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COMPARISON OF CELL SIZES OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* WITH PRESENCE AND ABSENCE OF THE MECA GENE

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Staphylococcus aureus is a pathogenic microorganism that causes a wide range of infectious diseases of humans and animals. Staphylococcus aureus produces a large number of toxins, in particular enterotoxins, which enter the body together with food and cause disorders in the gastrointestinal tract. Moreover, S. aureus has several mechanisms of antibiotic resistance, which greatly complicates prevention of bacteria spread as community-acquired and nosocomial infections. The ***aim*** *of the work was to determine and compare the differences in size of methicillin-resistant strains of S. aureus with different resistance mechanisms by scanning electron microscopy (SEM).* ***Methods.*** *Disc diffusion method was used to establish the mechanism of antibiotic resistance of the obtained isolates. After description of antibiotic resistant and selection of S. aureus isolates with resistance to penicillin and oxicilin, an SEM of the strains and a further comparison of their morphological characteristics, in particular cell size, with the help of Djmaizer v.5.1.10 software was carried out.* ***Results.*** *54 isolates of S. aureus, obtained from various environmental objects, dairy farms and food products, were tested. PCR revealed sequences of the mecA gene, which is responsible for bacteria resistance to beta-lactams. We determined the cells size of S. aureus isolates resistant to penicillin and oxycillin and performed a comparative analysis of their morphological characteristics using SEM.* ***Conclusions.*** *In the course of the study, it was found that S. aureus isolates with mecA gene (mecA+) have smaller cell size than S. aureus isolates without mecA gene (mecA-).*

Keywords: MRSA, radius of bacterial cells, mecA+, mecA-, scanning electron microscopy.

Staphylococci are gram-positive bacteria, which are a part of opportunistic microflora of the skin and mucous membranes of humans and animals. In this case, among them is a particularly pathogenic species – *Staphylococcus aureus*, which causes various purulent inflammatory infectious diseases of the skin (abscesses, carbuncles, furuncles and impetigo), upper respiratory tract and genitourinary system infections, osteomyelitis, endocarditis, sepsis and toxic shock syndrome. About one third of the world's population suffers from diseases caused by this microorganism; another one third of the cases are asymptomatic. It is also very important that *S. aureus* is recognized as one of the most dangerous food pathogens. This microorganism can cause food intoxication, as it produces a number of dangerous to the human body endotoxins in dairy and meat products. The main sources of *S. aureus* in food products are raw material of animal origin, technological equipment and workers of processing enterprises [1–3].

However, one of the important problems associated with *S. aureus* is its antibiotic resistance. This is due to prolonged and uncontrolled use of antibiotics in medicine and livestock.

The researchers are particularly concerned about the resistance of staphylococci to β-lactams (including penicillin and cephalosporin). β-lactam antibiotics are currently most commonly used in medicine for antimicrobial chemotherapy because they have high clinical efficacy and low toxicity. Nosocomial and non-hospital staphylococcal infections are mostly caused by methicillin-resistant staphylococci. Methicillin is an antibiotic that belongs to the penicillin series.

At the international level, these staphylococci are designated by the acronym MRSA (MRSA – methicillin-resistant *S. aureus*). Currently, MRSA has spread due to the uncontrolled use of antibiotics in various fields of human activity. MRSA was originally identified as a healthcare-associated MRSA (HA-MRSA) causative agent, and since the 90s of the twentieth century it has been found to be responsible for the spread of community-associated MRSA (CA-MRSA) and livestock-associated MRSA (LA-MRSA) infections [4–7].

Currently, there are increasing reports of MRSA interspecies transmission between animals and humans, leading to a growing awareness that MRSA is now a problem both in medicine and in animal production (including veterinary and all stages of processing of animal products) [6, 8]. In the scientific literature, there are reports of MRSA strains that have been isolated from different raw materials of animal origin (raw milk, poultry, beef, pork), herbs and objects of livestock farms and processing plants [1, 9–11].

Staphylococci have two mechanisms of resistance to antimicrobial preparations of the β-lactam group. The first is related to the presence of blaZ gene, which encodes the β-lactamase enzymes. In turn, β-lactamases hydrolyze preparations of the β-lactam group. The second mechanism of resistance is associated with the presence of the genetic mobile cassette SCCmec, which includes the mecA gene. This gene encodes a PBP2a protein that has low affinity for β-lactam antibiotics and allows the cell wall to be built in their presence. The low affinity of PBP2a to β-lactams allows MRSA to grow as a result of the synthesis of peptidoglycan in the presence of high concentrations of β-lactams that can inactivate the transpeptidase activity of PBPs. PBP2a belonging to PBP contains a transpeptidase domain and a non-penicillin-binding protein [7, 12].

