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Overcoming Antibiotic Resistance to Tetracycline in *Staphylococcus aureus* by Gold and Silver Nanoparticles

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Abstract—The use of tetracyclines in medicine, veterinary medicine, and stock raising has led to the spread of bacterial resistance to tetracycline, particularly among dangerous representatives of *Staphylococcus aureus*. Therefore, the analysis of resistance to tetracycline and the creation of approaches to overcome it are extremely relevant. Studies of 64 clinical isolates of *S. aureus*, which were characterized by moderate biofilm formation, showed that 33 of them contained plasmid DNA. A significant spread (in 96% of studied isolates) of the known transmissible tetracycline resistance genes *tet*(*K*) and *tet*(*M*) was shown in the examined plasmid-containing doxycycline-resistant clinical isolates of *S. aureus*. Plasmid-containing doxycycline-resistant clinical isolates with *tet*(*K*) and *tet*(*M*) genes lost resistance to the tetracycline antibiotic doxycycline after treatment of their cells with gold nanoparticles of 30 nm in a concentration of 3.2–9.6 μg/mL or medium-sized silver of 30 nm in a concentration of 20–40 μg/mL. Elimination of *tet* genes responsible for acquired resistance was confirmed by PCR.

Keywords: antibiotic resistance, *tet*(*K*) and *tet*(*M*) genes, *Staphylococcus aureus*, gold or silver nanoparticles, tetracycline, biofilm, R-plasmids

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Staphylococci are pathogens that are dangerous to humans and belong to microorganisms associated with common infections with high lethality, survivability, and antibiotic resistance. The critical situation with the spread of antibiotic resistance requires new approaches to both the development of antibiotics and the monitoring of bacterial resistance. To be one step ahead of bacteria in "the race antibiotics" will help a comprehensive study of bacterial resistance (Crofts et al., 2017). Antibiotic resistance is most often associated with extrachromosomal elements, such as plasmids, transposons, and integrons. Antibiotic resistance mediated by plasmid genes has long been the main focus of the study of epidemiologically important mechanisms of antibiotic resistance (Alekshun et al., 2007).

Currently, it is recognized that antibiotic resistance of bacteria is rapidly forming in medical practice, in veterinary medicine, and in agricultural production (livestock). Therefore, overcoming this dangerous phenomenon is relevant for all the listed industries (Zaheer et al., 2019). Resistance to tetracycline is a problem in the production system of beef, pork, and chicken in Ukraine and in Europe in general (Græsbøll et al., 2019). Tetracycline is one of the antibiotics that is widely used against various bacterial infections of humans and animals. Currently, a significant spread of tetracycline resistance genes has been shown in Ukraine *tet*(*M*) in bacteria, which may be a consequence of the widespread use of the antibiotic in agricultural production (Shevchenko et al., 2019). It is known that tetracycline resistance genes *tet*(*M*) and *tet*(*K*) most often localized on plasmids. Thus, up to 95% of cases of detection of such genes in *S. aureus* bacterial cells demonstrate precisely plasmid associa-

tion. PCR is used to detect antibiotic resistance genes to tetracyclines (Khoshbakht et al., 2018).

The most important feature of chronic staphylococcal infection is the ability of bacteria to grow in the form of biofilms: a community of bacterial cells immobilized on the surface that are embedded in an extracellular polymer matrix. The formed staphylococcal biofilms give such pathogens unique properties in the sense of resistance to generally accepted therapeutic doses of antimicrobial agents. Biofilm significantly increases the possibility of transfer of antibiotic resistance genes in microbial populations. Biofilm formation and resistance to antibiotics are interrelated; therefore, detection of the degree of biofilm formation in clinical isolates is a modern solution in the therapy of staphylococcal infections (Manandhar et al., 2018). Assessing the potential of biofilm formation by strains can help counteract the mechanisms involved in biofilm formation, determine rational control strategies for *S. aureus*, and counteract the transport of antibiotic resistance genes and transfer of antibiotic resistance genes (Rodríguez-Lázaro et al., 2018).

