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Evaluation of TD test for analysis of persistence or tolerance in clinical isolates of *Staphylococcus aureus*

Hana Kotková[a], Marie Cabrnochová[b], Irena Lichá[a], Jan Tkadlec[b], Libor Fila[c], Jana Bartošová[d], Oto Melter[b,*]

[a] Department of Genetics and Microbiology, Faculty of Science, Charles University, Albertov 6, 128 00 Prague 2, Czech Republic
[b] Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University, V Úvalu 84, 15006 Prague 5, Czech Republic
[c] Department of Pneumology, 2nd Faculty of Medicine, Charles University and Motol University Hospital, 15006 Prague 5, Prague, Czech Republic
[d] Department of Pediatrics, 2nd Faculty of Medicine, Charles University, V Úvalu 84, 15006 Prague 5, Czech Republic

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ABSTRACT

Besides natural and acquired mechanisms of resistance, bacteria can cope with presence of antibiotics by using complex mechanisms such as persistence or tolerance.

The main purpose of this study was to evaluate the suitability of newly developed Tolerance Disk Test (TDtest) (Gefen et al., 2017) to detect persistent or tolerant bacterial cells in clinical isolates of *Staphylococcus aureus*. The principle of the test is to resuscitate the subpopulation of persistent or tolerant bacterial cells following a disk diffusion test by glucose.

Results of the TDtest were evaluated using time killing experiments for three pairs of consecutive *S. aureus* isolates from lower respiratory airway samples of three cystic fibrosis patients with chronic staphylococcal infections.

TDtest enabled semi-quantitative detection of persistent or tolerant bacterial populations in all analyzed isolates for oxacillin, vancomycin, and ciprofloxacin to which isolates studied were susceptible. Therefore, TDtest is a promising method for rapidly determining persistence/tolerance in clinical isolates of *S. aureus*.

1. Introduction

Persistence to antibiotics is defined as the ability of a subpopulation, within susceptible bacterial population, to survive exposure to high concentrations of an antibiotic (Balaban et al., 2004, Gefen and Balaban, 2009, Zhang, 2014). In contrast, tolerance is more generally describe as the ability, whether inherited or not, of the whole population to survive transient exposure to high concentrations of an antibiotic without a change in the minimum inhibitory concentration (MIC), which is often achieved by slowing the basic bacterial metabolism (Brauner et al., 2016).

The formation of persistent subpopulations and persistence of living bacterial cells could complicate treatment of bacterial infections and also cause the chronicity of the infection (Conlon, 2014). It has been shown that a persistent subpopulation is a reservoir for the emergence of bacterial resistance to antibiotics as well (Cohen et al., 2013, Harms et al., 2016). Earlier studies show that various proportions of persistent subpopulations resulted from exposure to different antibiotics that may correlate with bacterial species and strains analyzed (Lewis, 2010 and Kint et al., 2012). Our previous study has also shown that environmental factors, such as salt stress, could increase the ability of *S. aureus* to generate persisters (Kubistova et al., 2017).

To prove persistence/tolerance to ampicillin in *Escherichia coli*, a Tolerance Disk Test (TDtest) was successfully designed by Balaban group (Gefen et al., 2017), which also currently confirmed epistasis between tolerance and resistance mutations (Levin-Reisman et al., 2019). In this study, we applied and assessed a modified TDtest for detection persistence/tolerance of *S. aureus* isolates ($n = 6$) of three cystic fibrosis (CF) patients to anti-staphylococcal antibiotics oxacillin, vancomycin, or ciprofloxacin. The latter was used in treatment of Gram-negative co-infection in the CF patients analyzed. The aim of our study was to evaluate the suitability of TDtest for analysis of clinical *S. aureus* isolates cultured from chronic infection, where a higher rate of persisters/tolerants formation is suspected (Suligoy et al., 2018). The modified TDtest introduced in the present study was able to detect persister cells in *S. aureus* clinical isolates that was evaluated and

* Corresponding author.
E-mail addresses: hana.kotkova@natur.cuni.cz (H. Kotková), mcabrnochova@seznam.cz (M. Cabrnochová), irena.licha@natur.cuni.cz (I. Lichá), jan.tkadlec@lfmotol.cuni.cz (J. Tkadlec), libor.fila@fnmotol.cz (L. Fila), Jana.Bartosova@fnmotol.cz (J. Bartošová), oto.melter@lfmotol.cuni.cz (O. Melter).