Thus, MRSA is a serious public health problem because of its prevalence and antibiotic resistance. It is especially dangerous because of the presence of methicillin-resistant *S. aureus* in animal raw materials and food products, which is another source of their entry into the human body. To prevent the spread of antibiotic resistant microorganisms, *S. aureus* requires constant and comprehensive study of their resistance characteristics, including changes in the morphological structure of bacterial cells [12–14].

One of the main methods for studying the morphological structure of microorganisms was and is light microscopy. Although this method is not powerful enough to examine many structures inside the cell, it is mainly used to identify bacteria in the first stage of bacteriological research, in order to detect the form of bacterial cells (cocci, rods, spiral shaped bacteria), and the thickness properties of microorganisms (by Gram staining and other staining methods) [1, 15, 16].

Another important approach for studying the morphology of microorganisms is the use of scanning electron microscopy (SEM) capabilities. SEM is widely used to study the structural details of bacterial cells surface and has several advantages. Among these, more important are: the ability to visualize microbial cells in comparison with the light microscope and the ability to measure and quantify the data in the form of photos, which can be collected and processed later, if necessary. In addition, the use of software for processing results allows to save images for further research and more objectively interpret the obtained data [17, 18]. In the past, the difficulties with sampling methods limited the use of SEM for routine microbiology. Today, many techniques for preparing microorganisms for research using SEM are available, in particularly a study of influence of different external factors on the morphology of bacteria [17–20].

In the studies of *S. aureus* resistance and sensitivity to vancomycin using electron microscopy, a direct relationship was established between the thickness of the cell wall of this microorganism and its antibiotic resistance [21].

Studies of *Klebsiella pneumoniae* found that bacteria resistant to cefotaxime had a larger size than those that did not have such resistance. The enlarged cells in cefotaxime resistant *K. pneumoniae* suggested that by increasing their size, the cells reduced the relative area of their cell envelope, the foremost target for the antibiotic [22].

Nevertheless, an analysis of literature showed that the determination of the size of bacteria was not used in the study of antibiotic-resistant MRSA and antibiotic-sensitive strains of this microorganism. Consequently, the detection and determination of additional characteristics of *S. aureus* antibiotic resistant strains may be useful for diagnostic purposes and, therefore, is a topical issue that needs to be investigated. The above analysis of literary sources showed that the question of comparing the morphological structure of *S. aureus* with some mechanisms of antibiotic resistance was not sufficiently investigated. In this regard, we conducted a study on the relationship between the size of MRSA and the manifestation of antibiotic resistance using classical microbiological

methods, SEM, polymerase chain reaction (PCR), and statistical analysis.

The **aim** of the work was to determine and compare by SEM the differences in size of methicillin-resistant strains of *S. aureus* with different mechanisms of resistance.

Materials and methods

Sampling for research. The total of 190 samples: 32 samples of raw milk, 38 swabs of udder skin, 38 samples of milk from cows with subclinical mastitis, 25 samples of different types of meat (minced meat chicken and turkey) and 57 environmental samples (10 swabs of milking machines, 13 swabs of milk tanks, 10 samples of animal feed, 24 swabs from floor of farm buildings) were collected for investigation in the period from 2014 to 2018 years.

Methods of isolating and identifying *S. aureus* from specimens. For detection of staphylococci all samples were plated on Baird-Parker Agar with Egg Yolk Tellurite Emulsion (Himedia, India) and cultivated at 35–37 ºC for 24–48 h in aerobic conditions. Typical staphylococcal colonies (black, convex, shiny colonies surrounded by clear zones) were used for microscopic examination, coagulase test with rabbit plasma and for production of hemolysin on Blood Agar to separate *S aureus* from other staphylococci. On microscopic examination all the *S. aureus* isolates were found to be gram-positive, non-spore forming, no motile cocci giving a clustered bunch of grape (Murray, Rosentthal, Pfaller Medical Microbiology, Elsever 2016).

