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Estimation of combined *ex vivo* effect of thermal ablation and vancomycin on the growth of *Staphylococcus aureus culture*

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ABSTRACT

Aim. To study the *ex vivo* effect of high temperature exposure (55–56 °C) combined with vancomycin on culture behavior of pathogenic *Staphylococcus aureus* (*S. aureus*).

Materials and methods. Liquid cultures of methicillin-resistant *S. aureus* (MRSA) strain 43300 were heated *ex vivo* at 55–56 °C for 0–60 min, either with or without vancomycin (20 μg/ml), followed by incubation at 37°C up to 120 min. A control suspension (100 or 250 microbial cells per 1 ml of isotonic saline) was maintained at 37°C. Then, cultures were seeded on solid agar medium, and colony-forming units (CFU) were calculated using computer morphometry after 48-h growth. Each experimental subgroup (growth control, thermal ablation, antibiotic, and thermal ablation + antibiotic) included at least three replicates.

Results. A semi-lethal heat exposure time (LD50) of 12.25 min was determined for a liquid microbial culture at 100 cells/ml. When the density was increased to 250 cells/ml, 30-min thermal ablation (55–56°C) was insufficient for MRSA growth suppression. Vancomycin (20 μg/ml) alone did not affect CFU output. However, combined heat and antibiotic treatment resulted in 28% bacteriostatic effect ($p < 0.001$) on agar medium.

Conclusion. The study revealed a bacteriostatic effect of combined use of high-temperature exposure with vancomycin, which were ineffective when used separately. The obtained results have practical significance for reconstructive surgery of bone tissue, but require additional studies to clarify the mechanisms of the discovered phenomenon.

Keywords: MRSA, liquid culture, high temperature exposure, antibacterial therapy, colony-forming units, bacteriostatic effect

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Compliance with the principles of ethics. The study was approved by the IACUC Committee of Siberian State Medical University (Minutes No. 1 dated April 3, 2024).

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Оценка комбинированного *ex vivo* воздействия термоабляции и ванкомицина на рост культуры *Staphylococcus aureus*

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РЕЗЮМЕ

Цель. Изучить *ex vivo* влияние высокотемпературного воздействия (55–56 °C) в сочетании с ванкомицином на поведение культуры патогенного золотистого стафилококка (*S. aureus*).

Материалы и методы. Жидкую культуру метициллинрезистентного *S. aureus* (MRSA) штамм 43300 нагревали *ex vivo* (55–56 °C, 0–60 мин) с или без добавления ванкомицина (20 мкг/мл), затем инкубировали (37 °C, до 120 мин). В качестве контроля использовалась микробная взвесь (100 или 250 микробных тел на 1 мл изотонического хлорида натрия) при 37 °C. После экстремального воздействия культуры *S. aureus* высевали на плотную питательную среду, через 48 ч определяли выход колониеобразующих единиц (КОЕ) методом компьютерной морфометрии цифровых изображений бактериальных культур в чашках Петри. Для каждой экспериментальной подгруппы (контроль роста; термоабляция; антибиотик; термоабляция + антибиотик) проводили не менее трех повторений.

Результаты. Отработан временной интервал высокотемпературного воздействия для определения полулетальной дозы нагревания (LD50) в отношении жидкой микробной культуры, который составил 12,25 мин при плотности разведения 100 микробных тел/мл растворителя. При увеличении плотности бактериальной культуры до 250 микробных тел/мл растворителя 30 мин ее нагревания до 55–56 °C недостаточно для подавления роста MRSA на агаре. Ванкомицин в терапевтической дозе 20 мкг/мл не влияет на выход КОЕ использованного патогенного штамма 43300. В то же время комбинированное *ex vivo* воздействие термоабляции и антибиотика оказывает бактериостатический эффект на уровне 28% ($p <$ 0,001) подавления роста бактерий на агаровой питательной среде.

