J Therm Anal Calorim (2011) 104:31–36
DOI 10.1007/s10973-010-1241-7

Chip-calorimetric evaluation of the efficacy of antibiotics and bacteriophages against bacteria on a minute-timescale

J. Lerchner · D. Mueller-Hagen · H. Roehr · A. Wolf · F. Mertens · R. Mueller · W. Witte · I. Klare

ISBCXVI Conference Special Issue

Abstract Rapid detection of antibiotic resistances of clinical bacterial strains would allow an early selective antibiotic therapy and a faster intervention and implementation of infection control measurements. In clinical practice, however, conventional antibiotic susceptibility tests of bacteria often need 24 h until the results are obtained. The metabolic heat production of bacteria is an excellent possibility to record their physiological activities and could therefore be used for a rapid discrimination of bacterial strains which are resistant or non-resistant to antibiotics and also to lytic bacteriophages, respectively. Unfortunately, conventional calorimeters suffer from need of comparably large volumes of bacterial suspensions are characterised by slow operation and high costs which restrict their application in clinical laboratories. The present paper demonstrates that a new type of calorimeters developed on silicon-chip technology enables the detection of antibiotic resistances on a minute-timescale. For this reasons, a prototype chip calorimeter was used which sensitivity is 20 nW related to the heat production of about 10^4 bacteria. For a clear discrimination of antibiotic resistance about 10^5 bacteria are required. The antibiotic resistances and susceptibilities of different strains of *Staphylococcus aureus* to cefoxitin and the sensitivities of *S. aureus* DSM 18421 and *E. coli* DSM 498 to a mixture of two bacteriophages were studied. Comparing the heat productions of cultures incubated with antibiotics or bacteriophages to those without these antibacterial preparations enabled a clear discrimination of resistant and non-resistant strains already after totally 2 h.

Keywords Chip calorimetry · Metabolic heat rate · Rapid susceptibility testing of bacteria · Antibiotics and bacteriophages

J. Lerchner (✉) · A. Wolf · F. Mertens
TU Bergakademie Freiberg, Institute of Physical Chemistry, Leipziger Str. 29, 09596 Freiberg, Germany
e-mail: johannes.lerchner@chemie.tu-freiberg.de

D. Mueller-Hagen · H. Roehr · R. Mueller
Polyphag GmbH, Viersen, Germany

W. Witte · I. Klare
Robert Koch Institute, Wernigerode Branch, Wernigerode, Germany

Introduction

Screening methods for the detection of antibiotic-resistant bacterial strains are well established. Particularly, early detection of multiresistant bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) infecting and/or colonising patients and early start of targeted antibacterial therapy can significantly reduce mortality. Furthermore, early detection of MRSA is important for efficient infection control.

Conventional methods of antibiotic susceptibility testing are often labour-intensive and time-consuming (results mostly obtained after 24 h). Recently developed DNA-based tests detect MRSA are more rapid, however, they are more costly [1]. A novel culture-based screening test for the detection of ciprofloxacin- and methicillin-resistant *S. aureus* which is implemented in the BacLite Rapid MRSA method [2] provides accurate results already after 5 h. The method comprises the extraction of MRSA applying magnetic beads coupled to specific antibodies and the selective lysis of the immobilised bacteria.

A more straight way to detect bacterial resistance towards growth inhibiting agents is the measurement of the

decrease of the metabolic heat production as an effect of the interaction with growth inhibitors such as antibiotics and lytic agents such as bacteriophages. Already more than 30 years ago, Mardh et al. [3] demonstrated that isothermal calorimetry can be used to determine antimicrobial susceptibilities. Recently, von Ah et al. [4] as well as Baldoni et al. [1] evaluated extensively the potential of modern calorimetric techniques for reliable MRSA detection. Both working groups used a conventional microcalorimeter TAM III (TA Instruments, New Castle, DE, USA) with a detection limit of 0.27 μW corresponding to a metabolic heat production of 1.35×10^5 bacteria. Baldoni et al. [1] investigated 30 genetically distinct clinical isolates of *S. aureus* and could identify 85% of MRSA after 3 h and 95% of MRSA after 5 h. High costs of the systems and large amounts of samples required are the bottlenecks for a wider application of the method. Potentially, the miniaturisation of the calorimeters could be the precondition for the establishment of this method for its routine use in clinical laboratories. Microfabrication techniques developed in recent years led to the invention of miniaturised silicon chip-based calorimeters (chip calorimeter). The volume of bacterial sample necessary is dramatically reduced to a few microlitres and the system costs are clearly decreased. At present time, chip calorimeters with nanowatt of heat flow detection are available which fulfil the requirements for microbial investigations [5, 6].