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Table 1
Clonal origin of six clinical isolates and reference strain of *S. aureus* (ATCC25923), and their minimum inhibitory concentration (MIC) to antibiotics tested.

Patient	Isolate	spa type/CC MLST	Patient Sex, Age	Patient treated	Spirometry (parameters within 2011–2013)	*S. aureus* infection & *P. aeruginosa* co-infection	MIC OXA (S ≤ 2 mg/L)	MIC VAN (S ≤ 2 mg/L)	MIC CIP (S ≤ 1 mg/L)
1	**Sa1**	t13495/CC8	Male 19/21 yrs	AMC, CIP	FVC 96%FEV1 90%	14/none	0.5	1	1
	Sa2								
2	**Sa5**	t13503/CC15	Male 19/	OXA, AMC, CXM, OFX, CIP	FVC 79%FEV1 64%	10/6 years	0.25	1	1
	Sa6	t3272*/CC15	21 yrs				0.5	2	1
3	**Sa23**	t13497/CC9	Female 16/18 yrs	AMC, CAZ, CFP, TZP, CPM, MEM, IPM, VAN, OFX	FVC 87%FEV1 69%	13/13 years	0.5	1	4
	Sa24								
Reference strain	**ATCC25923**	t064/CC8	ND	ND	ND	ND	0.06	1	0.125

Legend: CC MLST – clonal complex of multilocus sequence type, AMC – amoxicillin/clavulanic acid, CIP – ciprofloxacin, OXA – oxacillin, CXM – cefuroxime, OFX – ofloxacine, CAZ – ceftazidime, CFP – cefoperazon, TZP – piperacillin/tazobactam, CPM – cefepime, MEM – meropenem, IPM – imipenem, VAN – vancomycin, FVC – forced vital capacity, FEV1 – forced expiratory volume in first second, ND – not determined, note: * spa type t3272 has one additional inverted repeat comparing with that of t13503 - both of them are clonally closely related isolates within CC15 clonal complex.

confirmed by time killing experiments, which were used to quantify persisters/tolerants by other authors (Lechner et al., 2012 and Levin-Reisman et al., 2019).

2. Material and methods

2.1. Patients, bacterial isolates and reference strain

Clinical isolates of *S. aureus* ($n = 6$) were obtained from respiratory airways samples of three cystic fibrosis patients. For each patient, two clonally related consecutive isolates of *S. aureus* were isolated during exacerbation within a period of 2 years (2011 − 2013). Clinical status and lung functions expressed by spirometry (best parameter values of FVC and FEV1 are indicated in Table 1) proved relative stability within period of the study in patients 1 and 2 (mild bronchiectasis and sporadic exacerbation with chronic purulent expectoration in the latter) but significant decline in lung functions in patient 3 with frequent exacerbation (Table 1) which revealed in her lung transplantation in January 2014 (FVC = 68% and FEV1 = 55%). Because *S. aureus* was isolated over a six months period from all the patients, the infections are classified as chronic staphylococcal infections. Patients 2 and 3 were also chronically co-infected with *Pseudomonas aeruginosa*. Clonal relatedness of *S. aureus* isolates was assessed by *spa*-typing as described on the website of the European Network of Laboratories for sequence based typing of microbial pathogens (http://www.seqnet.org). The MLST clonal complexes (CC) were inferred from the spa-type using information available in the spa-server database (http://spaserver.ridom.de). As control for TDtest and time killing experiments, *S. aureus* ATCC25923 strain was used. All the antibiotics from the groups of evaluated antibiotics (beta-lactams, quinolones and glycopeptides) are also indicated in Table 1. Ciprofloxacin was used in treatment of Gram negative co-infections (i.e. *Klebsiella pneumonia*, *Achromobacter xylosooxidans* in patient 1 and *P. aeruginosa* in patients 2 and 3). Numerous antibiotics from other groups were also repeatedly used in treatment of patient 1 (sulfamethoxazole-trimethoprim, azitromycine, doxycycline and tobramycine), patient 2 (sulfamethoxazole-trimetoprim, azitromycine, clarithromycine and colistin) and patient 3 (sulfamethoxazole-trimethoprim and colistin) probably without any impact on evolving persistence/tolerance in the analyzed isolates.