Method of antibiotic sensitivity evaluating of *S. aureus* isolates. Antibiotic sensitivity of *S. aureus* isolates was tested by the standard disk diffusion method on Mueller-Hinton Agar (Oxoid, UK) according to the guide. Based on the inhibition zone size results were recorded as "Susceptible," "Intermediate," or "Resistant" according to the interpretive criteria specified in CLSI [23]. 4 antimicrobial agents (Oxoid, UK): methicillin (10 μg/disk), erythromycin (15 μg/disk), gentamicin (10 μg/disk), and tetracycline (30 μg/disk) were used.

The method of the mecA gene detecting. The mecA PCR typing was carried out with 2 primers according to technique described by Zhang and McClure (2005). MecA147-F (GTG AAG ATA TAC CAA GTG ATT) and MecA147-R (ATG CGC TAT AGA TTG AAA GGA T) were used for it. More detailed information on this method is given in the article [14].

Scanning electron microscopy technique. For this purpose, daily cultures of *S. aureus* were used. Cells of microorganisms were washed off from the surface of the nutrient medium with a small amount of distilled water.

2.5 % glutaraldehyde in phosphate buffer solution was used to fix *S. aureus*. The phosphate buffer – $NaH_2PO_4 + Na_2HPO_4$ ($H_2PO_4^- / HPO_4^{2-}$ is a weak acid / conjugated base) was used in the study.

S. aureus was washed in a tube from the surface of the culture medium, centrifuged; supernatant was poured; fixative agent was added a in the ratio of 1:10 to the contents of the test tube and suspended. The samples were kept sealed for 45 min and then centrifuged, supernatant was poured out. Re-fixation was carried out in the same way as the previous one.

The samples were washed from the fixator with a buffer solution, which was added in a ratio of 1:10. The contents of the test tube were suspended, kept for 5 min, centrifuged, and a supernatant was poured out. The washing was repeated twice.

A series of solutions with increasing concentration of ethyl alcohol (30, 50, 70, 96 and 100 %) were used for dehydration of *S. aureus*. Alternatively, to 1 ml of culture of cells, 9 ml of alcohol were added, suspended, centrifuged, poured out supernatant and added alcohol of the following concentration. The sample was kept for 10 min in 30, 50 and 70 % alcohol concentrations and 15 min in 96 and 100 % alcohol concentrations. Dehydration with 100 % alcohol was carried out twice.

The prepared bacterial suspension of *S. aureus* was applied to a metallic carbon plate and dried in air before coated with a layer of silver by installing VUP-5 ("SEMI", Ukraine). After the sample was prepared, images were taken at REM-106 ("SELMI", Ukraine), which works at an accelerating voltage of 20 kV.

Methods of data processing. Quantitative measurements of the radius of the individual *S. aureus* bacteria obtained from the cell image were processed using the Djmaizer v.5.1.10 program. The radius, area and length of the bacterial cells circle were determined for further processing. Statistical processing of the obtained quantitative measurements was carried out using the software Satistica v.12.

Results

The study of methicillin-resistant *S. aureus* cells size using SEM

As a result of the research, 64 isolates of *S. aureus* were obtained, 41 of which (64.1 %) were classified as MRSA using the disc diffusion method.

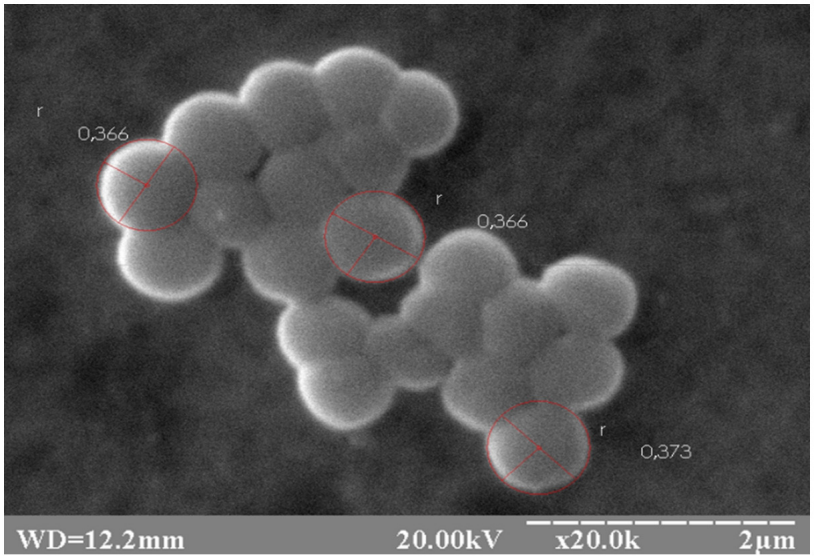
According to PCR results, 24 isolates (59.3 % of the total number of MRSA) were found to be positive for the mecA gene (mecA+). Methicillin-resistant isolates, in which the mecA gene was not detected by PCR, were classified for further studies as mecA-. After preparation the samples of MRSA isolates for electron microscopy and its execution, measurements of the *S. aureus* cells size were performed.

