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## DETERMINATION OF BIOFILM FORMATION AND ASSOCIATED GENE DETECTION IN *STAPHYLOCOCCUS* GENUS ISOLATED FROM THE ORAL CAVITY UNDER INFLAMMATORY PERIODONTAL DISEASES

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**Background.** Inflammatory periodontal diseases, including chronic periodontitis, are accompanied by a chronic persisting inflammatory process. The latter process and biofilm-forming potential are the factors that contribute to the formation of antibiotic-resistant microorganism strains. The purpose of our work was to study the biofilm-forming ability of antibiotic-resistant biofilm-forming *Staphylococcus* genus bacteria isolated from the oral cavities of patients suffering from periodontitis, and check the presence of the genes associated with biofilm formation in these bacteria.

**Material and methods.** Isolates were obtained from patients suffering from chronic periodontitis using differentially diagnostic nutrient media. Chemotaxonomic identification of the antibiotic-resistant isolates was performed on the Microflex LT device. Biofilm-forming potential was studied in plastic Petri dishes by spectrophotometric method. The antibiotic sensitivity of the bacteria and microscopic fungi was identified by the disc diffusion method. The presence of *icaA*, *icaD*, *icaC*, *icaB*, *agrA*, *srtA* genes was

detected in two multiplex polymerase chain reactions (mPCR). *16S rRNA* gene and *icaR* genes were amplified in a separate PCR.

**Results.** Opportunistic microbial associations dominate in the microbiom of the oral cavity under conditions of periodontitis; besides, in the majority of cases, *Staphylococcus* genus bacteria were present in associations. In 73 % (131/180) of cases, bacteria from *Staphylococcus* genus represented by six species – *S. aureus*, *S. haemolyticus*, *S. saprophyticus*, *S. epidermidis*, *S. xelosus*, and *S. hominis* were isolated from the nidus during the inflammatory process. From the oral cavities of the patients with inflammatory periodontitis, 51 biofilm-forming strains of *S. aureus*, 29 strains of *S. haemolyticus*, 12 strains of *S. saprophyticus*, and 12 strains of *S. epidermidis* were isolated. It was established that antibiotic-resistant isolates of *S. aureus* possess the genetic determinants which are now believed to be connected with *icaA*, *icaC*, *icaB*, *icaA*, *icaR*, *agrA*, and *srtA* biofilm-forming potential. Two non-biofilm forming strains of *S. aureus* were proven to lack the genes responsible for the biofilm-forming ability.

**Conclusion:** It has been established that microbial associations with a prevailing share of *Staphylococcus* genus bacteria, most of which have a biofilm-forming potential, dominate in the microbiom of the oral cavity under conditions of periodontitis.

**Keywords:** antibiotic resistance, microbial associations, *Staphylococcus* genus bacteria, biofilm formation

## INTRODUCTION

The rapid development of microorganisms' resistance to antibiotics is a challenging issue in contemporary biology, medicine and ecology. The problem of antibiotic resistance appeared as soon as the first antibiotics were synthesized; however, during the recent several decades it has turned into a menacing socio-economic issue [2, 11, 23]. Microorganisms resistant to several antibacterial medical preparations arise a special concern. Multiresistant microorganisms (MRM) are mainly bacteria resistant to the action of one or several antibiotic classes. *Staphylococcus* genus bacteria occupy top positions in the formation of chronic persisting inflammatory diseases. With that said, especially dangerous are bacteria of the given genus that are resistant to methicillin (oxacillin) and demonstrate multiple resistance to antibiotics [5, 8]. *Staphylococcus* strains resistant to antibiotics are, as a rule, agents of hospital infections. The role of coagulase-negative staphylococcus species in the formation of opportunistic infections and their multiple resistance to antimicrobial preparations have also been established [25, 27]. Today, the assertion that microorganisms exist in the form of biofilms – organized communities of microorganisms consisting of both actively functioning and dormant cells confined in the extracellular matrix – is a proven fact [4, 17]. Studies have shown that 91 % of the isolates from the oral cavity have a biofilm-forming potential. The frequency of detection of biofilm-forming properties among antibiotic resistant isolates is high.

Chronic periodontitis is a multifactorial disease, pathogenesis of which includes a number of inflammatory and dystrophic-inflammatory processes causing disbalance between the aggression factors (periodontopathogens) and organism protection factors, viz. of the oral cavity and the periodontal complex [12]. At the initial stages of an inflammatory process in periodontal tissues, the infection factors play the key role. The listed representatives of the oral cavity microbiota belong to periodontopathogens: *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*,

*Prevotella intermedia*, *Campylobacter rectus*, *Eikenella corrodens*, and *Fusobacterium nucleatum*. At the initial stages of periodontitis, significant disorders of local immunity of periodontal tissues are observed accompanied by the action of such anaerobic Gram-negative bacteria as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythensis*. These bacteria take part in the formation of periodontal (gingival) pocket, destruction of the connective tissue, alveolar bone resorption (alveoloclasia), etc. The formation of inflammatory infiltrate is accompanied by various immunopathologies [4, 6, 20].

Apart from that, complications and relapses of periodontal inflammatory diseases, including periodontitis, may be caused by the transformation of the microbial coenosis and domination of opportunistic microorganisms in the microbial associations of the oral cavity. The colonization of the mucous membrane with transitory and allochthonous representatives of the microbiota, which are often characterized by multiple resistance to antibiotics, leads to continuous recurrence and a chronic course of the inflammatory process. A number of authors consider periodontitis as an opportunistic infection accompanied by the presence of opportunistic and pathogenic bacteria in the oral cavity [4].