At the same time, with the relevance of research into the epidemiologically important mechanisms of antibiotic resistance to the most common antimicrobial drugs, the question of overcoming such antibiotic resistance arises. A promising direction in the development of ways to overcome antibiotic resistance of pathogenic bacteria is the elimination of R-plasmids from bacterial cells with the help of chemicals. Indeed, ethidium bromide leads to loss by bacteria *Staphylococcus* plasmid-associated tetracycline resistance gene. R-plasmids of bacteria *Escherichia, Enterobacter, Proteus, Staphylococcus*, and *Yersinia* are eliminated when the cell is exposed to some heterocyclic substances that can bind to DNA (Spengler et al., 2006). At the same time, such substances are toxic to humans and animals, so they cannot be used to overcome antibiotic resistance.

A study of interaction of gold and silver nanoparticles with plasmid DNA of bacterial strains *E. coli* showed that these nanoparticles effectively eliminate R-plasmids (Dybkova et al., 2014).

The purpose of the work was detection of epidemiologically important transmissible tetracycline resistance genes in *S. aureus* clinical isolates isolated from samples of complicated wounds patients that underwent diagnostics in the Laboratory of Microbiology and Chemotherapy of the Institute of Traumatology and Orthopedics (National Academy of Medical Sciences of Ukraine) studying their potential for biofilm formation and the possibility of overcoming antibiotic resistance in such isolates using biosafe gold and silver nanoparticles 30 nm in size.

MATERIALS AND METHODS

We used 64 *S. aureus* clinical isolates isolated in the Laboratory of Microbiology and Chemotherapy (Institute of Traumatology and Orthopedics, National Academy of Medical Sciences of Ukraine) from patients (with informed consent) with purulent-inflammatory and periprosthetic joint infections and control strain *Staphylococcus aureus* ATCC 25923.

For the screening of plasmid DNA in bacterial cells, plasmid DNA was isolated by the alkaline lysis method (Bimboim et al., 1979).

The resistance of the studied strains was determined by the disk-diffusion method (Balouiri et al., 2016) to antibiotics: doxycycline, tigacil, amoxiclav, rifampicin, erythromycin, ciprofloxacin, cefoxitin, clindamycin, and amikacin.

Detection of tet genes using PCR. Studies of transmissible genes of resistance to tetracycline *tet*(*K*) and *tet*(*M*) in *S. aureus* clinical isolates were carried out using polymerase chain reaction of total DNA of relevant clinical isolates. Specific oligonucleotide primers were used in the study (Stromminger et al., 2003):

tet (K)-F 5' GTAGCGACAATAGGTAATATAGT

tet (K)-R 5' GTAGTGACAATAAACCTCCTA

tet (M)-F 5' AGTGGAGCGATTACAGAA

tet (M)-R 5' CATATGTCCTGGCGTGTCTA

(specific primers for gene detection of *tet*(*K*) and *tet*(*M*)).

ST-F 5' CAGCTCGTGTCGTGAGATGT

ST-R 5' AATCATTTGTCCCACCTTCG

(primers specific for *S. aureus,* positive control). PCR was performed in a reaction mixture with a volume of 25 μL, which included 0.5 μg of DNA and 10 pmol of the corresponding primers. The amplification reaction was carried out for 1 cycle of 93.5°C 8 min and 40 cycles of 93.5°C 35 s; 55°C 35 s; 72°C 40 s. PCR products were separated in a 1.8% agarose gel followed by visualization of PCR products with ethidium bromide solution. The size of the fragment that corresponds to the gene *tet*(*K*) is 360 bp, *tet*(*M*) is 158 bp, and positive control on *S. aureus* is 420 bp.