In the present report we used a microcalorimeter based on chip technology that allows a rapid heat detection based on a very low sample volume. Our results demonstrate that this system is suitable for a rapid detection of bacterial susceptibilities to antibiotics and phages. Additionally, first experiments are presented which show the applicability of chip calorimetry to analyse phage-host systems.

Experimental

Bacterial strains

E. coli DSM 498 (strain 1); *S. aureus* DSM 18421, SPA-type t1518, minimal inhibitory concentration to oxacillin, $MIC_{OXA} \leq 1$ mg/L (strain 2); *S. aureus* NCTC 8325, SPA-type t211, international antibiotic-susceptible control strain, $MIC_{OXA} \leq 0.5$ mg/L (strain 3); *S. aureus* 96-01678, SPA-type t032, $MIC_{OXA} > 4$ mg/L (strain 4); *S. aureus* 07-00244, SPA-type t004, MIC_{OXA} 1 mg/L (strain 5); *S. aureus* 03-02112, SPA-type t002, MIC_{OXA} 4 mg/L (strain 6); *S. aureus* 08-00171, SPA-type t003, MIC_{OXA} 4 mg/L (strain 7); *S. aureus* 09-02069, SPA-type t036, MIC_{OXA} 4 mg/L (strain 8); *S. aureus* 06-01700, SPA-type t030, MIC_{OXA} 4 mg/L (strain 9). *S. aureus* strains which are resistant to oxacillin are also resistant to methicillin and all cephalosporins, including cefoxitin (= MRSA), and on the other hand, oxacillin-sensitive strains of *S. aureus* are also sensitive to the antibiotics mentioned before. MICs were determined in antibiotic susceptibility testing by broth microdilution according to German DIN standard [7].

Bacteriophages

The bacteriophage mixture DSM 18723/DSM 18722 was provided by PolyPhag GmbH (Viersen, Germany). For the production of high-titre bacteriophage, 500 μL of a bacterial overnight culture of the strains *S. aureus* DSM 18421 and *E. coli* DSM 498, respectively, in Luria–Bertani (LB) broth (5 g/L yeast extract, 10 g/L Trypton, 5 g/L NaCl), adjusted to $OD_{600} = 0.8$, were mixed with 100 μL bacteriophage suspension in phosphate-buffered saline (PBS: 80 g/L NaCl, 2 g/L KCl, 6.1 g/L Na_2HPO_4, 2 g/L KH_2PO_4, pH 7.4) containing10^5 plaque forming units (PFU) per mL. This mixture was incubated for 20 min at 37 °C. The mixture was transferred into 100 μL LB broth with 10 mM $MgSO_4$. Following incubation at 37 °C for 6 h, the culture was centrifuged for 15 min at 8,000 rpm and the supernatant was sterile filtrated.

Bacteriophage lysis assays

Twenty millilitre of LB broth were inoculated with 100 μL of an overnight culture of the corresponding bacterial test strain in LB broth (*S. aureus* DSM 18421 and *E. coli* DSM 498, respectively) and 100 μL of phages suspension in PBS and cultured in an incubation shaker at 37 °C and 200 rpm. At indicated intervals probes were taken and analysed (see Fig. 4).