At the same time, the continuously growing tendency to form antibiotic resistance, especially when opportunistic microbiota are part of the biofilm, explains the need to constantly monitor the circulation of polyantibiotic-resistant isolates [22], analyze the effectiveness of antibacterial preparations, and develop new complex approaches and correction techniques with regard to the oral cavity microbiota affected by an inflammatory process.

The purpose of our work was to study the biofilm-forming ability of antibiotic-resistant biofilm-forming *Staphylococcus* genus bacteria isolated from the oral cavities of patients suffering from periodontitis, and the presence of the genes associated with biofilm formation in these bacteria.

## MATERIALS AND METHODS

The level of microorganisms' resistance to antimicrobial preparations was examined under the persisting chronic inflammatory process (chronic generalized periodontitis). The study was conducted on the basis of the University of Dentistry. The biological material was taken from the oral cavity of human patients suffering from chronic generalized periodontitis at stages III and IV. The material for the study was taken from 180 patients of both genders aged from 35 to 65. The material was obtained from the nidus of the inflammatory process – the periodontal pockets – using a sterile transport system (a test-tube with gel and an applicator for biological liquids produced by FLmedical, Italy). The mucous membrane possessed the following features of an inflammatory process: hyperemia, angiostaxis, and release of serous and purulent exudate. Patients with no signs of periodontitis and inflammatory periodontal processes ( $n = 50$ ) formed the control group. The material was plated according to Gold onto the following nutrient media: Sabouraud Dextrose Agar, HiCrome™ Candida Differential Agar (Himedia) for cultivation of microscopic fungi; blood agar – for haemolytic microflora, in particular *Streptococcus* and *Neisseria* genera bacteria; Endo and Ploskoreva agar (Farmaktiv, Ukraine) – for *Enterobacteriaceae* genera bacteria; Mannitol Salt Agar (Biolid-Italia) – for *Staphylococcus* genus bacteria; *Enterococci* were identified with Bile esculin agar (Biolid-Italia). We identified the bacteria and yeasts based on macromorphology, micromorphology, physiological and biochemical tests using ENTERO-test, STREPTO-test, and STAPHYLO-test

manufactured by Erba Lachema. The chemotaxonomic identification of the antibiotic-resistant isolates was performed on the Microflex LT device (Bruker Daltonics GmbH, Leipzig, Germany), Flex Control (version 3.0). The spectrums obtained from each isolate were imported to the Biotyper software, version 3.0 (Bruker Daltonics GmbH, Leipzig, Germany, version 3.3.1.0), and analyzed with using the database of reference spectrums of medically important bacteria. Values within the range of 2.300 and 3.000, were considered a highly reliable identification of species; values between 2.000 and 2.999 were estimated as a reliable identification of a genus and a probable identification of species; values between 1.700 and 1.999 were considered as a probable identification of a genus; and values below 1.7 were considered an unreliable identification.

The antibiotic sensitivity of the bacteria and microscopic fungi was identified by the disc diffusion method according to Order No. 167 of the Ministry of Public Health of Ukraine of 05/04/2007 and EUCAST (European Committee on Antimicrobial Susceptibility Testing).

**Determination of the density of the obtained biofilms.** Into sterile disposable Petri dishes were introduced 5 cm<sup>3</sup> of meat-peptone broth and 1 cm<sup>3</sup> of daily test-culture of microorganisms in the concentration of 10<sup>5</sup> CFU/cm<sup>3</sup> and incubated at the temperature of 37 °C for 24–48 hours. Following the incubation, the dishes were three times cleaned off the plankton (floating) microorganisms with a phosphate buffer, dried off, and the resulting biofilms were fixed with 96° ethyl alcohol for 10 minutes. Then they were dyed for 10 minutes with a 0.1% solution of crystal violet. 3.0 cm<sup>3</sup> of 96-percent ethyl alcohol was poured into the Petri dishes and left for 20–30 min, and stirred regularly. The optical density of the alcoholic solution was measured spectrophotometrically at 570 nm wave length [22]. In case of optical density (OD) of the scrub solution from biofilms up to 0.50 OD units, the density of the formed biofilms was considered low; from 0.51 up to 1.00 OD units – intermediate; and above 1.01 OD units – high [14].

**DNA extraction.** Bacterial strains of *Staphylococcus aureus* were cultivated in 5 ml TSB (tryptic soy broth) for 24 h at 37 °C with continuous shaking at 130 rpm (New Brunswick Classic C2 Platform Shaker). The bacterial genomic DNA was extracted with a High Pure PCR Template Preparation Kit (Roche) according to the manufacturer protocol. The DNA concentration and purity were determined spectrophotometrically by measuring A260 and A280 using NanoDrop 8000 Spectrophotometer (Thermo Scientific). The genomic DNA samples were stored at -20 °C until use. The genes associated with biofilm formation were determined with specific primers as listed in Table 1. The primers used for the PCR were selected from the sequence data library or designed to facilitate the concomitant amplification of all putative PCR products in a single reaction. Therefore, all oligonucleotide had similar melting temperatures of approximately 61 °C and yielded PCR products of 75 to 211 bp (**Table 1**).

The presence of *icaA*, *icaD*, *icaC*, *icaB*, *agrA*, *srtA* genes was detected by two multiplex polymerase chain reactions (mPCR). The 16S *rRNA* gene and *icaR* gene were amplified by a separate PCR. mPCR amplification was performed using 20 µL of a reaction mixture containing DreamTaq Green Master Mix (2X) (Thermo Scientific), 0.25 µM of each primer and 10 ng of the template DNA.