Analysis of biofilm formation. The ability of bacteria *S. aureus* clinical isolates for biofilm formation was studied according to (Piechota et al., 2018). Each strain was grown for 18 h on meat peptone agar (MPA). Then, in sterile test tubes, a 10^8 CFU/mL-suspension of bacteria was diluted in meat-peptone broth (MPB) (total volume 1 mL) and cultivated for 48 h at 37°C. After cultivation, the culture liquid was drained, and the biofilms formed on the walls of the test tubes were washed with a sterile phosphate buffer (pH 7.4). After washing, biofilms were fixed with methanol for 15 min, treated with a 1% solution of crystal violet for 5 min, washed with distilled water, and dried in air. Next, 0.5 mL of 96% ethanol was added to the test tubes, the biofilm was removed from

the walls, and the optical density was analyzed using a KFC-2 photocolorimeter at a wavelength of 492 nm. The index of ≥0.12 indicated biofilm formation; <0.2, 0.2–0.4, and >0.4, indicated weak, moderate, and strong biofilm formation, respectively (Piechota et al., 2018).

Elimination of plasmid DNA using nanoparticles. To eliminate plasmid DNA, a colloidal gold solution with spherical gold nanoparticles with an average size of 30 nm (38.6 μg/mL by metal) and a colloidal silver solution with spherical particles with an average size of 30 nm (80 μg/mL by metal) were used. Night culture bacteria of *S. aureus* clinical isolates was diluted with MPB in a ratio of 1 : 50, incubated on a rocker for 2 h to a cell titer of $1–2 \times 10^9$. We placed $1 \times 10^4–1 \times 10^5$ cells in test tubes with 2 mL of MPB, where the initial drug of gold or silver nanoparticles was previously added in an amount that provided a gold content of 3.2 or 9.6 μg/mL and silver 20 or 40 μg/mL by metal. Cultures were grown at 37°C for 20–24 h. Later, bacterial cultures were sown in 0.1 mL on plates with MPA and sensitivity to antibiotics was determined by the disk diffusion method. In parallel, in all of the cells treated with metal nanoparticles of all studied clinical isolates, the presence of plasmids was visualized by electrophoresis in a 1% agarose gel and total DNA was isolated to detect tetracycline resistance genes.

RESULTS AND DISCUSSION

S. aureus is usually a representative of the normal microflora of healthy people but can cause life-threatening conditions, mainly in debilitated persons or patients who have undergone surgical interventions. They are one of the main causes of nosocomial infections and are known for their tendency to develop resistance to many antimicrobial agents. Of particular concern is the constant increase in the number of multiresistant *S. aureus* strains. In staphylococci, antibiotic resistance genes are mainly associated with mobile genetic elements, such as plasmids and transposons (Kwong et al., 2017).

Study of 64 clinical isolates *S. aureus,* selected from patients with purulent-inflammatory and periprosthetic joint infections in the Laboratory of Microbiology and Chemotherapy of the Institute of Traumatology and Orthopedics (National Academy of Medical Sciences of Ukraine) showed that 33 *S. aureus* clinical isolates contained plasmid DNA, and given the significant potential of antibiotic resistance among bacterial plasmids of *S. aureus* clinical isolates (Mores et al., 2021), an assumption was made about the presence of antibiotic resistance genes on such visualized extrachromosomal elements in the studied isolates.

All these 33 clinical plasmid-containing *S. aureus* isolates were found to be methicillin-resistant (MRSA). It is known that MRSA is the cause of severe staphylococcal infections that are difficult to treat due to their resistance to some antibiotics (Liu et al., 2011). An important characteristic of *S. aureus* (MRSA) is their ability to cause hospital (nosocomial) infections.