Заключение. Выявлен бактериостатический эффект комбинированного применения высокотемпературного воздействия с ванкомицином, неэффективных по отдельности. Полученные результаты имеют практическую значимость для реконструктивной хирургии костной ткани, однако требуют проведения дополнительных исследований для уточнения механизмов обнаруженного феномена.

Ключевые слова: MRSA, жидкая культура, высокотемпературное воздействие, антибактериальная терапия, колониеобразующие единицы, бактериостатический эффект

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is the most common pathogenic microorganism in surgical hospitals. In diseases caused by *S. aureus*, the duration of hospitalization, cost of treatment, and mortality are 2 times higher than in infections caused by other types of microorganisms. The frequency of isolation of methicillin-resistant (or oxacillin-resistant) strains of *S. aureus* (MRSA) from inflammation foci has increased sharply (from 1–40 (according to various sources) to 54%) since the beginning of the 21st century [1].

The problem of treating MRSA-associated infectious diseases attracts great attention worldwide. For the query “MRSA and treatment”, the PubMed database of the US National Institutes of Health (https://pubmed.ncbi.nlm.nih.gov/) showed 25,642 studies published from 1955 to 2024. The maximum number of articles was published in 2021 (1,596 results).

S. aureus, including MRSA, are the main causative agents of implant-associated infections (IAI; up to 50–80%) [2–4], which include periprosthetic infections [5, 6] and infections associated with bone fractures [7]. According to other sources, *S. aureus* and *P. aeruginosa* are isolated in 60% of clinical cases of all IAI [8].

Vancomycin (VMN) is one of the leading antibacterial drugs used experimentally and clinically for the prevention and pharmacotherapy of osteomyelitis caused by MRSA [9]. In general, MRSA strains are highly sensitive to VMN [1]. However, a single administration of VMN, even in high doses (up to 1 g), sometimes is insufficient to suppress MRSA-associated infections [10].

Hyperthermia is considered as one of the promising approaches in the combination therapy of infections caused by MRSA [11]. Nevertheless, only 69 publications related to the topic were found in the PubMed database, starting from 2003 (https://pubmed.ncbi.nlm.nih.gov/?term=mrsa%20and%20hyperthermia&timeline=expanded; query date is October 21, 2024). The query “MRSA and thermal ablation” revealed only 3 results since 2016 related to photodynamic therapy (https://pubmed.ncbi.nlm.nih.gov/?term=mrsa+and+thermal+ablation; query date is October 21, 2024).

In this regard, the *in vitro* study of the effect of high-temperature exposure (thermal ablation at 55–56 °C) in combination with vancomycin on the MRSA culture is of absolute relevance.

MATERIALS AND METHODS

Freshly prepared (according to the attached manufacturer’s instructions) nutrient agar for the cultivation of microorganisms (NACM) (BTN-agar, Biotekhinnovatsiya, Russia) in plastic Petri dishes with a lid (MiniMed, Belarus) with a diameter of 90 mm was tested for sterility of the medium by a thermostat programmed at 37 °C for 24 hours. NACM showed the absence of microbial growth (sterility).

The original MRSA culture (strain 43300) was obtained from the Bacteriological Laboratory of Siberian State Medical University, which has a sanitary and epidemiological inspection report (dated July 20, 2015 No. 70.TS.06.000.M.000268.07.15) for manipulations with pathogens of infectious diseases of III–IV pathogenicity groups.

The original MRSA culture was diluted to achieve a bacterial concentration of 100 or 250 microbial bodies in 1 ml of a sterile 0.9% sodium chloride solution in plastic sterile conical tubes with a cap of 15 ml (MiniMed, Belarus).

General heating of the microbial suspension in test tubes was carried out in two electric dry-air thermostats ShS-80N (SG-term, Russia), set to the required temperature of 37 °C or 55–56 °C. The exposure time was selected based on the determination of the semi-lethal time of heating the liquid MRSA culture in a preliminary experiment.