The concentration of primers in the 16S *rRNA* and *icaR* determination in a separate PCR was 0.5 µM. The PCR reaction was carried out in a thermocycler (Mastercycler nexus, Eppendorf) using the following program: the initial step at 95 °C for 3 min and 35 cycles at 95 °C for 30 s, 61 °C for 30 s and 72 °C for 15 s; the final extension step at 72 °C for 10 min was added.

Table 1. Primers used for PCR

Таблиця 1. Праймери, використані для ПЛР

Genes	Nucleotide sequence of primers (5'–3')	Amplicon size (bp)
<i>icaA</i> (Intercellular adhesion)	F: CTTGCTGGCGCAGTCAATAC R: GTAGCCAACGTCGACAACCTG	75
<i>icaD</i> (Intercellular adhesion)	F: ACCCAACGCTAAAATCATCG R: GCGAAAATGCCCATAGTTTC	211
<i>icaC</i> (Intercellular adhesion)	F: CTTGGGTATTTGCACGCATT R: GCAATATCATGCCGACACCT	209
<i>icaB</i> (Intercellular adhesion)	F: ATACCGGCGACTGGGTTTAT R: ATCCAAATCGTGGGTATGTGT	141
<i>agrA</i> (Gene expression regulator)	F: TCGTAAGCATGACCCAGTTG R: AAATCCATCGCTGCAACTTT	96
<i>srtA</i> (Gene encoding sortase A)	F: GTGGTACTTATCCTAGTGGCAGC R: GCCTGCCACTTTCGATTATC	183
<i>16S_rRNA</i> (Internal standard gene)	F: CTACAATGGACAATACAAAGGGC R: TCACCGTAGCATGCTGATCT	141
<i>icaR</i> (Intercellular adhesion regulator)	F: TTTTCGACATCGAAGAAAGG R: TTCCAGAAAATTCCTCAGGC	72

The amplified fragments were visualized by electrophoresis in 2.5 % agarose gel stained with GoodView™ Nucleic Acid Stain (Ecoli). The results were captured with digital imaging system (Kodak Gel Logic 100, Kodak). The PCR probe containing DNA of *S. aureus* strain CNN 4223 and all PCR components was used as a positive control. The PCR probe that included sterile water and all PCR components was used as a negative control.

## RESULTS AND DISCUSSION

The results of our previous study [15] showed that during periodontitis, dysbacteriosis characterized by a growing number of opportunistic microorganisms was formed in the oral cavity. In particular, dysbiotic changes were observed in all 180 periodontitis patients investigated. At the same time, an increase in the population level of the microorganism association was detected in 81 % of cases; in 46 % of cases, these associations consisted of two species of microorganisms, and in 35 % they consisted of three species of microorganisms. In 73 % of cases, bacteria of *Staphylococcus* genus were isolated both in the mono-infection and in the structure of microbial associations.

The analysis of the taxonomic structure of microbial associations under periodontitis showed that in 73 % (131/180) of cases *Staphylococcus* genus bacteria were represented by the following six species: *S. aureus*, *S. haemolyticus*, *S. saprophyticus*, *S. epidermidis*, *S. xelonus* and *S. hominis*, which had been isolated from the nidus of the inflammatory process. *S. aureus* was isolated from 58 patients; 16 of those isolates were methicillin-resistant. In the case of domination of microbial associations of opportunistic microorganisms in the oral cavity, clinical features of the disease were better expressed and correlated with clinical manifestations of the inflammatory process and dystrophic changes of the mucous membrane.



It was established that under a chronic persisting inflammatory process, associations of opportunistic microorganisms dominated in the oral cavity of patients suffering from periodontitis. The analysis of the taxonomic structure of the isolates proved the domination of *Staphylococcus* genus bacteria. In the case of mono-infection, 19 isolates of *Staphylococcus* genus bacteria were collected; the others (112) were isolated within associations. Microorganisms isolated from patients with the most acute symptoms of the disease are presented in **Table 2**. It is noteworthy that *Staphylococcus* genus bacteria were present in all associations. The most frequently isolated bacteria were *S. aureus* + *E. faecalis*, *S. haemolyticus* + *E. cloacea*, *S. aureus* + *E. faecalis* + *C. albicans*, *S. epidermidis* + *E. cloacea*, etc.

**Table 2.** Formation of biofilms by *Staphylococcus* genus bacteria, isolated from the oral cavity of patients suffering from periodontitis, absolute number of isolates, n = 131

**Таблиця 2.** Формування біоплівки бактеріями роду *Staphylococcus*, ізольованих із ротової порожнини хворих з пародонтитом, кількість ізолятів, n = 131

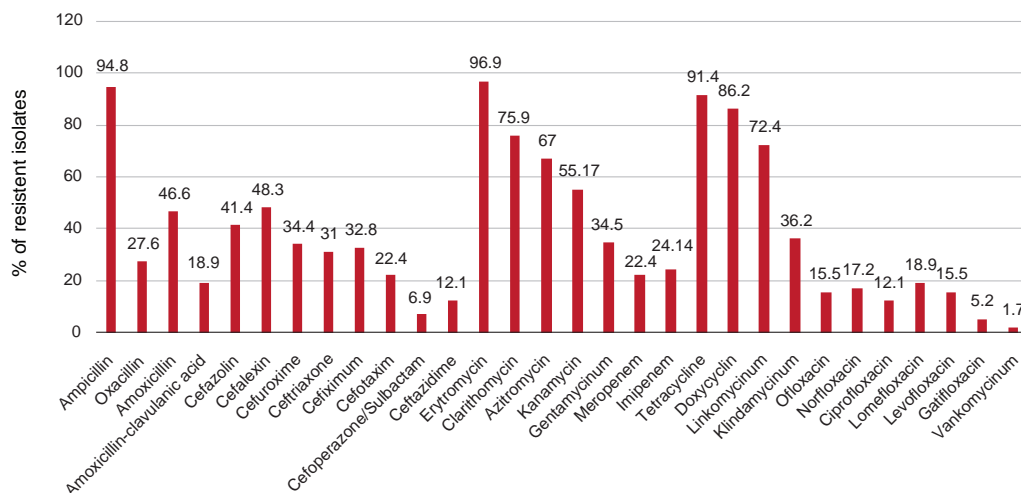
Microorganisms	Non-biofilm-forming	Number of microorganisms that formed biofilm with the indicated density:		
		low	moderate	high
<i>S. aureus</i>	7	0	3	48
<i>S. haemolyticus</i>	8	0	7	29
<i>S. saprophyticus</i>	7	-	-	-
<i>S. epidermidis</i>	8	0	2	10
<i>S. xelosus</i>	1	-	-	-
<i>S. hominis</i>	1	-	-	-

