofilm formation [4].

Bacteria living in a biofilm usually have significantly different properties from free-floating bacteria of the same species, being protected by a dense biofilm structure, which allows them to cooperate and interact in different manners. The main features of the biofilms are their high resistance to disinfectants and antimicrobial drugs; whereas the thick extracellular matrix and the outer layer cells protect the interior of the community [5].

Most microorganisms form biofilms as a means of response to a number of factors, including cellular recognition of specific or non-specific attachment sites on a surface nutritional index, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics [6, 7].

It is estimated that microbial biofilms play a major role in over 80% of infections. Sixty percent of healthcare-associated infections are due to biofilm formation on medical implants. Moreover, many chronic diseases are associated with biofilms, such as infectious endocarditis, cystic fibrosis pneumonia, periodontitis, chronic rhinosinusitis, trophic ulcers and otitis media [8].

Staphylococci, predominantly *Staphylococcus aureus* and *Staphylococcus epidermidis*, are the disease-causing agents in a series of infections, which are often associated with chronicity, difficulty to eradicate and antimicrobial resistance [9]. Staphylococci are ranked first among the etiological fac-

tors of bacterial infections, along with the annual increase in the number of methicillin-resistant staphylococci (MRS) strains and the occurrence of new antibiotic-resistant bacterial strains, which place this pathology among the emerging infectious diseases [10].

Staphylococcus aureus is an opportunistic pathogen, commonly involved in skin and soft tissue infections. It could be detected in the nasopharynx, skin, eyes, intestine and urogenital tract as part of the normal flora; although in some cases, it might pass through the skin barriers of wounds or surgical incisions, causing infections. In addition, it has the property to adhere and form biofilms on tissues or medical devices. Coagulase-negative staphylococci (CoNS) are considered saprophytic, avirulent or low-virulent microorganisms. However, over the past three decades there has been an increase in human infections caused by CoNS, particularly of *S.epidermidis* [11].

Levuriform fungi of the genus *Candida* are found as part of the normal flora in healthy individuals and are involved in the etiology of opportunistic infections, resulting in high mortality rates, particularly in immunocompromised individuals [12]. *Candida* species are most commonly associated with human diseases due to both virulence factors and biofilm-forming ability. *Candida* spp. causes systemic diseases and is the fourth most common cause of hospital-acquired blood infections. *Candida albicans* is the most commonly found species in fungal infections, whereas other species are involved to a lesser extent. However, the increased rate of non-Candida albicans isolation and antimicrobial resistance has become a major challenge for clinicians over the recent years [13].

Most infections caused by *Candida* spp. are related to biofilm formation on the mucosal surfaces and contaminated medical devices. Some study results revealed that the biofilms, formed by *Candida* spp. may become resistant to antifungal drugs, including amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole [14].

Therefore, a current *in vitro* study of the biofilm-forming ability associated with the antimicrobial resistance patterns of *Candida* spp. and *Staphylococcus* spp. strains isolated from various clinical biosubstrates is required for the efficient management of these infections.

Material and methods

There have been examined 134 strains of *Staphylococcus* spp. (88 – *S. aureus,* 46 – *S. epidermidis*) and 147 strains of *Candida* spp. (75 – *C. albicans,* 24 – *C. glabrata,* 22 – *C. krusei,* 14 – *C. parapsilosis,* 12 – *C. tropicalis*), isolated from clinical biosubstrates (blood, trophic ulcers, infected wounds, and vaginal secretions) and which have been identified by standard microbiological techniques [15].

Antimicrobial susceptibility testing and the result interpretation were carried out according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing) by using both qualitative methods (Kirby-Bauer disk diffusion assay) and quantitative methods determining the minimum inhibitory concentration (E-test, Vitek 2 Compact) [16].

Staphylococcus spp. strains were tested for benzylpenicillin, gentamicin, norfloxacin, cefoxitin, chloramphenicol, erythromycin, clindamycin, tetracycline, rifampicin, linezolid and vancomycin, whereas *Candida* spp. strains were assessed to fluconazole, itraconazole, amphotericin B, micafungin and flucytosine.