Quantitative measurements of the radii of *S. aureus* bacteria with different resistance mechanisms were performed in the course of the study. In each group (mecA+, mecA-), the sample contained 125 individual cells randomly selected from different isolates (Fig. 1). The measurement results are presented in Table 1.

Analysis of the data showed that the quantitative indicators of both groups differ significantly. As we can see from Table 1, the average value of the *S. aureus* radiuses with the existing mecA gene is smaller compared to *S. aureus* without mecA gene. The statistical analysis of quantitative data showed that the measurement error is for mecA+ ± 0.0677, for mecA- ± 0.0578.

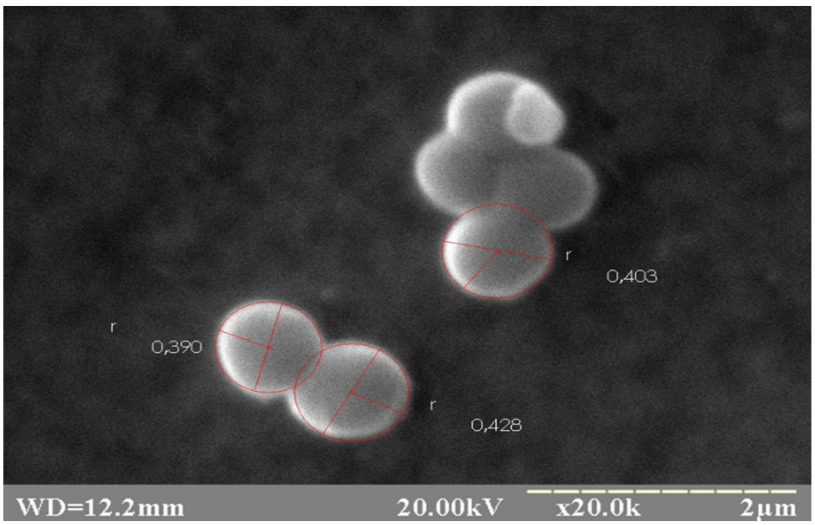
Figures 2 and 3 allow to estimate the distribution of quantitative data obtained from the studied groups of microorganisms and their relation to the expected normal. According to these calculations, both samples correspond to normal distribution, and deviations from normal are not significant. Rule 3 sigma – practically all values of normally distributed random variables lie in the interval [$\bar{x}$ - 3σ; $\bar{x}$ + 3σ]. More

A



F i g. 1, a. Dimensions of MRSA with existing mecA gene (scanning electron microscopy)

B



F i g. 1, b. Dimensions of MRSA without mecA gene (scanning electron microscopy)

Table 1

Quantitative measurements of *S. aureus* cells radius

Statistical indicators	*S. aureus* with available mecA gene, μm	*S. aureus* with missing mecA gene, μm
Number of measured cells	125	125
Maximum cell radius	0.591	0.613
Minimum cell radius	0.291	0.306
Average cell radius	0.397	0.431
Measurement error	0.068	0.058

precisely – not less than with 99.7 % confidence, the value of a normally distributed random variable lies in the specified interval (provided that the value $\bar{x}$ is definitely known, but not obtained as a result of processing the sample). Within 1 σ should be 68.3 % option, within 2σ – 95.5 %. For mecA+, the distribution is 69.6 %, 95. 2 % and 100 %. For mecA- – 94.4 %, 94.4 % and 99.2 % respectively.