2.2. Antibiotic susceptibility testing

Antimicrobial susceptibility of the isolates and reference strain (ATCC25923) were determined by the disk diffusion method (DDT) for oxacillin (1 μg), vancomycin (5 μg), and ciprofloxacin (1 μg) according to the EUCAST guidelines (EUCAST - European Committee on Antimicrobial Susceptibility Testing, 2019). Minimum inhibitory concentrations (MIC) were determined using M.I.C. Evaluator Strips (Thermo Scientific Oxoid, UK). For disc diffusion tests (DDT) and persistence/tolerance test (TDtest) Muller-Hinton (MH) agar (cat. No. PO0152) was used as a standard medium to test antibiotic susceptibility in clinical laboratories together with frequently used enriched culture media: Columbia agar with sheep blood (cat. No. PB0123), chocolate agar with vitox (cat. No. PO5090A), and brain heart infusion (BHI) agar (cat. No.CM1136). All the culture media used were produced by Thermo Scientific Oxoid (UK). Antibiotics used to perform time killing experiments were oxacillin (Prostaphylin, Bristol-Meyers Sqiubb, UK), vancomycin (MD Biomedicals, UK), and ciprofloxacin (Sigma Aldrich, UK).

2.3. TDtest

Modified TDtest (Gefen et al., 2017) was applied and evaluated for important antistaphylococcal drugs oxacillin and vancomycin, along with broad-spectrum drug ciprofloxacin, which is often in the case of cystic fibrosis, used in treatment of serious *Pseudomonas aeruginosa* co-

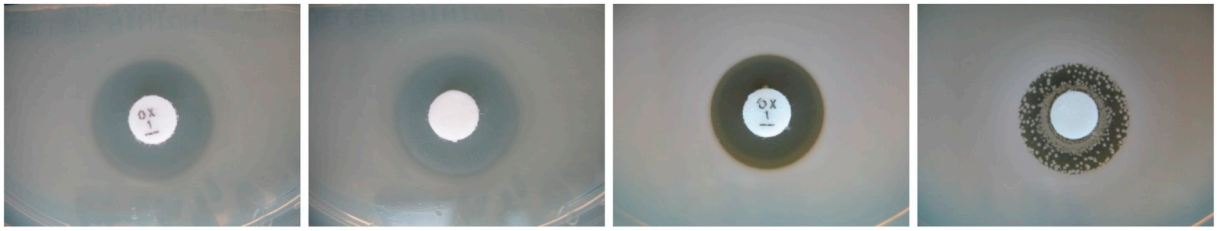


Fig. 1. Representative figures of TDtest evaluation on Mueller-Hinton (MH) and brain heart infusion (BHI) agar.
Legend: both media inoculated by the isolate Sa1 are presented (A - MH agar and 1 μg oxacillin disk, B – MH agar and overnight glucose resuscitation, C - BHI agar and 1 μg oxacillin disk, D - BHI agar and overnight glucose resuscitation.

infection. Bacterial inoculum (100 μl) consisting of 10^9CFU/ml (5 McFarland) was inoculated and streaked by loop in duplicates on MH, Columbia agar with sheep blood, chocolate agar, and BHI to perform DDT solely to oxacillin, vancomycin, or ciprofloxacin. After overnight cultivation, all antibiotic disks within inhibition zones were replaced by sterile blank disks (Thermo Scientific Oxoid, UK) soaked with 20 μl of 40% sterile glucose. Growing colonies of resuscitated viable bacterial cells were observed after 24 h and 48 h incubation at 35 °C.