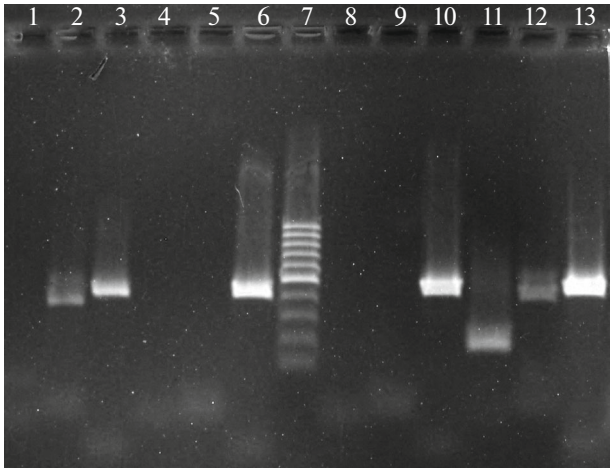

Fig. 1. Electrophoregram of PCR products of *tet*(*K*) and *tet*(*M*) gene detection: (1–3) doxycycline-resistant clinical isolate no. 110 ((1) *tet*(*M*); (2) *tet*(*K*); (3) ST); (4–6) control strain *Staphylococcus aureus* ATCC 25923 ((4) *tet*(*M*); (5) *tet*(*K*); (6) ST); (8–10) doxycycline-sensitive clinical isolate no. 126 ((8) *tet*(*M*); (9) *tet*(*K*); (10) ST); (11–13) doxycycline-resistant clinical isolate no. 418 ((11) *tet*(*M*); (12) *tet*(*K*); (13) ST); (7) Generunner 100-bp molecular weight marker.

The study of antibiotic resistance profiles showed that 24 of 33 clinical plasmid-containing *S. aureus* isolates showed resistance to the antibiotic of the tetracycline series: doxycycline. Resistance to *S. aureus* tetracycline is caused by the ribosome modification mechanism encoded by a widespread *tet*(*M*) gene or the mediated efflux mechanism encoded by the *tet*(*K*) gene. From the variety of different *tet* genes encoding efflux mechanisms, *tet*(*K*) is most often found in *S. aureus* (Schmitz et al., 2001). It is known that *tet*(*K*) and *tet*(*M*) tetracycline resistance genes, which have plasmid localization, are very common in Europe and Asia (Khoshbakht et al., 2018). Therefore, *tet*(*K*) and *tet*(*M*) gene detection was carried out in plasmid-containing *S. aureus* clinical isolates using PCR. Figure 1 shows one of the electrophoregrams for the separation of PCR products obtained during DNA research of bacteria of clinical isolate no. 110, 126, and 418 and the control strain *Staphylococcus aureus* ATCC 25923.

The availability of epidemiologically important transmissible genes of resistance to tetracycline in bacteria of 23 *S. aureus* clinical isolates was shown. In general, it is shown that 11 of 24 doxycycline-resistant plasmid-containing *S. aureus* clinical isolates had the gene *tet*(*M*), four isolates contained the gene *tet*(*K*), eight had and *tet*(*M*) and *tet*(*K*), while the bacteria of one clinical isolate did not contain either *tet*(*M*) or *tet*(*K*) genes.

Table 1. Assessment of the potential for biofilm formation in *S. aureus* clinical isolates

Group of clinical isolates	Indicator of optical density at 492 nm	Degree of biofilm formation
31 isolates, the bacteria of which do not have plasmids	0.21–0.24	Moderate
9 plasmid-containing doxycycline-sensitive isolates	0.20–0.25	Moderate
24 plasmid-containing doxycycline-resistant isolates	0.21–0.30	Moderate
Carriers of the gene *tet*(*K*)	0.22–0.24	Moderate
Carriers of the gene *tet*(*M*)	0.21–0.27	Moderate
Gene carriers *tet*(*K*) and *tet*(*M*)	0.21–0.30	Moderate
Isolates that do not have genes *tet*(*K*) and *tet*(*M*)	0.21–0.26	Moderate

Nine doxycycline-sensitive plasmid-containing clinical isolates were also examined for the presence of *tet*(*K*) and *tet*(*M*) genes. It was shown that no doxycycline-sensitive clinical isolate had genes *tet*(*K*) and *tet*(*M*).