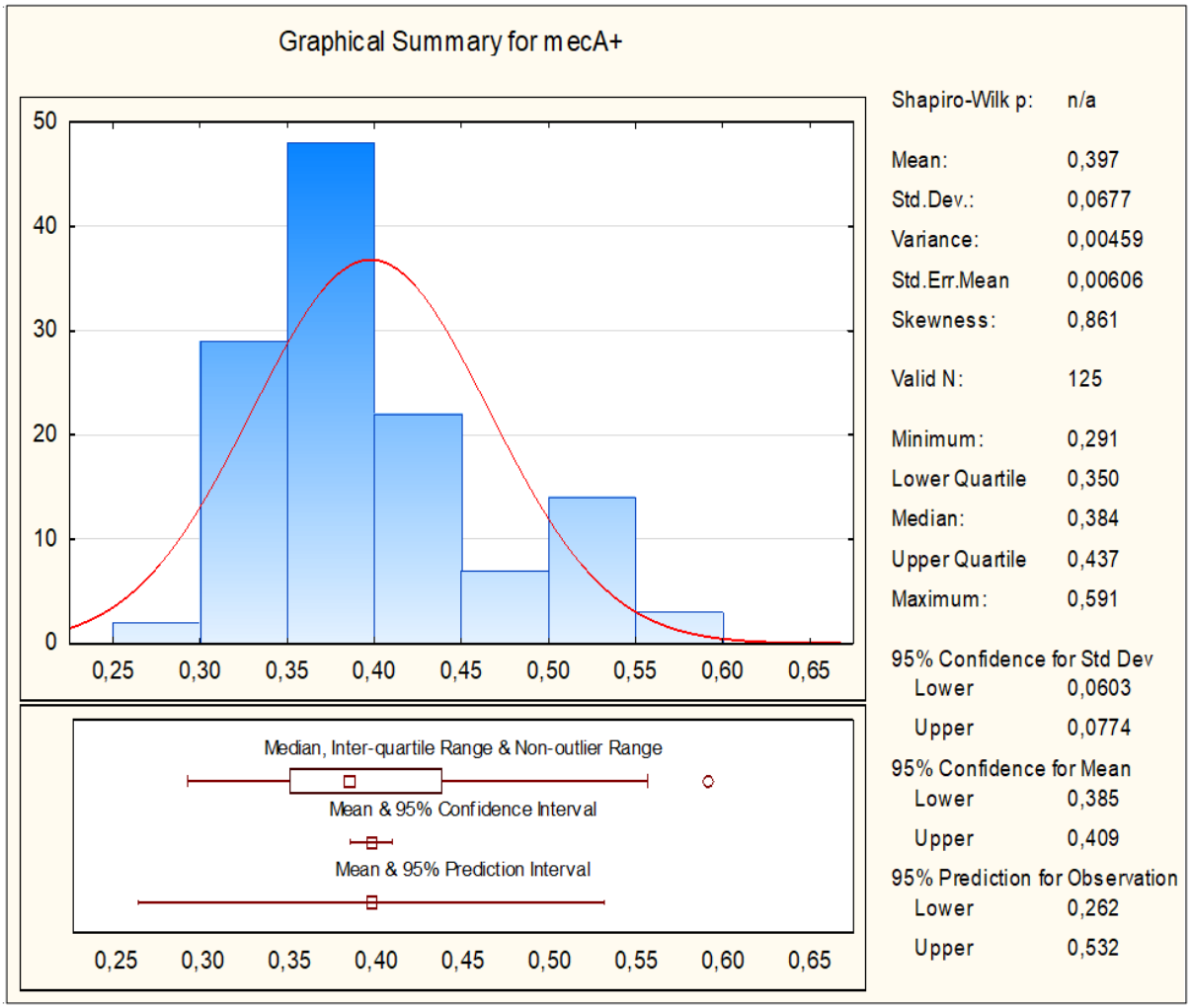
The point diagram (Fig. 4) illustrates the degree of correlation between the two indicators. In this case, the system of points with a slope from the bottom left corner to the upper right corner indicating a positive correlation. The more consistency of these two sets, the greater the concentration is concentrated in the immediate vicinity of the identity line; if two sets of data are numerically identical, the spread is identical to the line of identity.

To compare the average values of the radius of cells, the parametric method, namely, the Student's criterion (t-criterion) was used.