After heating the liquid MRSA cultures, 0.2 ml (20 or 50 microbial bodies) of the bacterial suspension were transferred to the NACM in Petri dishes. At least 3 tubes/Petri dishes with staphylococcal culture were used for each experimental subgroup (growth control at 37 °C; thermal ablation at 55–56 °C; antibiotic at 37 °C; and thermal ablation at 55–56 °C + antibiotic vancomycin (VMN, Belmedpreparaty, Belarus) 20 μg/ml).

MRSA agar cultures on Petri dishes showed an increase in colony-forming units (CFUs) after just 24 hours of cultivation at 37 °C and 100% humidity. However, small (dust-like) CFUs made morphometric studies difficult. Therefore, microbial cultures were left to grow for another 24 hours (total cultivation time was 48 hours), after which the potential bacteriostatic effect was assessed by reducing the area of CFUs grown on NACM.

The relative area of bacterial cultures was assessed according to the computer morphometry method of digital images [12] using ImageJ software tools version 1.38 (National Institutes of Health, USA;

http://www.rsb.info.nih.gov/ij). Digital photographs were taken using a Canon PowerShot A2200 camera (Canon Inc., China) with a resolution of 14.1 megapixels.

Due to the massive but uneven growth of MRSA on a solid nutrient medium (agar), the following method was used for a more accurate calculation of the relative (specific) area (RA) of bacterial cultures:

1. Each Petri dish was conditionally divided into 8 segments (Fig. 1).2. In each segment, the area of bacterial cultures (*S*c) was calculated in square millimeters.3. The area of each segment (*S*s) was calculated in square millimeters.4. The relative area of bacterial cultures (*S*r) was calculated for each segment using the formula *S*r = *S*c/*S*s, and the calculated shares were summed up.

As a result of the calculations, the RA in the Petri dish occupied by bacterial cultures was obtained.

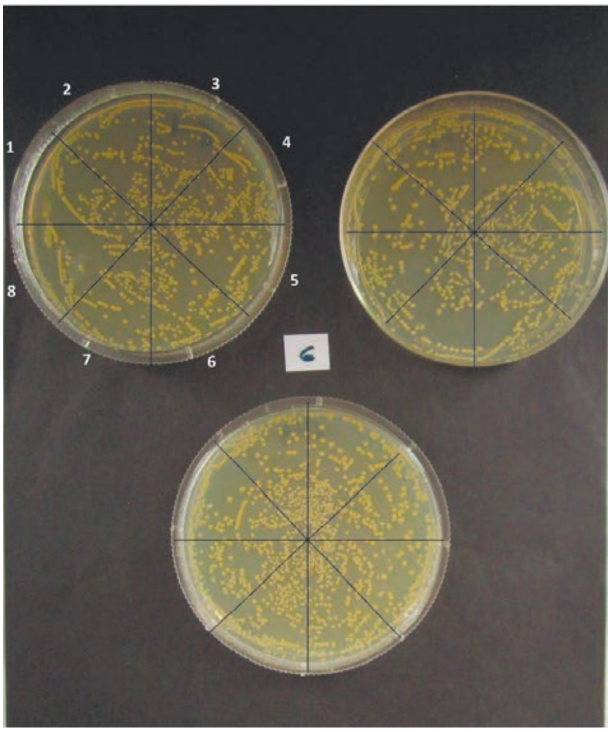


Fig. 1. Examples of dividing a Petri dish into segments when counting the area of grown MRSA colonies

The prepared liquid suspensions of MRSA in isotonic NaCl solution after mixing were cultured (with or without vancomycin) in the following time interval (in the main experiment): 2 hours (120 min) at 37 °C; 30 min at 55–56 °C + 90 min at 37 °C.

The total time interval (120 min) for pre-incubation of liquid cultures of *Staphylococcus aureus* was chosen in accordance with previous studies [13], as well as taking into account a preliminary study of the semi-lethal dose of thermal ablation at 55–56 °C (see below).