Therefore, the presented data allow us to draw a conclusion about the significant spread of transmissible genes of resistance to tetracycline *tet*(*K*) and *tet*(*M*) in the examined plasmid-containing doxycycline-resistant *S. aureus* clinical isolates; 96% of such isolates had at least one of these genes.

Since the detection of pathogenic strains forming biofilms is extremely important in diagnostic laboratories and in view of the increased danger of the transfer of antibiotic resistance genes in biofilms, we investigated potential for biofilm formation in all 64 *S. aureus* clinical isolates presented. Such isolates were represented by three groups: 31 isolates, the bacteria of which do not have plasmids; 24 plasmid-containing doxycycline-resistant isolates; nine plasmid-containing doxycycline-sensitive isolates. In the group of 24 plasmid-containing doxycycline-resistant isolates, we monitored and systematized data on the ability to form biofilms in bacteria that had tetracycline resistance genes: *tet*(*K*) and *tet*(*M*).

The results of assessing the potential for biofilm formation are presented in Table 1.

Thus, research carried out regarding potential for biofilm formation in the presented 64 *S. aureus* clinical isolates demonstrated a moderate biofilm-forming ability of staphylococci of plasmid-containing isolates and isolates that did not have plasmids. This fact is cause for concern since the bacteria studied were clinical isolates isolated from patients with purulent-inflammatory and periprosthetic joint infections and, therefore, there is an extremely high risk of spreading plasmid genes of resistance to tetracycline in hospital populations of biofilm-forming bacteria *Staphylococcus aureus*.

A study was conducted on the elimination of plasmid-associated antibiotic resistance genes in clinical isolates of *Staphylococcus aureus* using gold and silver nanoparticles. We used gold nanoparticles with an average size of 30 nm in the form of a colloidal solution obtained by hydrothermal synthesis: gold hydrochloric acid ($HAuCl_4$ $3H_2O$) reduced with sodium citrate in the presence of potassium carbonate and silver nanoparticles with an average size of 30 nm in the form of a colloidal solution obtained by the condensation method by reducing silver nitrate with tannin.

Treatment of plasmid-containing bacterial cells of *S. aureus* clinical isolates, which demonstrated the presence of the studied *tet*(*K*) and/or *tet*(*M*) genes with gold or silver nanoparticles, was carried out by incubating bacteria with nanoparticles. The indicated nanoparticles of metals were introduced into the culture medium in the form of sterile aqueous solutions in an amount that ensures the content of gold nanoparticles in the medium of 3.2–9.6 μg/mL and silver of 20–40 μg/mL per metal. Thus, to the culture of bacteria of clinical isolates in the MPB with a cell titer of $1–2 \times 10^9$, the initial drug of gold or silver nanoparticles was added to the concentrations indicated above and cultivated at 37°C for 20–24 h.

According to the disco-diffusion method of studying sensitivity to the tetracycline antibiotic doxycycline, all bacteria treated with silver or gold nanoparticles with an average size of 30 nm lost resistance to this antibiotic. Bacterial inoculation of each treated strain on medium with doxycycline showed no growth on medium with this antibiotic.

Short-term cultivation of bacteria that lost resistance to doxycycline on MPA (approximately 18 h) followed by inoculation on a medium with an antibiotic showed no reversal of antibiotic resistance to doxycycline.

To visualise plasmids in all metal nanoparticle-treated clinical isolates of *Staphylococcus*, electrophoresis in a 1% agarose gel was performed. The absence of plasmids was shown in all clinical isolates that underwent plasmid elimination using gold or silver nanoparticles with an average size of 30 nm.

PCR analysis of such eliminators for the presence of previously identified *tet*(*K*) and *tet*(*M*) genes in them showed the absence of such genes after treatment of bacterial cells with nanoparticles (Fig. 2).