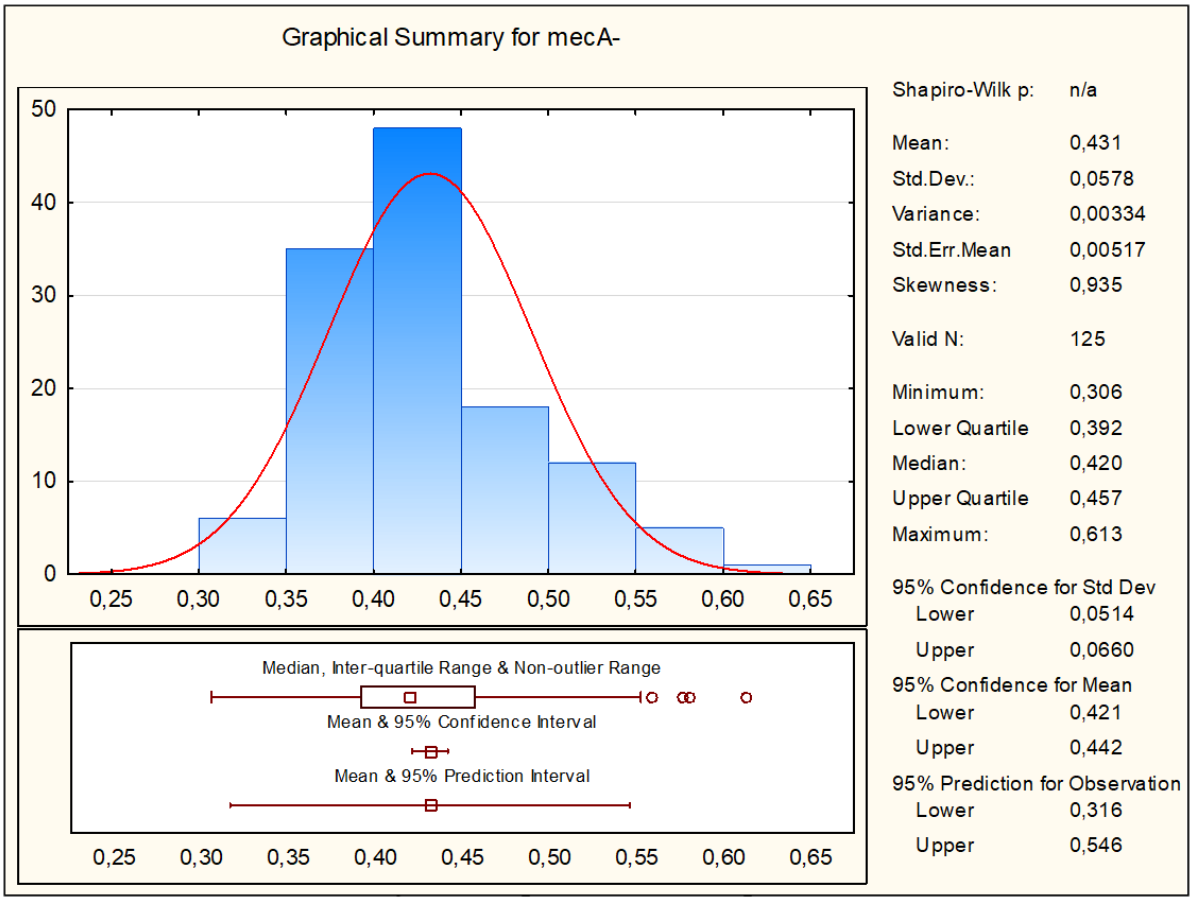
To apply this method, two parameters need to be implemented:

a) the values of the signs in the comparable groups should have a normal distribution;