Bacteria that showed resistance to at least one preparation out of three or more antimicrobial groups were identified as multidrug resistant strains (MDR) in accordance with the guidelines recommended by the joint initiative of the European Center for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) [17]. The methicillin-resistant or methicillin-sensitive (MSS) patterns of *Staphylococcus* spp. strains were determined according to the inhibition zone diameters of cefoxitin disk (30mg), based on EUCAST: MSS if the diameter is at least 22 mm; MRS if less than 22 mm. A double disc diffusion test (D test) was used for detecting inducible resistance to clindamycin. The erythromycin (15mg) and clindamycin (2mg) discs are placed at a distance of 12-20 mm measured from the edges of the discs. A flattening of the zone of inhibition around the clindamycin disk (D test positive) is reported as a clindamycin-resistance [18].

Biofilm production by isolated strains was quantitatively determined using the microtiter plate method [19]. For the purpose of study, 150µl of peptonate broth and 15µl of bacterial suspension were added to a 96 well plate and adjusted to the 0.5 McFarland turbidity standard (respectively $1.5x10^8$ 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 *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 10231) and *Candida tropicalis* (ATCC 750) were used for quality control. EpiInfo 2000 was used in statistical data analysis.

Results

The antimicrobial susceptibility testing results of 134 strains of *Staphylococcus* spp., revealed that 92 (68.6%) were polyresistant to antibiotics, 69 (51.5%) were methicillin-resistant, and 32 (23.9%) were D-test positive.

Staphylococcus spp. strains showed the highest sensitivity levels to vancomycin (100%), followed by tetracycline (88.8%), linezolid (83.6%) and chloramphenicol (82.8%) (fig. 1).

Invasive candidiasis is usually treated with five main groups of antifungal drugs, including azoles, polyenes, allylamines, echinocandins and pyrimidine analogues [20]. A study, conducting a susceptibility testing for *Candida* species to fluconazole, voriconazole, itraconazole, ketoconazole and flucytosine, showed that most *Candida* spp. strains were sensitive to fluconazole and flucytosine [21].

The studied *Candida* spp. strains showed different levels of susceptibility to the tested antimycotics. The data analysis showed the highest level of resistance to itraconazole (87.7%) and fluconazole (87.1%), followed by amphotericin B (10.9%) and micafungin (2.7%). All tested strains were found to be sensitive to flucytosine (fig. 2).

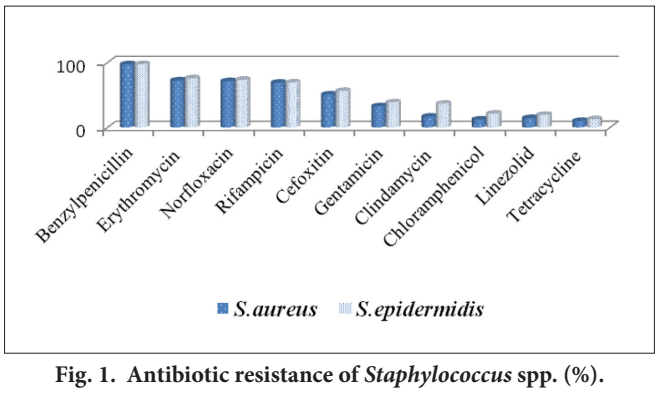

Fig. 1. Antibiotic resistance of *Staphylococcus* spp. (%).

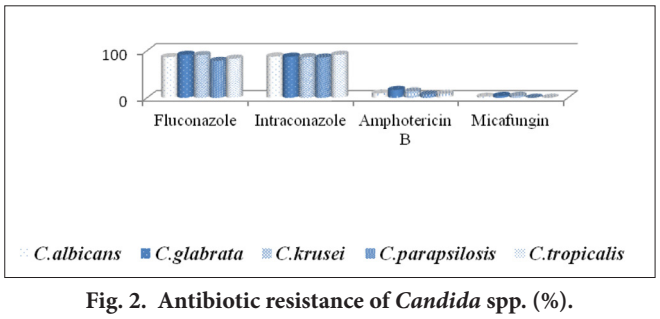

Fig. 2. Antibiotic resistance of *Candida* spp. (%).