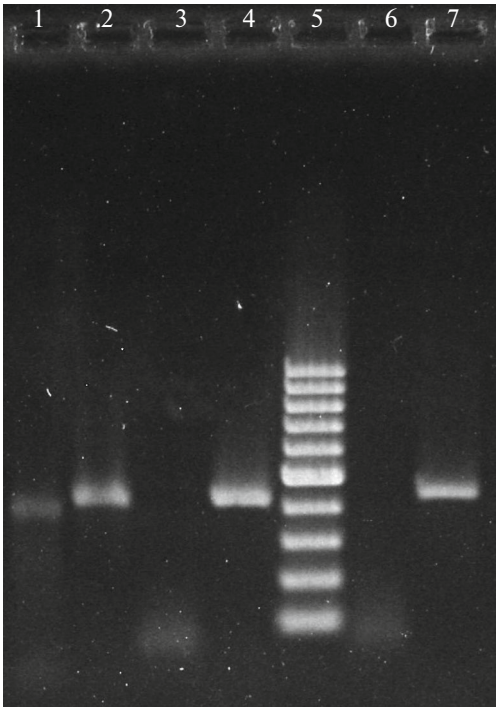

Fig. 2. Electrophoregram of PCR products of gene detection *tet*(*K*) in DNA clinical isolate *S. aureus* no. 110: (1, 2) before treatment with nanoparticles, (3, 4) after treatment with 30-nm gold nanoparticles (3.2 μg/mL), (5) Generunner 100-bp molecular weight marker, (6, 7) after treatment with 30-nm silver nanoparticles (20 μg/mL) ((1, 3, 6) tet(K)-F + tet(K)-R primers, (2, 4, 7) ST-F + ST-F primers, positive control *S. aureus*).

It was noted that bacteria of the same strain, in which no *tet*(*K*) and *tet*(*M*) genes were detected also lost resistance to doxycycline, and, therefore, it can be assumed that other genes for resistance to the specified antibiotic are also located on the plasmid.

The mechanism of elimination of R-plasmids from the studied clinical isolates can be explained based on the data that the studied nanoparticles of gold or silver with a size of 30 nm penetrate into the bacterial cell by means of electroporation induced by the membrane potential (Ulberg et al., 2014). At the same time, there is an interaction inside the cell of charged nanoparticles with a charged molecule of plasmid DNA, which leads to a change in the structure of the plasmid and the elimination of such a plasmid from the cell.

Therefore, all representatives of 24 plasmid-containing doxycycline-resistant clinical isolates lost their resistance to the tetracycline antibiotic doxycycline under the influence of treatment of their cells with gold nanoparticles of 30 nm in a concentration of 3.2–9.6 μg/mL or medium-sized silver of 30 nm in a concentration of 20–40 μg/mL. PCR analysis of clinical isolates, which contained the studied genes *tet*(*K*) and *tet*(*M*) before treatment with metal nanoparticles, showed the absence of such genes in eleminants. Elimination of tetracycline resistance genes in plasmid-containing *S. aureus* isolates with the known plasmid localization of the studied *tet*(*K*) and *tet*(*M*) genes testifies to the effectiveness of the demonstrated method of overcoming antibiotic resistance of pathogens of purulent-inflammatory and periprosthetic infections of human joints with the help of metal nanoparticles. However, the possibility was shown for the first time of overcoming the antibiotic resistance of the causative agents of purulent-inflammatory and periprosthetic infections of human joints to the widespread antibiotic tetracycline, which is used for the treatment and prevention of these diseases, by eliminating plasmid DNA with the help of gold or silver nanoparticles with an average size of 30 nm obtained by chemical condensation methods.

FUNDING

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Bioethics Commission of the Ovcharenko Institute of Biocolloidal Chemistry of the National Academy of Sciences of Ukraine on 21 May 2024 (protocol no. 9). Informed consent was obtained from each participant of the study.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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