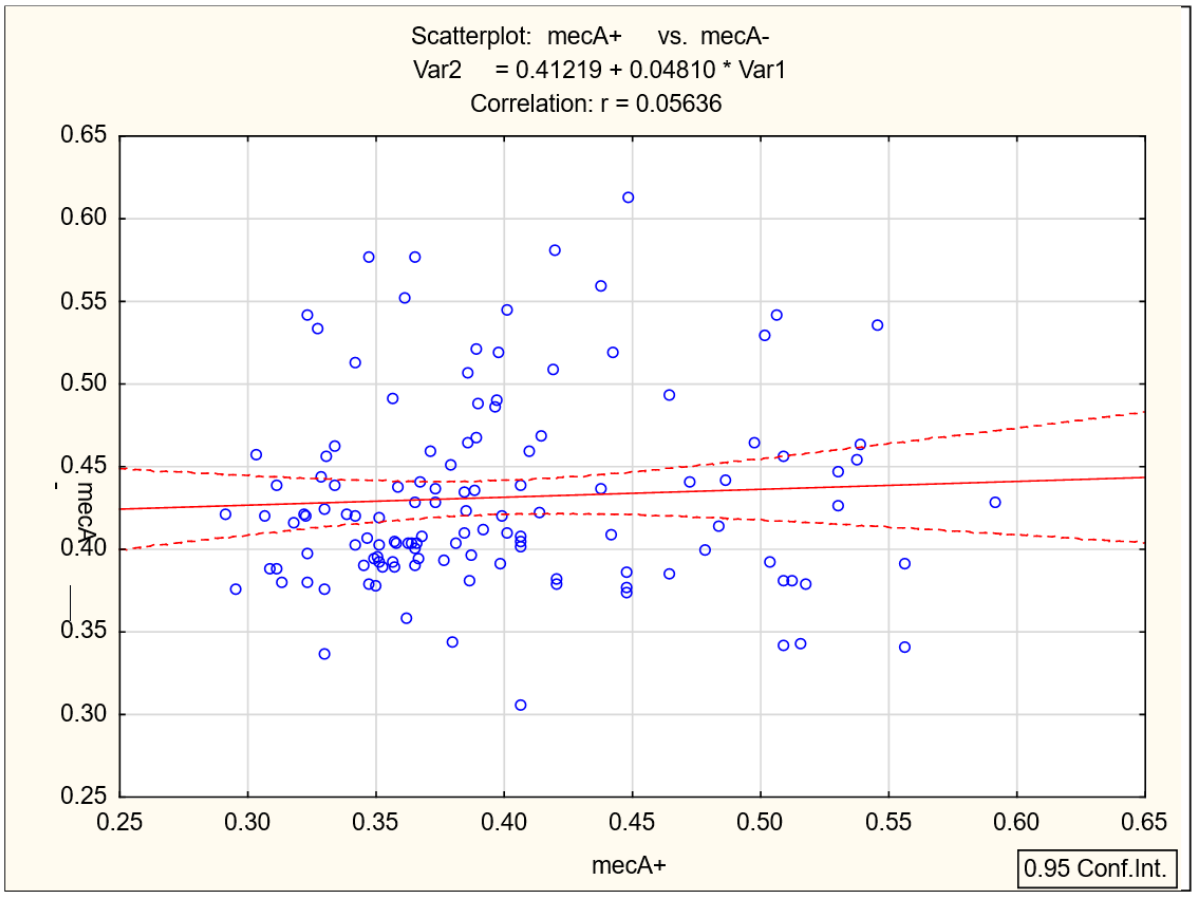
b) the dispersion of the distribution of features in the two compared groups should be equal.



F i g. 2. Results of statistical processing of quantitative values of *S. aureus* (mecA+)



F i g. 3. Results of statistical processing of quantitative values of *S. aureus* (mecA-)



F i g. 4. Diagram of scattering of quantitative values of *S. aureus* mecA- and mecA+ groups

In the course of the study it was found that the values of the radii of cells of the two comparable groups have normal distribution (Fig. 2, 3). Levin's test showed that the reliability was $p > 0.05$ (Table 2). This testifies to the equality of the dispersion of the distribution of the sign in the comparable groups. Thus, to establish the reliability of the difference between the mean values of two independent samples, the parametric method – the t-criterion was used.

Table 3 shows the calculation of Student's criterion, for which $p = 0.001$ (according to statistical rules instead of the value of $p = 0.000024$, $p = 0.001$ is used). This indicates the high reliability of the results of the study.

Further statistical processing of the data obtained in the course of the study showed that at least 5 radii of random sample should be carried out for the reliable determination of mecA+ *S. aureus*.

Table 2

The value of Lewin's test

Group 1 vs. Group 2	T-test for Independent Samples		
	Levene F (1, df)	df Levene	p Levene
mecA+ vs. mecA-	3.450953	248	0.064401

Legend: Variables were treated as independent samples.

Table 3

Student's criterion calculation (t-criterion)

Group 1 vs. Group 2	T-test for Independent Samples				
	Mean Group 1	Mean Group 2	t-value	df	p
mecA+ vs. mecA-	0.397028	0.431287	-4.30114	248	0.000024

Legend: Variables were treated as independent samples.

Discussion

Studying the morphological structure of methicillin-resistant *S. aureus* using SEM

The intensive use of antibiotics in medicine and veterinary practice has led to the spread of antibiotic resistance of bacterial strains and the emergence of a global problem worldwide [25–26].

Disco-diffusion method and a method of serial dilutions are known conventional laboratory methods for determining antibiotic resistance of microorganisms. In addition to them modern method of polymerase chain reaction (PCR) is used, whose purpose is to determine the presence of genes of antibiotic resistance in microorganisms [16, 23, 24].