Statistical analysis of the data was conducted in the RStudio environment (ver. 2024.04.2+764) in the R programming language (version 4.4.1) using the MVN [14], PMCMRplus [15], and brunnermunzel [16] packages. The normality of distribution of quantitative variables was checked by the Shapiro–Wilk test with the Royston correction AS R94 for large samples ($3 \leq n \leq 5{,}000$) [17]. The description of normally distributed quantitative characteristics is given as the mean value and standard deviation ($M \pm SD$); non-normally distributed quantitative variables and ordinal characteristics were in the form of the median and the first and third quartiles Me (Q_1; Q_3).

For quantitative independent data that do not comply with the normal distribution, the comparison was performed using the Brunner–Munzel test [18, 19]. The peculiarity of the Brunner–Munzel test is that it does not require the assumptions: (a) equal deviations and (b) equal distributions, while the Mann–Whitney *U*-test is quite sensitive to their violations. At the same time, met, the Brunner–Munzel test corresponds to the Mann–Whitney *U*-test, which shows its reliability and universality [20, 21].

For quantitative non-normal independent data, multiple comparisons were performed using the Van der Waerden test. The choice of the Van der Waerden test is based on the fact that it provides high power (at the ANOVA level, at which the assumptions of normality are met) [22], and at the same time has sufficient reliability [23, 24]. The Dunn's post-hoc test was used as a one-versus-all (control-versus-all) a posteriori test.

RESULTS

The visual picture of bacterial CFU cultivation on a solid nutrient medium (agar) showed a significant inhibitory effect of thermal ablation on the growth of MRSA (Fig. 2).

Statistical processing of the results of MRSA colony growth on agar after preliminary heating of liquid microbial cultures made it possible to establish that at 55–56 °C, a statistically significant drop in the CFU area was observed already after 15 minutes of extreme exposure (5.5% (11/198 mm^2/dish) of the control); and by minute 30, the growth of MRSA colonies on agar was completely absent (Fig. 3).

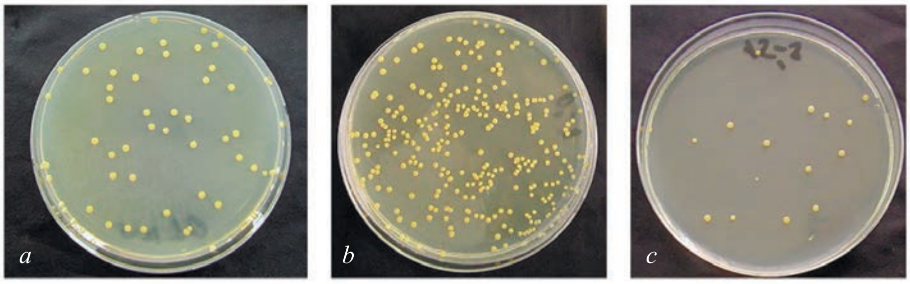


Fig. 2. Examples of 48-hour growth of MRSA colonies on agar in Petri dishes after preliminary extreme effects on liquid bacterial culture (concentration of 100 microbial bodies per 1 ml of 0.9% sodium chloride solution; 20 microbial bodies per Petri dish) at different temperatures: *a* – 37 °C, 120 min; *b* – 37 °C, 120 min; *c* – 55–56 °C 15 min + 105 min at 37 °C

The exposure dose of thermal energy causing 50% death of MRSA CFUs *in vitro* (LD50) was determined using calculated curves with reliable ($p < 0.001$) exponential (Fig. 3) approximation of the data. The calculated LD50 when heating a liquid MRSA culture (100 microbial bodies per 1 ml of 0.9% sodium chloride solution) to 55–56 °C was 12.25 min (Fig. 3).