The next stage of the study determined the biofilm formation ability of *Staphylococcus* spp. and *Candida* spp. Of the 134 tested staphylococcus strains, 77 (57.5%) produced detectable biofilms. The biofilm status referred to 27 (35.1%) of isolates, which produced strong biofilms, 32 (41.6%) – moderate biofilms and 18 (23.4%) – weak biofilms (fig. 3).

Candida spp. strains produced detectable biofilms in 59.2%. The highest level of biofilm formation ability was recorded in *C.glabrata* strains (95.8%), followed by *C. parapsilosis* (57.1%), *C. krusei* (54.5%), *C. albicans* (52.0%) and

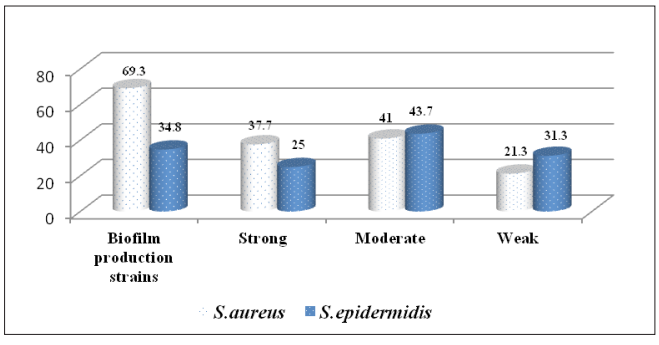

Fig. 3. The biofilm formation capacity of *Staphylococcus* spp. (%).

C. tropicalis (41.7%). 44 (50.6%) of *Candida spp*. strains produced strong biofilms, 29 (33.3%) – moderate biofilms and 14 (16.1%) – weak biofilms (fig. 4).

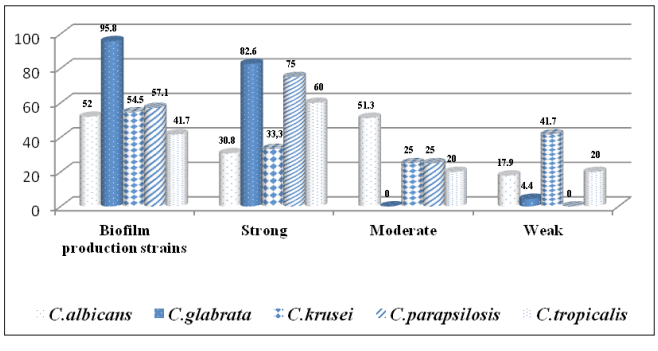

Fig. 4. Biofilm formation capacity of *Candida* spp. (%).

It is a well-known fact that bacterial populations in biofilms are considerably more resistant to antibiotics than planktonic cells [22]. Thus, biofilm-producing *Staphylococcus* spp. strains showed a higher antibiotic resistance compared to non-producing strains: benzylpenicillin (100% vs. 94.7%), gentamicin (61.0% vs. 0%), erythromycin (94.8 % vs 45.6%), tetracycline (19.5% vs 0%), cefoxitin (68.8% vs 42.1%), clindamycin (38.9 vs 3.5%), norfloxacin (90.9% vs 47.4%), chloramphenicol (27.3% vs 0%), rifampicin (81.7% vs 29.8%) and linezolid (25.9% vs 3.5%). All strains were found sensitive to vancomycin (tab. 1).

Comparison of biofilm formation ability between methicillin-resistant (MRS) and methicillin-sensitive (MSS) isolates of *Staphylococcus* spp. was carried out. The quantitative and qualitative results showed higher biofilm formation ability in MRS strains for both *S.aureus* and *S. epidermidis* strains compared to MSS bacteria. Biofilm-producing strains revealed a higher antibiotic resistance, which may lead to treatment failures in MRS infections (tab. 2).

The studies on *Candida* spp. strain resistance to antifungal drugs, as well as biofilm formation capacity, showed a statistical correlation between biofilm formation capacity and antifungal susceptibility ($p < 0.05$) (tab. 3).

Flucytosine is known to inhibit both ribonucleic acid and deoxyribonucleic acid synthesis [23] and was the most effective antifungal agent against biofilm-producing *Candida* strains, tested within the present study.