2.4. Time killing experiments

Time killing experiments were performed as previously described (Kubistova et al., 2017) with minor modification. Exponential growth phase (OD_{600} = 0.5–0.6) culture of *S. aureus* isolate in BHI broth (cat. No.CM1135, Thermo Scientific Oxoid, UK) was exposed to selected antibiotic (oxacillin, vancomycin, ciprofloxacin) in concentrations corresponding to the 100-fold of MIC for tested isolate. To estimate the number of persisters, 100 μl samples of the bacterial culture were taken before the antibiotic challenge (t_0 = 0 h) and in one-hour intervals for five hours (t_{1-5}). Samples were centrifuged, and the pellet was re-suspended in 2% NaCl (1 ml) to remove residual antibiotic. To estimate CFU/ml, the 10-fold dilution series was prepared from each sample and 100 μl aliquots were plated on BHI agar. After incubation for 24–48 h at 34 °C, colonies were counted. To be able to compare data from different experiments, values of CFU/ml at each time (CFU t_x) were divided by the value of CFU/ml at t_0 according to the equation CFU t_x relative = $CFUt_x/CFUt_0$. For CFU at t_0, t_0 relative was then equal to 1.

3. Results and discussion

3.1. Susceptibility to antibiotics

All of the isolates analyzed, including the reference *S. aureus* strain (ATCC25923), were sensitive to all antibiotics tested with DDT and MIC according to the EUCAST guidelines, except for isolates Sa23and Sa24 that were resistant to ciprofloxacin (Table 1).

3.2. TDtest results

In this study we screened for persistence or tolerance of the clinical isolates of *S. aureus* using modified TDtest introduced by Gefen (Gefen et al., 2017), when after antibiotic exposure, viable bacterial cells were resuscitated by glucose. Culture media used were Muller-Hinton, which is the standard medium to test antibiotic susceptibility in clinical laboratories, together with three enriched culture media; BHI agar, Columbia blood agar, and chocolate agar (see also Materials and Methods). Comparing all tested media, clearly visible distinct colonies originated from persistent/tolerant cells after resuscitation by glucose were detected only on BHI agar (Fig. 1).

Resuscitated persisters were detected inside antibiotic inhibition zone (IZ) either as (A) the colonies growing in the vicinity of glucose disk, (B) homogeneously dispersed colonies within the IZ, or (C) colonies growing on the edge of the IZ. Representative diagrams and figures are presented in Fig. 2. To check if the colonies growing within IZ represent persistent/tolerant and not to the tested antibiotics