The identification showed that the isolated bacteria of *Staphylococcus* genus belonged to the following six species: *S. aureus*, *S. haemolyticus*, *S. saprophyticus*, *S. epidermidis*, *S. xelosus* and *S. hominis*. Representatives of *S. aureus* species were isolated from 58 patients, 16 of those isolates were methicillin-resistant; in associations with other opportunistic microorganisms were isolated 45 strains of *S. aureus*.

The analysis of antibiotic susceptibility of the representatives of *Staphylococcus* genus showed the following percentage of resistance (**Fig. 1, Fig. 2**).

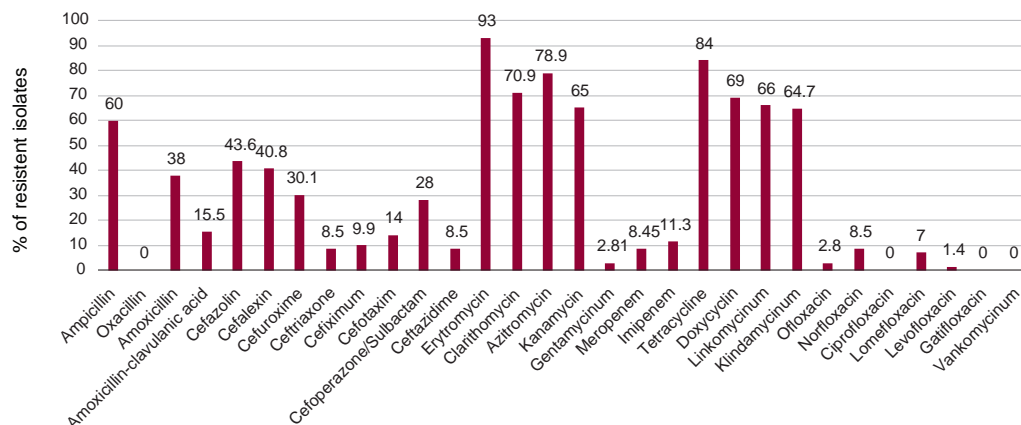
The analysis of susceptibility to  $\beta$ -lactam antibiotics revealed the highest level of resistance to ampicillin and amoxicillin. The highest percentage of susceptibility was observed to amoxicillin/clavulonate. *Staphylococcus* genus bacteria demonstrated high resistance to the group of macrolides, especially erythromycin, clarithromycin and azithromycin. The same tendency was observed for tetracyclines.

For staphylococci, the highest level of susceptibility was observed against phthorcinolons, especially levofloxacin, ofloxacin and gatifloxacin. The resistance of *Staphylococcus* genus bacteria to cephalosporins was as follows (**Figs 1–2**). A high level of the isolates resistance to such first and second generation cephalosporins as cefazolin, cephalexin and cefuroxime was observed. Species of *Staphylococcus* genus bacteria, were most susceptible to the third generation cephalosporins, such as ceftriaxone, cefixime and cefotaxime, and to the fourth generation cephalosporins: cefoperazone/sulbactam and ceftazidime – 12 %.



**Fig. 1.** Antibiotics resistance levels of coagulase-positive species of *Staphylococcus* genus bacteria isolated under conditions of periodontitis

**Рис. 1.** Рівень антибіотикорезистентності коагулазопозитивних видів бактерій роду *Staphylococcus*, ізолюваних в умовах пародонтиту



**Fig. 2.** Antibiotics resistance levels of coagulase-negative species of *Staphylococcus* genus bacteria isolated under conditions of periodontitis

**Рис. 2.** Рівень антибіотикорезистентності коагулазонегативних видів бактерій роду *Staphylococcus*, ізолюваних в умовах пародонтиту

The comparative analysis of susceptibility to antibiotics of coagulase-positive and coagulase-negative species of *Staphylococcus* genus bacteria showed that the percentage of resistant isolates was lower among the coagulase-negative isolates. In particular, no strains resistant to oxacillin or gatifloxacin were identified among the coagulase-negative strains. It was found that 31 % of the coagulase-positive strains were resistant to ceftriaxone, whereas this percentage among the coagulase-negative strains was only 8.5 %. Besides, 15.5 % of *S. aureus* isolates proved resistant to ofloxacin, while among the coagulase-negative strains the share of ofloxacin-resistant strains was only 2.8 %.

Resistance to carbapenems was low, but the share of resistant isolates was higher by 10 % among the coagulase-positive isolates.

Over 60 % of the isolates – both coagulase-positive and coagulase-negative – were resistant to linkozamins and tetracyclines.

The highest level of susceptibility of *Staphylococcus* genus bacteria was established to fluoroquinolones and cephalosporins: cefoperazone/sulbactam, ceftazidime, ceftriaxone; among betalactamines – to amoxicillin/clavulonate.