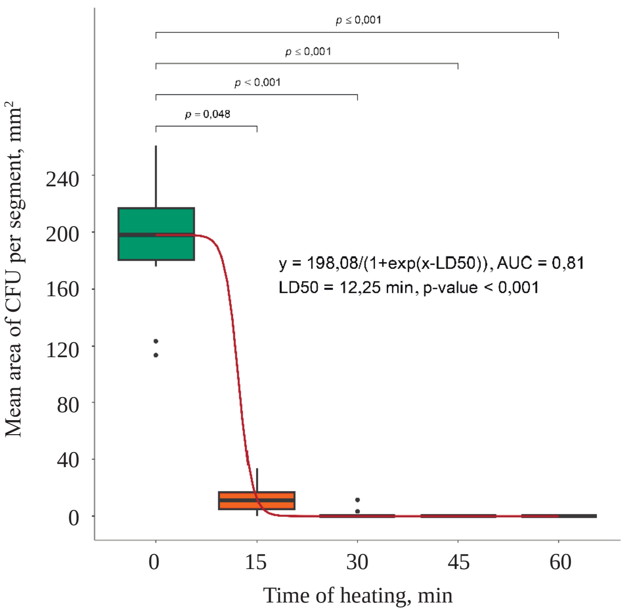


Fig. 3. Graphical approximation of experimental data on the area of CFUs (mm^2) grown in a 48-hour MRSA culture on agar after heating the liquid bacterial culture at 55–56 °C to determine (by the time of extreme exposure) the exposure semi-lethal dose (LD50) of thermal energy. The results are expressed as *Me* (Q_1; Q_3). Multiple comparisons, Van der Waerden test (p<0.001); pairwise comparisons, Dunn's test (p = 0.048 between the time 0 and 15 min)

Thus, heating to 55–56 °C quickly inhibited the vital activity of pathogenic bacteria at a concentration of MRSA in a liquid culture of 100 microbial bodies per 1 ml of 0.9% sodium chloride solution, which was expressed in a significant bacteriostatic effect on a solid nutrient medium (Fig. 2, *c*). At the same time, when MRSA was seeded on an agar medium from 20 microbial bodies subjected to heating in a liquid culture, only 11 CFUs germinated, there was a risk of a false positive result with the combined inhibitory effect of thermal ablation and antibiotic.

In addition, the control culture of staphylococcus at a concentration of 100 microbial bodies and a temperature of 37 °C gave an unstable yield of CFUs (Fig. 2, *a, b*). At the control points (0 min of liquid culture thermal ablation; 2-hour preincubation of bacteria in liquid culture, and 48-hour growth at 37 °C) with the same bacterial culture seeding density (20 microbial bodies per Petri dish), the CFU growth density in the controls differed by 3.33 times (CFU area 59.31 (20.47; 80.38) mm^2/dish (Fig. 2, *a*) and 198.08 (180.85; 216.97) mm^2/dish, Fig. 2, *b*).

In connection with the listed circumstances, the main experiment was carried out on the MRSA culture with a density of 250 microbial bodies per 1 ml of liquid suspension in 0.9% NaCl solution, 50 microbial bodies in 0.2 ml on the agar medium of Petri dishes. The thermal ablation time of liquid bacterial cultures at 55–56 °C was 30 min, which corresponds to LD100 at a culture density of 100 microbial bodies per 1 ml of sodium chloride (Fig. 3).

Nevertheless, a paradoxical effect of MRSA growth stimulation on agar was obtained, since after heating the liquid bacterial suspension, the proportion of grown colonies increased to 0.22 (22% of the Petri dish area) from 0.13 (13%) in the control (37 °C), respectively ($p < 0.001$; Brunner–Munzel test, Fig. 4).

Perhaps the increased (from 100 mt/ml to 250 mt/ml) density of the microbial liquid culture is important, which during hyperthermia can lead to an increase in the survival rate of MRSA due to a decrease in the amount of absorbed heat flux (thermal energy per unit surface area of microbial bodies).