There are studies that show changes in the morphological structure of microorganisms under the influence of antibiotic substances. Cells that were treated with antibiotic were increased in size compared to untreated cells. As mentioned above, the enlarged mutant cells of *K. pneumoniae* were resistant to cefotaxime [22]. One of the most common phenotypic changes observed in vancomycin-intermediate *S. aureus* (VISA) is the thickened cell wall with reduced peptidoglycan cross-linking. In addition to thickened cell wall, VISA strains exhibit other phenotypic changes including reduced autolytic activity, reduced hemolytic activity and slow growth *in vitro* [27]. In our study, only the morphology of untreated cells is compared.

In order to evaluate the morphology of *S. aureus*, SEM was performed and summarized. The results showed that MRSA cells have altered their morphology in comparison with MSSA (methicillin-sensitive *S. aureus*). The morphology of MSSA (0.343±0.023) cells was apparently normal, but enlarged and malformed in the MRSA (0.453±0.0316) cells [28]. In the course of this study it was found that *S. aureus* isolates with different mechanisms of antibiotic resistance (with an existing (mecA+) or absent (mecA-) gene) differ in cell size. Thus, isolates of *S. aureus* with mecA+ have a smaller cell size (average value of the radius of the bacterial cell was 0.397 ± 0.068 μm) than *S. aureus* mecA- isolates (mean value of the radius of the bacterial cell was 0.431 ± 0.058 μm).

Conclusions. The authors concluded that there is a convincing relationship between the size of bacteria and their resistance to antibiotic drugs. The correlation between antibiotic resistance and the size of bacterial cells may be considered in other research. Further study of the correlation between the genetic structure and the specificity

of the antibiotic-resistant strains can expand the explanation of the reason for this relationship.

This study can be considered as a promising direction for studying the mechanisms of antibiotic resistance of microorganisms. The ability to use this method in medical, veterinary laboratories with the need to quickly identify antibiotic-resistant *S. aureus* for effective treatment of patients, organizations involved in monitoring the safety of food raw materials and food quality require further consideration.

ПОРІВНЯННЯ РОЗМІРІВ КЛІТИН МЕТИЦИЛІН-РЕЗИСТЕНТНИХ ІЗОЛЯТІВ *STAPHYLOCOCCUS AUREUS*, В ЯКИХ ПРИСУТНІЙ ТА ВІДСУТНІЙ ГЕН MECA

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Резюме

Staphylococcus aureus – патогенний мікроорганізм, який викликає широкий спектр інфекційних захворювань у людей та тварин. *Staphylococcus aureus* продукує велику кількість токсинів, зокрема ентеротоксинів, які потрапляють в організм разом з їжею та викликають розлади в шлунково-кишковому тракті. Більше того, *Staphylococcus aureus* має кілька механізмів стійкості до антибіотиків, що значно ускладнює запобігання поширенню як позалікарняних, так і внутрішньолікарняних інфекцій. **Метою** роботи було визначити та порівняти відмінності у розмірах метицилін-резистентних штамів *S. aureus* з різними механізмами стійкості до антибіотика за допомогою скануючої електронної мікроскопії (СЕМ). **Методи.** Для встановлення механізму антибіотикорезистентності отриманих ізолятів, визначення чутливості до антибіотичних препаратів застосовували дискдифузійний метод. Після опису штамів, стійких до антибіотиків, та виділення ізолятів *S. aureus*, резистентних до пеніциліну та оксициліну, була проведена СЕМ отриманих культур та подальше порівняння їх морфологічних характеристик, зокрема розміру клітин, за допомогою програмного забезпечення Djmaizer v.5.1.10. **Результати.** Нами було проаналізовано 54 ізоляти *S. aureus*, виділені з різних об'єктів навколишнього середовища, молочних ферм та харчових продуктів. В результаті постановки ПЛР виявлено послідовність гена mecA, який відповідає за стійкість бактерій до бета-лактамів. В результаті скануючої електронної мікроскопії виділених ізолятів *S. aureus*, резистентних до пеніциліну та оксициліну, встановлені розміри їх клітин та проведено порівняльний аналіз цих морфологічних характеристик. **Висновки.** В результаті проведених досліджень було встановлено, що ізоляти *S. aureus,* в яких присутній ген mecA (mecA+), мають менший розмір клітин, ніж ізоляти *Staphylococcus aureus*, в яких відсутній ген mecA (mecA-).

Ключові слова: MRSA, радіус клітин бактерій, mecA+, mecA-, скануюча електронна мікроскопія.

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