One of the factors that potentiates the formation of antibiotic resistance is biofilm formation by bacteria. Both coagulase-positive and coagulase-negative species of *Staphylococcus* genus bacteria are known to form biofilms.

We isolated 51 biofilm-forming strains of *S. aureus*, 36 strains of *S. haemolyticus*, 12 strains of *S. epidermidis* from the oral cavity of patients with inflammatory periodontitis. 99 (75.6 %) out of 131 isolated strains had a biofilm-forming potential (**Table 2**). In addition, most of them were part of microbial associations. A prevailing majority of the isolates formed high-density biofilm; e.g., out of 51 strains of *S. aureus*, 48 formed high-density biofilm ( $3.95 \pm 0.15$ ) (**Table 3**). Among the *S. epidermidis*, 10 isolates formed high-density and only two – moderate-density biofilm.

**Table 3. Optical density of the suspension of biofilm forming bacteria of *Staphylococcus* genus, isolated from patients suffering from periodontitis,  $M \pm m$ ,  $n = 131$**

**Таблиця 3. Оптична густина суспензії біоплівкотвірних бактерій роду *Staphylococcus*, ізольованих у пацієнтів з пародонтитом,  $M \pm m$ ,  $n = 131$**

Microorganisms	Density of biofilm forming, OD ( $\lambda = 570$ nm), $M \pm m$		
	low	moderate	high
<i>S. aureus</i>	$1.50 \pm 0.05$	$2.90 \pm 0.3$	$3.95 \pm 0.15$
<i>S. haemolyticus</i>	0	$2.75 \pm 0.25$	$3.90 \pm 0.10$
<i>S. epidermidis</i>	0	$2.85 \pm 0.10$	$3.85 \pm 0.20$
<i>S. xelusus</i>	-	-	-
<i>S. hominis</i>	-	-	-

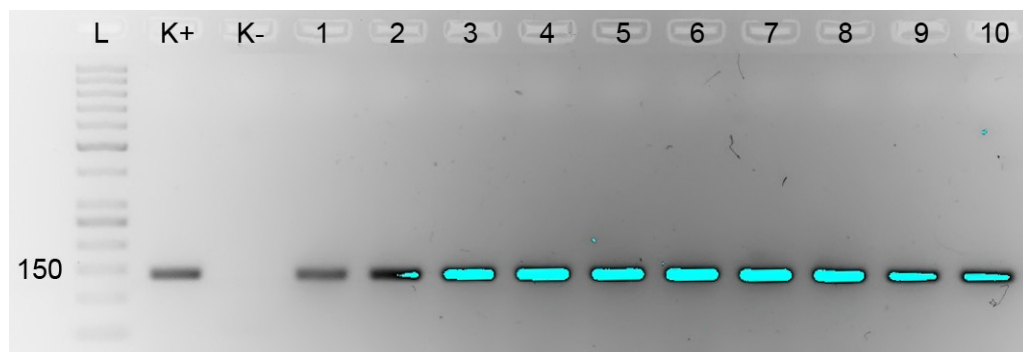
We selected 10 isolates with a high level of resistance to antimicrobial preparations (resistant to 10 and more antibiotics), performed their chemotaxonomic identification and analysis of the genes associated with biofilm formation: 1, 3–5, 7–10 – biofilm forming *S. aureus* 2, 6 – non-biofilmforming *S. aureus*.

It was established that all *S. aureus* isolates possessed genetic determinants that are connected with *icaA*, *icaB*, *icaC*, *icaD* and *icaR* biofilm-forming potential. Lack of genes responsible for biofilm formation was established for two strains of *S. aureus* that did not form biofilm in polystyrene (**Figs 3–6**).

Opportunistic allochthonous microbiota are characterized by a high resistance level to antibacterial preparations at the background of a chronic inflammatory process – chronic periodontitis. Biofilm formation by microorganisms referred to agents of opportunistic infections substantially aggravates the course of the inflammatory process and makes it difficult to treat due to the limited bioavailability of antimicrobial preparations and the formation of antibiotic resistance [15]. We investigated that under chronic generalized periodontitis, opportunistic microbial associations dominated in the microbiocenosis of the oral cavity; in addition to that, in a prevailing majority of cases *Staphylococcus* genus bacteria were present in associations. It was found that 75.6% of the isolates

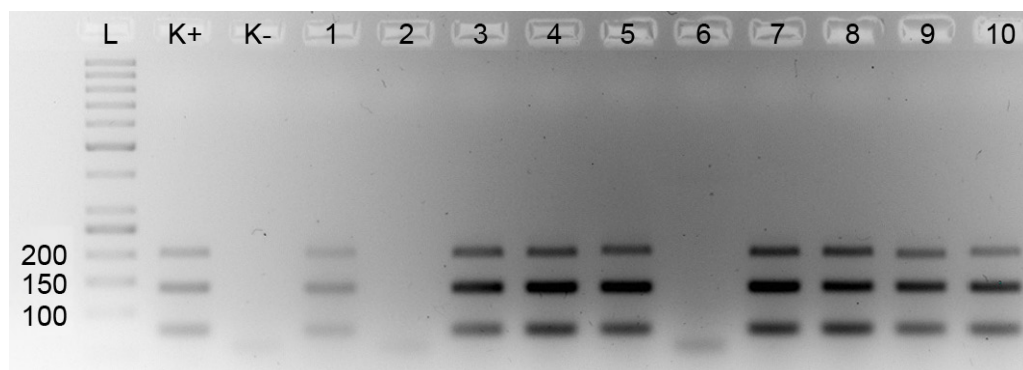


of staphylococci were biofilm-forming. At the same time, the biofilm-forming isolates were extra-antibiotic-resistant. We selected 10 isolates characterized by a high resistance level to antibiotics, and detected the genes associated with biofilm formation. It was demonstrated that eight of them had all identified genes in their genome.