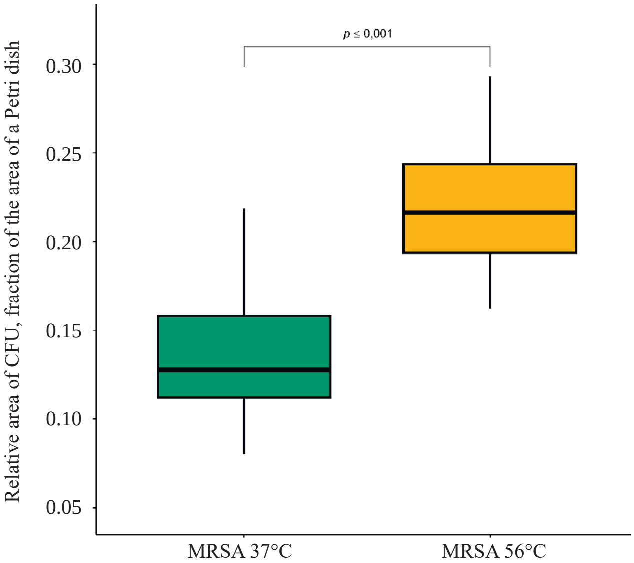


Fig. 4. Comparison of MRSA colony growth on agar in Petri dishes after preliminary heating of liquid bacterial cultures to different temperatures. Pairwise comparison, the Brunner–Munzel test

Notably, VMN at a therapeutic concentration (20 μg/ml) under conditions of 2-hour co-cultivation with a liquid bacterial culture at a temperature of 37 °C did not have an inhibitory effect on the growth of MRSA on agar. Visually, the number of CFUs did not differ from that in the control culture (Fig. 5).

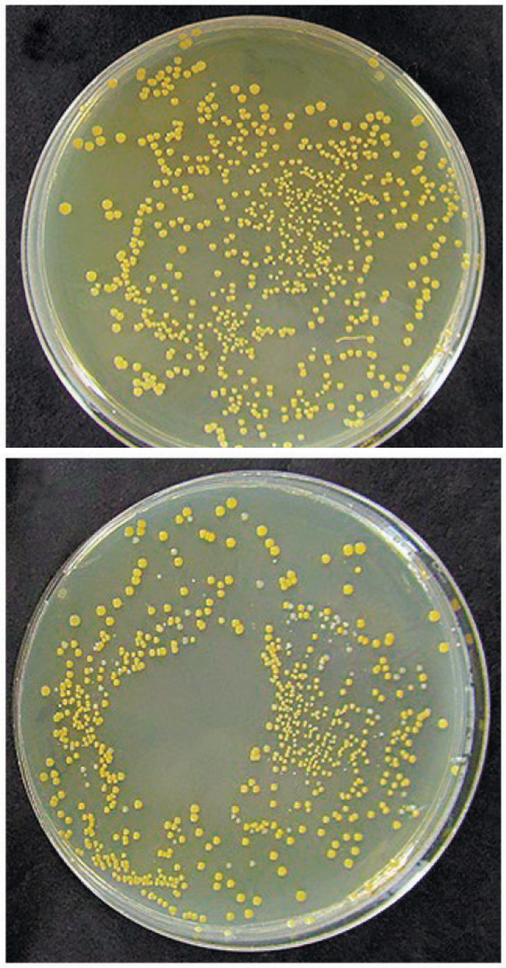
Computer morphometry of digital images of MRSA CFUs on agar also did not show statistically significant changes after exposure to VMN to a liquid microbial culture at 37 °C (Table). In other words, the used MRSA culture was resistant to a single 2-hour exposure to VMN at a dose of 20 μg/ml at 37 °C.

Table

Area (% of the Control) of MRSA Colony-Forming Units after Extreme Effects in Liquid Culture Followed by 48-Hour Cultivation on Agar in Petri Dishes at 37 °C

Group, $n = 3$	Sample size in each group, n_1	Relative area of bacterial culture, *Me* (Q_1; Q_3)	Pairwise comparison, the Brunner–Munzel test
MRSA 37 °C, growth control	24	100.00 (87.58; 123.44)	5.13; $p = 0.16$
MRSA + VMN 37 °C	24	97.23 (81.95; 116.85)	