Plaque assays

An Erlenmeyer flask with 20 mL LB was inoculated with *S. aureus* DSM 18421 (and with *E. coli* DSM 498, respectively) up to an OD_{600} of 0.2 and then cultured in an incubation shaker (37 °C, 200 rpm) until an OD_{600} of 0.8 was reached. Then 100 μL of this bacterial culture were mixed with 100 μL of 10^{-4} to 10^{-7} diluted phage solution and incubated for 20 min at 37 °C. After addition of 3 mL overlay-agar, the mixture was poured over LB agar and incubated overnight at 37 °C.

CFU assays

To calculate the MOI (Multiplicity Of Infection), overnight cultures from *S. aureus* DSM 18421 or *E. coli* DSM 498 were diluted up to 10^{-7} in LB broth. One hundred

microlitres of the dilutions 10^{-4}–10^{-8} were streaked onto LB agar and incubated overnight at 37 °C.

For determination of CFU during phage infection, 100 µL of the assay were removed at preset intervals and diluted to 10^{-7} in LB broth. One hundred microlitres of dilutions 10^{-4}–10^{-8} were streaked onto LB agar and incubated overnight at 37 °C.

Antimicrobial susceptibility testing by chip calorimetry

The following incubation procedures in chip calorimetry had been applied.

Test (A)

4.5 mL of Tryptic Soy Broth (TSB; with and without cefoxitin, 4 mg/L) and Mueller–Hinton Broth (MHB; with and without cefoxitin, 4 mg/L), respectively, were inoculated with each 0.5 mL from a fresh overnight culture of the tested staphylococcal strains, grown in TSB and MHB, respectively. The different medium variants of (A) were incubated for 2 h at 37 °C and 10 min before the end of incubation 1% of glucose was added to each test variant and incubated again for 10 min at 37 °C. Then 0.5 mL of each of these test variants were used for the chip calorimetric measurements.

Test (B)

Instead to use an overnight culture of the corresponding strain in TSB for inoculation of the test (B) variants, it was tried to perform this test with a small volume of a suspension of only *one fresh single colony* of the corresponding strains as inoculum. For this reason one fresh typical staphylococcal colony which was grown overnight on a blood agar plate, was picked up and suspended in 220 µL of TSB. Each 100 µL of this suspension was used as inoculum of 900 µL 1% glucose-supplemented TSB (with and without 4 mg/L cefoxitin, respectively). After incubation of these test variants at 37 °C for 90 min, 0.5 mL of each variant were used for the chip calorimetric measurements.

Test (C)

An Erlenmeyer flask containing 20 mL LB broth was inoculated with 20 µL of bacterial overnight culture of *S. aureus* DSM 18421 and *E. coli* DSM 498, respectively, and either 100 µL of phage mix (4×10^{10} PFU/mL; PFU, plaque forming units) or 100 µL PBS in case of a control experiment. The Erlenmeyer flask was incubated at 37 °C with 200 rpm. At indicated time points (see Figs. 4, 5), 0.5 mL probes were taken for measurement.

Calorimetry

Thermal heat measurement was performed in a prototype chip calorimeter developed by the group of Lerchner at the TU Bergakademie Freiberg, Germany. The calorimetric device was described in detail in a former publication [5]. The main component is a silicon chip with integrated thin film thermopiles which consists of 118 BiSb/Sb thermocouples (Fig. 1a). It converts the heat rate produced by bacteria inside the measurement chamber into a voltage signal. The measurement chamber is made from polymethylmethacrylate (PMMA). The inner size of the flow channel is 20.0 mm in length, 1.0 mm in width, and 1.2 mm in height resulting in an inner volume of 24 µL. The complete chip module is enclosed in a high precision thermostat. Prior to injection of the bacterial suspension the measurement system is thermally equilibrated in heat exchangers close to the chip module. The fluidic system used for sample dosage consists of a membrane pump (50 µL), a stepping motor driven piston pump (280 µL), several microvalves, and a sample loop with a volume of 60 µL.