**Fig. 3.** Visualization of the amplified DNA fragments of *16S rRNA* gene (141 bp) of *S. aureus* isolates (lanes 1–10) from the oral cavity of patients with inflammatory disease. “K+” – *S. aureus* CNN 4223 – positive isolate; “K-” – negative control; L- = 50 bp DNA ladder

**Рис. 3.** Візуалізація ампліфікованих ДНК фрагментів *16S rRNA* гена (141 bp) в ізолятів *S. aureus* (лінії 1–10) із ротової порожнини хворих з запальними захворюваннями. “K+” – *S. aureus* CNN 4223 – позитивний контроль; “K-” – негативний контроль; L- = 50 bp DNA ladder



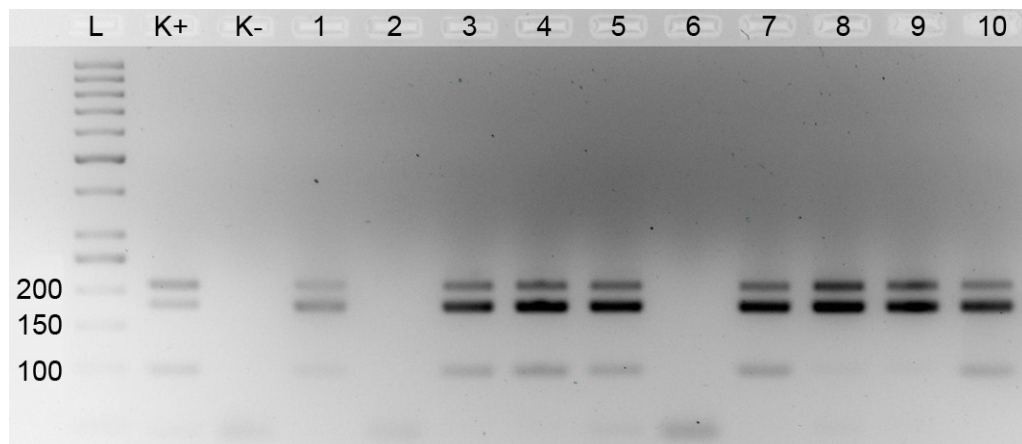
**Fig. 4.** Visualization of the amplified DNA fragments of *icaA* (75 bp), *icaB* (141 bp) and *icaC* (209 bp) genes of *S. aureus* isolates (lanes 1–10) from the oral cavity of patients with inflammatory disease. “K+” – *S. aureus* CNN 4223 – positive isolate; “K-” – negative control; L- = 50 bp DNA ladder

**Рис. 4.** Візуалізація ампліфікованих ДНК фрагментів *icaA* (75 bp), *icaB* (141 bp) та *icaC* (209 bp) генів у ізолятів *S. aureus* (лінії 1–10) із ротової порожнини хворих з запальними захворюваннями. “K+” – *S. aureus* CNN 4223 – позитивний контроль; “K-” – негативний контроль; L- = 50 bp DNA ladder

It was experimentally proven that biofilm formation is controlled by a number of genes, however, their role has not been fully clarified so far. Changes of gene expression during biofilm formation coordinated the formation of the structure, and the rise of antimicrobial tolerance [13]. In that case, the changes in gene expression were coordinated by specific signal molecules. The presence of such molecules activated, and then suppressed the genes that controlled virulence factors. It was established that

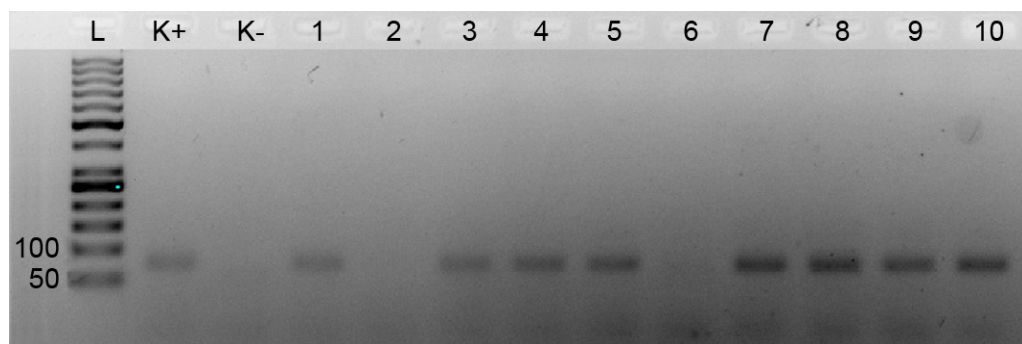
*Staphylococcus* genus bacteria are an etiological factor of chronic persisting processes for humans and animals [13, 16].

We showed for the first time that *S. aureus* isolated from the oral cavity affected by periodontitis had both the phenotypic signs of the forming biofilm and the associated biofilm-forming genes. As most microorganisms in the oral cavity are part of the biofilm, our results may indicate that *S. aureus* bacteria play a significant role in the formation of oral biofilm in conditions of oral cavity dysbacteriosis under periodontitis. The presence of opportunistic microorganisms in the biofilm structure may play a significant role in the formation of antibiotic-resistant populations of staphylococci.