N o t e. VMN is vancomycin, 20 **μ**g/ml; *n* is the number of Petri dishes in each group, divided into 8 segments in each dish (n_1) for more accurate determination of the parameter



a

b

Fig. 5. Examples of 48-hour growth of MRSA colonies on agar in Petri dishes after 2-hour cultivation of the liquid culture at 37 °C with antibiotic: *a* – MRSA growth control; *b* – MRSA + VMN 20 μg/ml

In turn, under conditions of 30-minute heating of the liquid bacterial culture at a temperature of 55–56 °C, VMN at a therapeutic concentration (20 μg/ml) visually reduced the number of CFUs grown on agar, compared to the control culture (Fig. 6).

Computer morphometry of digital images showed a statistically significant ($p < 0.001$) decrease in the yield of MRSA CFUs on agar (by 28% compared to the control) after the exposure to VMN on the liquid microbial culture under conditions of its thermal ablation at 55–56 °C (Fig. 7).

Thus, heating liquid cultures of MRSA for 30 minutes at a temperature of 55–56 °C significantly increased the colony-forming ability of MRSA on a solid nutrient medium (agar-agar, Fig. 4). Addition of vancomycin (20 μg/ml) to the liquid culture without heating did not affect the yield of microbial CFUs on agar (Table).

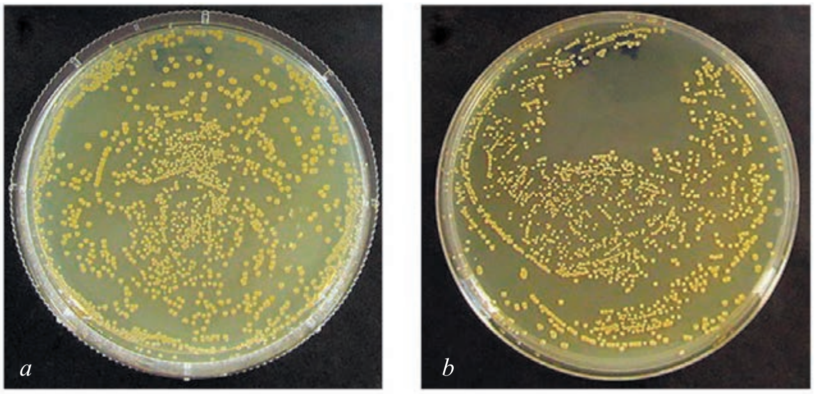


Fig. 6. Examples of 48-hour growth of MRSA colonies on agar in Petri dishes after preliminary thermal ablation of the liquid culture at 55–56 °C: *a* – MRSA growth control without antibiotic; *b* – MRSA + VMN 20 μg/ml

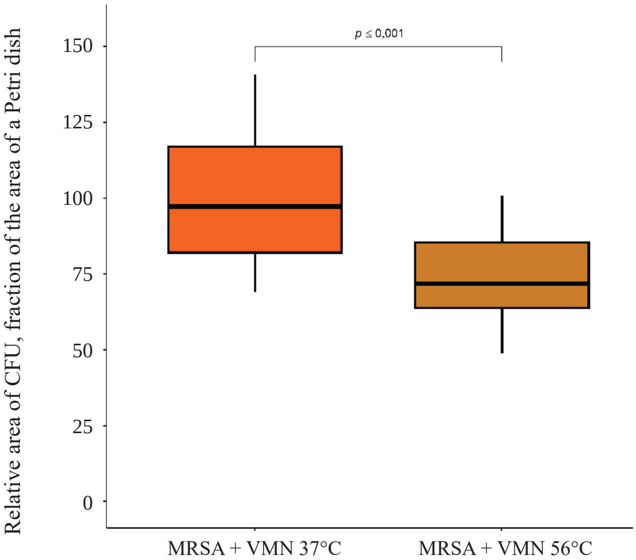


Fig. 7. Graphic representation (% of the control) of the areas of MRSA CFUs after adding vancomycin (20 μg/ml) and heating the liquid culture at 37 °C or 55–56 °C, followed by culturing for 48 hours on agar in Petri dishes at 37°C. Pairwise comparison, the Brunner–Munzel test.