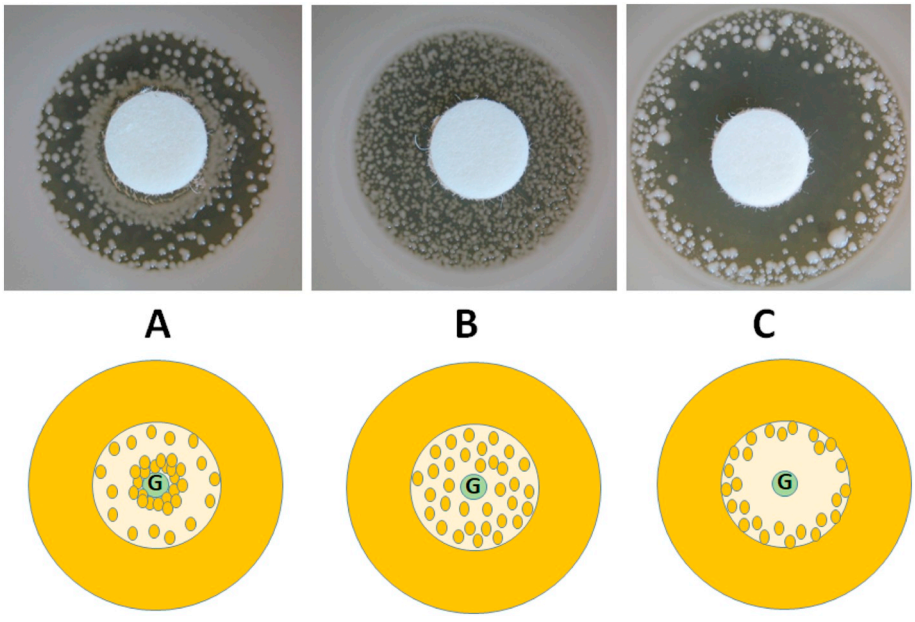


Fig. 2. Diagram and representative figures of persistence/tolerance TD growth pattern after overnight resuscitation using glucose disk.
Legend: Glucose concentration dependent growth of resuscitated Sa1 isolate after previous exposure to oxacillin (A), glucose concentration independent growth of resuscitated Sa5 isolate after previous exposure to oxacillin (B), growth on the edge of antibiotic inhibition zone (IZ) of resuscitated Sa5 isolate after previous exposure to ciprofloxacin (C).

Table 2
Classification of persistence/tolerance frequency and TDtest pattern on BHI agar.

Strain isolate	Oxacillin			Vancomycin			Ciprofloxacin		
	DDT OXA	P/T frequency	TD pattern	DDT VAN	P/T frequency	TD pattern	DDT CIP	P/T frequency	TD pattern
ATCC25923	25 mm	Low	B + C	11 mm	Low	B	27 mm	Low	B + C
Sa1	16 mm	High	A	12 mm	Low	B	16 mm	High*	B + C
Sa2	16 mm	High	A	12 mm	Low	B	16 mm	High*	B + C
Sa5	15 mm	High	B	11 mm	Medium	B	17 mm	High*	B + C
Sa6	15 mm	High	B	12 mm	Low	B + C	16 mm	High*	B + C
Sa23	15 mm	High	B	12 mm	Medium	B	6 mm	ND	ND
Sa24	16 mm	High	B	12 mm	Medium	B	6 mm	ND	ND

Legend: Persistence/tolerance (P/T) frequency is classified as low – 1 to 10 colonies inside IZ; medium – 10 to 100 colonies; and high - >100 colonies. The correspondence of the location of growth colonies to the TD pattern is as follows; if majority of grown colonies were located in the vicinity of glucose disk (TD pattern A – glucose concentration dependent growth), homogeneously within the IZ (TD pattern B – glucose concentration independent growth), on the edge of antibiotic IZ (TD pattern C - growth on the edge of antibiotic inhibition zone). Note: *interpretation of sporadic persistant/tolerant colonies was not influenced even by resistant colonies inside IZ with ciprofloxacin. ND – not determined because the isolates were resistant to CIP using DDT.

resistant bacterial population, five colonies were streaked on media without antibiotics and susceptibility to the tested antibiotic was determined by DDT.

Quantity of persistent/tolerant colonies inside the IZ were classified into three categories as was previously suggested (Gefen et al., 2017) - (1) low − 1 to 10 colonies, (2) medium – 10 to 100 colonies, (3) high - >100 colonies. The results of all isolates and antibiotics tested on BHI agar are summarized in Table 2.

3.3. Evaluation of TDtest results by time killing experiments

In parallel, we performed time killing experiments (see also Methods), which can be considered as a quantitative determination of ability to form persisters to confirm the accuracy of the TDtest. Experiments were performed for all strains and antibiotics tested. The number of viable cells in treated populations decreased by two to six orders of magnitude at the end of the experiment (5 h), depending on drug and isolate tested (Fig. 3).

These experiments with several clinical isolates confirmed general observations that various isolates may generate different proportion of persisters or tolerants after the application of diverse antibiotics. Interestingly, closely clonally related isolates within pairs of consecutive single patient isolates tended to have a similar curves dynamic of time killing experiments.