**Fig. 5.** Visualization of the amplified DNA fragments of *icaD* (211 bp), *srtA* (183 bp), *agrA* (96 bp) genes of *S. aureus* isolates (lanes 1–10) from the oral cavity of patients with inflammatory disease. "K+" – *S. aureus* CNN 4223 – positive isolate; "K-" – negative control; L- = 50 bp DNA ladder

**Рис. 5.** Візуалізація ампліфікованих ДНК фрагментів генів *icaD* (211 bp), *srtA* (183 bp), *agrA* (96 bp) у ізолятів *S. aureus* (лінії 1–10) із ротової порожнини хворих з запальними захворюваннями. "K+" – *S. aureus* CNN 4223 – позитивний контроль; "K-" – негативний контроль; L- = 50 bp DNA ladder



**Fig. 6.** Visualization of the amplified DNA fragments of *icaR* gene *S. aureus* isolates (lanes 1–10) from the oral cavity of patients with inflammatory disease. "K+" – *S. aureus* CNN 4223 – positive isolate; "K-" – negative control; L- = 50 bp DNA ladder

**Рис. 6.** Візуалізація ампліфікованих ДНК фрагментів *icaR* гена у ізолятів *S. aureus* (лінії 1–10) із ротової порожнини хворих з запальними захворюваннями. "K+" – *S. aureus* CNN 4223 – позитивний контроль; "K-" – негативний контроль; L- = 50 bp DNA ladder

The formation of bacterial biofilm by *Staphylococcus* genus bacteria is a sophisticated process controlled by a variety of genes. Recent works have shown that *ica* operon is usually found in biofilm-forming bacteria [10], and it is directly related to the formation of bacterial biofilm as its important factor. The operon consists of four genes (*icaA*, *icaD*, *icaB* and *icaC*); the product of their expression is Polysaccharide Intercellular Adhesion protein (PIA) that plays a considerable part in *Staphylococcus* adhesion during biofilm formation [9, 13]. This work also showed that among the 116 isolated bacteria of *Staphylococcus* genus, 41 strains had *icaAD* genes, and 51 strains – *icaBC* genes.

During the biofilm-forming process, the development of the mature stage of biofilm mainly depends upon polysaccharide adhesion, which is a prerequisite for accumulation of bacteria. The *ica* ADBC operon takes part in biofilm formation by coding the enzymes participating in PIA synthesis. Besides, the following genes take part in the process: the regulatory gene (*sar*), the additional regulatory gene (*agr*) and *sigB* [3]. The agglutinating factor (Clfa) and fibronectin-binding proteins (Fnbp) mediate the bacterial integration.

PIA biosynthesis is performed by the products of the *ica* gene locus responsible for expression of N-acetylglucosamine transferase (*icaA* i *icaD*), and PIA diacetylase (*icaB*) [3, 10, 18]. The *ica* gene locus expression is regulated by a significant number of factors and regulatory proteins. It points to the correlation between the presence of *icaA* and *icaD* genes and their biofilm-forming potential. It was established that *icaD* plays a considerable role in the maximum expression of N-acetylglucosamine transferase, which results in the expression of capsule polysaccharide [9, 25]. The *sigB*, *sar* and *agr* genes also fulfill important functions in the processes of bacterial activity. Mutations in this sector lead to a substantial decrease in *ica* operon expression and PIA synthesis intensity [26].

The work [19] described an analysis of 176 isolates of coagulase-negative bacteria of *Staphylococcus* genus. It was established that *S. epidermidis* isolates with *icaAD* genes expression had a low degree of adhesion, while those with *icaACD* expression were characterized by a high degree of adhesion. Thus, the study showed that the isolates with three expressed genes were highly virulent due to producing Polysaccharide Intercellular Adhesion protein (PIA). It was found that the presence of one gene was not sufficient to form biofilm. Expression of *icaA* caused a weak fermentative activity and an insignificant amount of PIA. The presence of *icaD* gene product promoted polysaccharide biosynthesis. Expression of *icaAD* or *icaACD* intensified biofilm formation. At the same time, it is probable that *icaB* determined diacetylase when forming PIA. In that case, even if these genes are expressed, biofilm can be formed only as a subject to their expression. The experiment showed that of the 176 studied strains only 41 strains expressed *icaAD*, and of the 116 biofilm-positive strains only 51 strains expressed *icaBC* [13].

Another study established that the percentage of availability of *icaA*, *icaB*, *icaC* and *icaD* in *S. aureus* isolates equaled 51.6 %, 45.1 %, 77.4 % and 80.6 %, respectively. In 12 (38.7 %) isolates, all biofilm genes were positive [21]. It was also found that the *icaR* gene encodes a transcriptional repressor involved in the environmental regulation of *ica* operon expression and biofilm formation in *S. epidermidis* [7, 18].

## CONCLUSIONS

Under conditions of generalized periodontitis, the domination of opportunistic microorganisms in the patients' oral cavity has been established. It is worth noting that bacteria of *Staphylococcus* genus were present in all associations. The most frequently isolated strains were *S. aureus* + *E. faecalis*, *S. haemolyticus* + *E. cloacea*, *S. aureus* + *E. faecalis* + *C. albicans*, *S. epidermidis* + *E. cloacea*, etc. The analysis of the taxonomic structure of microbial associations under periodontitis showed that in 73 % (131/180) of cases were isolated *Staphylococcus* genus bacteria represented by the following six species: *S. aureus*, *S. haemolyticus*, *S. saprophyticus*, *S. epidermidis*, *S. xelosus*, and *S. hominis*. *S. aureus* was isolated from 58 patients, of which 29 isolates were methicillin-resistant. The highest level of susceptibility of *Staphylococcus* genus bacteria was observed for fluoroquinolones and cephalosporins – to cephoperason/sulbactam, ceftazidimum and ceftriaxone; among betalactams – to amoxicillin/clavulanat. The analysis of the taxonomic structure of the isolates proved the domination of *Staphylococcus* genus bacteria.