Table 1

Antibiotic resistance of biofilm-producing and non-producing *Staphylococcus* spp.

Antimicrobials	Biofilm-producing strains (N=77)	Biofilm-nonproducing strains (N=57)	p-value
	n (%)	n (%)	
Penicillins			
Benzylpenicillin	77 (100)	54 (94.7)	$p>0.05$
Aminoglycosides			
Gentamicin	47 (61.0)	0 (0)	$p<0.0001$*
Macrolides			
Erythromycin	73 (94.8)	26 (45.6)	$p<0.0001$*
Tetracyclines			
Tetracycline	15 (19.5)	0 (0)	$p>0.05$
Cephalosporins			
Cefoxitin	53 (68.8)	24 (42.1)	$p<0.05$*
Lincosamides			
Clindamycin	30 (38.9)	2 (3.5)	$p>0.05$
Fluoroquinolones			
Norfloxacin	70 (90.9)	27 (47.4)	$p<0.0001$*
Miscellaneous agents			
Chloramphenicol	21 (27.3)	0 (0)	$p<0.05$*
Rifampicin	76 (81.7)	17 (29.8)	$p<0.0001$*
Oxazolidinones			
Linezolid	20 (25.9)	2 (3.5)	$p>0.05$
Glycopeptides			
Vancomycin	0 (0)	0 (0)	NA

Note: *Statistically significant ($p<0.05$); NA – not applicable.

Table 2

Biofilm formation capacity of MRS and MSS *Staphylococcus* spp.

Biofilm production	S.aureus			S.epidermidis		
	MRSA n (%)	MSSA n (%)	Total n (%)	MRSE n (%)	MSSE n (%)	Total n (%)
Strong	16 (26.2)	7 (11.5)	23 (37.7)	4 (25.0)	0 (0)	4 (25.0)
Moderate	17 (27.9)	8 (13.1)	25 (41.0)	6 (37.5)	1 (6.3)	7 (43.7)
Weak	7 (11.5)	6 (9.8)	13 (21.3)	3 (18.7)	2 (12.5)	5 (31.3)

Note: MRSA – methicillin resistant *S. aureus*; MSSA – methicillin sensitive *S. aureus*; MRSE – methicillin resistant *S. epidermidis*; MSSE – methicillin sensitive *S. epidermidis*.

Table 3

Antimicrobial resistance of biofilm- producing and non-producing *Candida* spp. strains

Antimicrobials n (%)		Biofilm-producing strains (N=87)	Biofilm-nonproducing strains (N=60)	p-value
		n (%)		
Azoles				
Fluconazole	87 (100)	41 (68.3)	$p<0.0001$*	
Intraconazole	87 (100)	42 (70.0)	$p<0.0001$*	
Polyenes				
Amphotericin B	15 (17.2)	1 (1.7)	NA	
Echinocandins				
Micafungin	4 (4.6)	0 (0)	$p>0.05$	
Pyrimidine analogue				
Flucytosine	0 (0)	0 (0)	NA	

Note: *Statistically significant ($p<0.05$); NA – not applicable.

Conclusions

The study results revealed a higher biofilm formation capacity in the clinical strains of *Staphylococcus* spp. and *Candida* spp. as well as higher rates of antimicrobial resistance in biofilm-producing strains compared to non-producing ones. The obtained data proves a strong correlation between biofilm formation capacity and antimicrobial resistance patterns. The implementation of the relevant antimicrobial susceptibility testing of biofilm-producing strains will improve the management of infections caused by these microorganisms, as well as provide feasible strategies to prevent their spread.

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Authors' contributions
GB designed the trial and interpreted the data. OB revised the manuscript critically.
Both authors revised and approved the final version of the manuscript.

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The trial was authors' initiative.
The authors are independent and take responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate
The strains used in this study were obtained from the routine analysis of clinical specimens. Sample collection did not involve direct contact with the patient, thus, no consent was required. The study was approved by the ethics committee of *Nicolae Testemitsanu* State University of Medicine and Pharmacy from the Republic of Moldova, proceedings No 65/12.04.2017 and No 67/12.05.2017.

Conflict of Interests
No competing interests were disclosed.