Comparing the results of the TDtest and the time killing experiment revealed that if the viable cell drop was six orders of magnitude at 5 h after antibiotic administration, the isolate was categorized as a weak persister in the TDtest e.g. strain ATCC25923 and oxacillin (Table 1 and Fig. 3A). If the decrease was only three orders of magnitude, TDtest categorized the isolate as a strong persister e.g. Sa24 and oxacillin (Table 1 and Fig. 3A).

Correlation of the curves course from the time killing experiments, which distinguish persistence from tolerance (Brauner et al., 2016) and localization of the growing colonies of persisters within the IZ, may indicate whether they are true persistent cells or being tolerant. Growth of the colonies throughout the area of the IZ almost corresponds to biphasic course of curves from time killing experiments typical for persisters (e.g. isolate Sa6 exposed to oxacillin as shown in Fig. 3A). However, when the colonies, after the removal of the antibiotics disks, grow predominantly on the edge of the inhibition zone the course of the time killing experiment curves were rather without the initial sharp decline of surviving cells after antibiotic treatment indicating tolerance to antibiotic tested (e.g. isolates Sa1, Sa5 exposed to ciprofloxacin as shown in Fig. 3C). Time killing experiment results of particular patients' isolates correlated with minor exceptions with TDtest but this our observation should be proved with more isolates tested.

Time killing experiment results of particular patients' isolates correlated with minor exceptions with TDtest. Therefore, the latter method is reliable for the determination of persistence or tolerance in clinical isolates of *S. aureus* since the implementation of routine killing experiments is unrealistic in clinical microbiology laboratories.

4. Conclusions

By correlating both methods, we demonstrated that the TDtest designed by Gefen et al. (2017) could also be used to test persistence or tolerance in clinical *S. aureus* isolates.

The above-described modified TDtest can be considered as a semi quantitative assay, which very well detects levels of low, medium or high bacterial persistence or tolerance to antibiotics. Moreover, localization of resuscitated colonies inside the inhibition zone could help to differentiate a persistent isolate from one being tolerant.

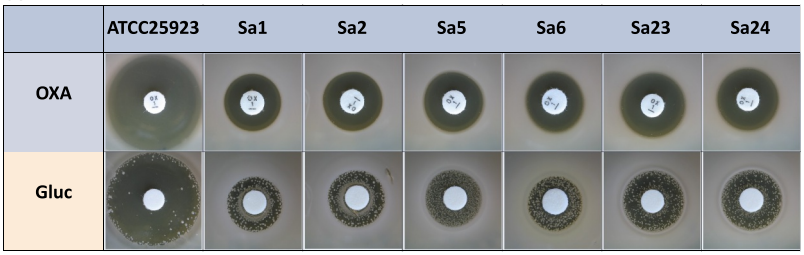
We believe that implementation of TDtest in clinical microbiology practice has the potential to improve treatment of hard-to-treat and chronic infection as its results could give treating physicians essential warning that the patient's antibiotic therapy (selection of an effective antibiotic, dosage and duration of therapy) should be modified to cope with presence of tolerant or persistent bacteria.

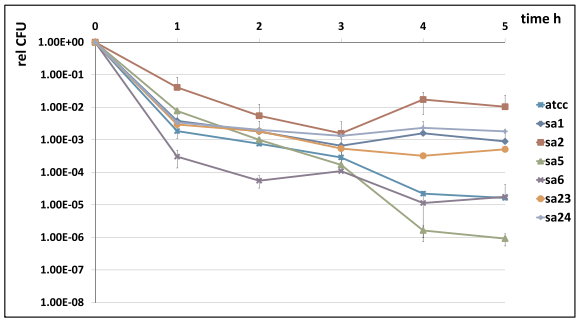
However, implementation of the modified above described TDtest in algorithm of regular antibiotic susceptibility testing requires evaluating the test on a larger collection of clinical isolates of *S. aureus* and possibly other microbial species. Therefore, other comprehensive studies with various bacterial species and various antibiotics are essential to validate the suitability of TDtest to be included in regular diagnostic algorithms of clinical microbiology practice.