From the oral cavity of patients with inflammable periodontal process, 51 biofilm-forming strains of *S. aureus*, 36 biofilm-forming strains of *S. haemolyticus*, 12 biofilm-forming strains of *S. epidermidis* were isolated. Out of the 131 isolates, 99 (76,5 %) had a biofilm-forming potential. In addition to that, most of them did not belong to microbial associations. It has been established that antibiotic-resistant biofilm-forming isolates of *S. aureus* possess genetic determinants connected with *icaA*, *icaC*, *icaB*, *icaA*, *icaC*, and *icaB* genes that define biofilm-forming potential. Lack of genes responsible for biofilm formation was established for two non-biofilm forming strains of *S. aureus*.

## CONFLICT OF INTEREST

The authors declare that there is no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Human Rights:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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## ВИЗНАЧЕННЯ БІОПЛІВКОУТВОРЕННЯ ТА ВИЯВЛЕННЯ АСОЦІЙОВАНИХ ГЕНІВ У ІЗОЛЯТІВ РОДУ *STAPHYLOCOCCUS* ІЗ РОТОВОЇ ПОРОЖНИНИ В УМОВАХ ЗАПАЛЬНИХ ЗАХВОРЮВАНЬ ПАРОДОНТУ

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**Вступ.** Стрімко зростаюча тенденція до формування антибіотикорезистентних штамів мікроорганізмів обумовлює актуальність робіт, спрямованих на моніторинг їхньої чутливості до антибіотиків. Запальні захворювання пародонту, зокрема, пародонтит, супроводжуються хронічним персистуючим запальним процесом, що потребує застосування антибактеріальних препаратів. Більшість мікроорганізмів ротової порожнини містяться у складі біоплівки. Хронічний запальний процес і здатність до біоплівкоутворення є чинниками, що сприяють формуванню антибіотикорезистентних штамів мікроорганізмів.

Метою роботи було дослідити здатність до утворення біоплівки та наявність генів, асоційованих з утворенням біоплівки у антибіотикорезистентних біоплівкотвірних бактерій роду *Staphylococcus*, ізолюваних із ротової порожнини хворих на пародонтит.

**Матеріали і методи.** Дослідження проведені на базі Ужгородського національного університету (Ужгород, Україна) та Кошицького університету ветеринарії та фармації (Кошице, Словачія). Виділення ізолятів проводили у хворих на хронічний пародонтит із використанням диференційно діагностичних поживних середовищ; ідентифікацію проводили з використанням ENTERO-test, STREPTO-test, STAPHYLO-test. Хемотаксономічну ідентифікацію антибіотикорезистентних ізолятів виконували на пристрої Microflex LT (Bruker Daltonics GmbH, Lipsko, Nemecko) Flex Control (verzia 3.0). Здатність до біоплівкоутворення вивчали способом культивування у пластикових чашках Петрі, з подальшим фарбуванням біоплівки 0,1% розчином кристал віолету та спектрометричним визначенням оптичної густини спиртового розчину після відмивання етиловим спиртом.

Чутливість бактерій до антибіотиків і мікроскопічних грибів визначали диско-дифузійним методом відповідно до наказу Міністерства охорони здоров'я України від 16.04.2007 № 167 та EUCAST (Європейський комітет з тестування сприйнятливості до антимікробних препаратів). Наявність генів *icaA*, *icaD*, *icaC*, *icaB*, *agrA*, *srtA* виявляли за допомогою двох мультиплексних полімеразних ланцюгових реакцій (мПЛР). Ген *16S rRNA* і ген *icaR* ампліфіковано за допомогою окремої ПЛР.

**Результати.** З'ясовано, що в умовах пародонтиту у мікробіоценозі ротової порожнини домінують умовно патогенні мікробні асоціації, у цьому разі в переважній більшості у структурі асоціацій наявні бактерії роду *Staphylococcus*. У 73 % (131/180) випадків із осередку запального процесу виділяли бактерії роду *Staphylococcus*, представлені 6 видами: *S. aureus*, *S. haemolyticus*, *S. saprophyticus*, *S. epidermidis*, *S. xelonus*, *S. hominis*. Найвищий рівень чутливості бактерій роду *Staphylococcus* виявлений для фторхінолонів і цефалоспоринов – цефоперазон/сульбактаму, цефтазидиму, цефтріаксону; серед беталактамів – амоксацилін/клавулонату. Із ротової порожнини людей із запальним процесом пародонту ізолювано 51 біоплівкоутворюючий штам *S. aureus*, 29 – *S. haemolyticus*, 12 – *S. saprophyticus* і 12 – *S. epidermidis*. Із 131 ізолятів 104 (79 %) ізолюваних штамів були здатними до утворення біоплівки, більшість із них входили до складу мікробних асоціацій. Встановлено, що біоплівкотвірні ізоляти *S. aureus* мають генетичні детермінанти, з якими на сьогодні пов'язують здатність утворення біоплівки *icaA*, *icaC*, *icaB*, *icaA*, *icaR*, *agrA*, *srtA*. Встановлено, що у двох полірезистентних ізолятів *S. aureus*, що не утворювали біоплівку, немає генів, які асоційовані зі здатністю до її формування.

**Висновки.** Встановлено, що у мікробіоценозі ротової порожнини у разі хронічного пародонту домінують мікробні асоціації, у складі яких переважають бактерії роду *Staphylococcus*, більшість з яких має здатність до утворення біоплівки та стійкість до ряду антибіотиків.

**Ключові слова:** антибіотикорезистентність, мікробні асоціації, бактерії роду *Staphylococcus*, біоплівкоутворення