In turn, heating the microbial suspension to a temperature of 55–56 °C (Fig. 7) in the presence of VMN has a noticeable bacteriostatic effect in comparison with the effect of the antibiotic at 37 °C. Presumably, the use of high-temperature exposure (above 55 °C) can improve the results of standard antibacterial therapy in the treatment and prevention of MRSA-induced osteomyelitis.

DISCUSSION

MRSA is resistant to all beta-lactam antibiotics, including inhibitor-protected penicillins and cephalosporins of all generations. Another clinically important feature of methicillin-resistant staphylococci is the high frequency of associated resistance to antibacterial drugs of different groups (aminoglycosides, macrolides, and lincosamides) [1]. Due to the high frequency of antibiotic use in the complex treatment of surgical patients, pathogenic MRSA strain 43300 was chosen for *in vitro* experiments.

Vancomycin (VMN) at a therapeutic concentration in human serum in the range of 10–20 μg/ml [25] is one of the leading antibacterial drugs used for the prevention and pharmacotherapy of osteomyelitis caused by MRSA [9].

However, the MRSA strain 43300 used in the work turned out to be insensitive to the therapeutic dose of VMN (20 μg/ml) at a temperature of 37 °C (Table), corresponding to the temperature of the human body, especially during inflammatory processes. The increased yield of bacterial CFUs at a relatively high density of the microbial liquid culture (250 microbial bodies per 1 ml) under conditions of 30-minute heating of the microbial suspension to 55–56 °C, which usually causes coagulation necrosis, turned out to be unexpected (Fig. 4).

A possible explanation for the obtained phenomenon is the fact that an increase in genetic diversity with an increase in the number of microbial bodies in the culture leads to the emergence and accumulation of bacteria carrying antibiotic resistance genes [26]. In turn, this increases the overall chances of the bacterial strain to survive in extreme conditions. In particular, there is significant variability in the thermosensitivity of *S. aureus* to 45 °C [27]. Similar to osteogenic cells, which are activated by moderate hyperthermia (~42 °C) due to hyperexpression of heat shock proteins [28], some “thermotolerant” MRSA may ensure the survival of the strain at high temperatures.

CONCLUSION

Antibiotic therapy is considered to be themainstay in the prevention and treatment of infectious complications in bone fractures and their surgical treatment [7]. However, the increasing antibiotic resistance of bacterial strains and the increasing contribution of MRSA to infectious inflammation make the development of new approaches to the treatment of infectious complications a key challenge in biomedicine.

In this regard, an important result of the *in vitro* study is the practical feasibility of the simultaneous administration of vancomycin and thermal ablation of liquid MRSA cultures, which are ineffective separately, but have an almost 30% bacteriostatic effect when used together. The obtained data have practical significance for specialists involved in the treatment of diseases of the skeletal bones and their complications. However, the obtained results require additional research to clarify the mechanisms of the discovered phenomenon.

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Author Contribution

All authors confirm that their authorship complies with the international ICMJE criteria (all authors made a significant contribution to the conception and design, conduct of the study, preparation of the manuscript, read, and approved the final version of the manuscript before publication). Gorokhova A.V. — morphometric processing, interpretation, and visualization of data. Nasibov T.F. – drafting and editing of the manuscript and statistical analysis. Mushtovatova L.S. — conception and design, conducting the experimental study. Bochkareva O.P., Bariev U.A. — conducting the experimental study. Anisenya I.I., Sitnikov P.K. — conducting the experimental study and experimental data interpretation. Leshenkova A.V. — conducting the experimental study and data visualization. Ryzhkova A.Yu. — conducting the experimental study, collecting and analyzing literary sources. Pakhmurin D.O. — final approval of the content for publication of the manuscript, conducting the experimental study. Khlusov I.A. — coordination of the study, writing and editing the article.

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