Author contributions

Hana Kotková - conducting experiments, preparation of the ms; Marie Cabrnochová - strain isolation, performing TDtes analysis, ms checking; Irena Lichá - supervisor of MSc. Kotková and Bc. Cabrnochová, designing of time killing experiments, ms writing and checking; Jan Tkadlec - antibiotic susceptibility test, desingning TDtest and time killing experiments; Libor Fila - selection of patients with staphylococcal infections, performing clinical and lung function evaluation, checking the ms; Jana Bartošová - selection of patients with staphylococcal infections, performing clinical and lung function evaluation, checking the ms; Oto Melter - P.D.Study designing, repeating TDtest experiments.

A





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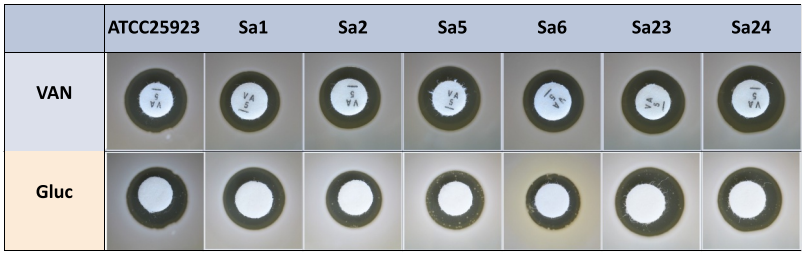

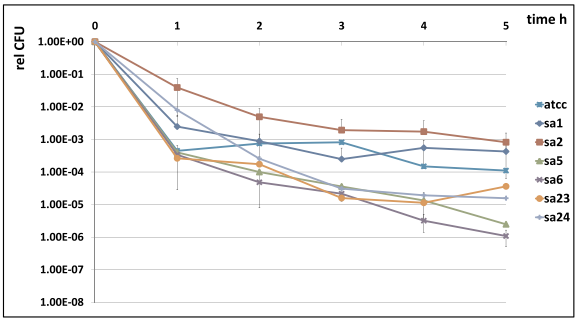

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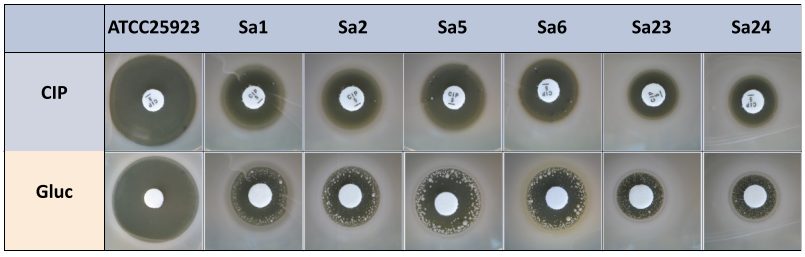

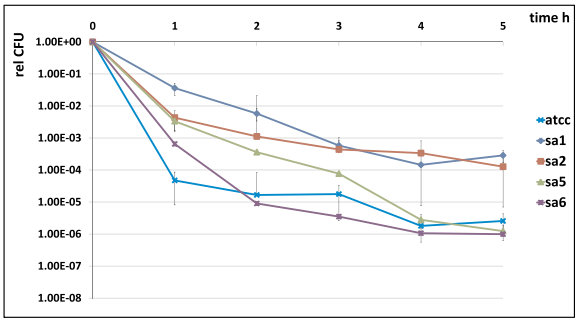

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Fig. 3. TDtests and killing curves of the analyzed clinical isolates and the reference strain.
Results of TDtest (upper part) and corresponding killing curves (lower part) of particular clinical isolates together with the reference S. aureus strain (ATCC25923) and antibiotics studied. Note: Gluc – glucose, OXA - oxacillin (A), VAN - vancomycin (B), CIP - ciprofloxacin (C).

Declaration of Competing Interest

No competing financial and personal interests exist.

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