Before the calorimetric measurements started, the fluidic system of the calorimeter had been chemically sterilised with a mixture of ethanol/water/sulphuric acid (1:0.41:0.01) for 30 min followed by cleaning with sterile distilled water and a final flushing with sterile saline (= 0.9% NaCl solution). At the beginning of a measurement cycle, the membrane pump was used to absorb the sample (bacterial suspension or saline), subsequently the sample loop was flushed until a defined bacterial concentration inside the system was achieved. The sample volume was 400 µL in all experiments. After flushing, the content of the sample loop was driven into the calorimeter using the piston pump, thereby replacing the saline. Usually, a volume flow rate of 50 µL/min was applied. The injection was stopped when the amount necessary for filling the measurement chamber with sample was reached. As soon as a steady-state output signal was achieved, the sample inside the chamber was removed by a second injection operation of the piston pump which replaced the sample with saline in order to prepare the system for the next measurement cycle. During the stop phase the membrane pump and the sample loop were flushed with saline to remove the remaining biomass. The time required for one measurement cycle was 15 min.

Figure 1b shows typical signals obtained from zero experiments and from the injection of a bacterial suspension. After relaxation of an endothermal injection effect caused by incomplete thermal equilibration of the fluid, an exothermal signal shift indicates a steady-state metabolic heat production by the bacteria. The baseline is restored after the second injection step due to removal of the bacteria from the measurement chamber. The signal shift can

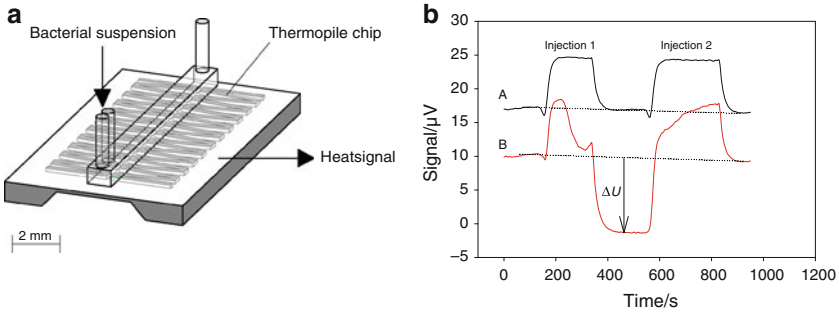

Fig. 1 Calorimetric signal generation. **a** Schema of the calorimetric chip module. **b** Typical shapes of signals from zero measurements and those with bacterial suspension. The signal shift ΔU is a measure of the metabolic heat rate

be measured with a resolution of about 100 nV according to 20 nW, which was determined by long-term measurements using saline (zero measurements).

Results

Chip-calorimetric measurements of antibiotic susceptibilities

Figure 2 demonstrates the different inhibitory effects of cefoxitin on the metabolic heat production of *S. aureus* NCTC 8325, *S. aureus* 96-01678, and *S. aureus* 07-00244 (strains 3–5), respectively. The consecutively measured heat signals of the samples incubated with and without cefoxitin, respectively, were compared and + characterised by differences between these three strains (Test A). The time period data on the abscissa were related to the start of incubation. The susceptibility of strain 3 towards cefoxitin was recognised already after less than total 2 h. Strain 4 was correctly indicated as resistant to cefoxitin, whereas strain 5 was slightly inhibited by the antibiotic which corresponds to the decreased cefoxitin susceptibility of this isolate (borderline resistance). Furthermore, it is noteworthy that the resistant strain 4 has an essentially more active metabolism. The results confirm the susceptibility (MICs) of strain 3 and the resistances of strains 4 and 5 to cefoxitin, see above).

A second measurement series was performed using Test B (based on use of only one single colony for the inoculum preparation). The results are similar to that of the first two series (Fig. 3). The strains 6–9 were known as cefoxitin-resistant isolates. It was confirmed by the calorimetric measurements which showed a loss in the metabolic activity by less than 10% after 3 h.

The inhibition of the strains *E. coli* DSM 498 and *S. aureus* DSM 18421, respectively, by the phage mixture DSM 18723/DSM 18722 is demonstrated in Fig. 4. No change in the metabolic activity in comparison to the control samples can be observed 170 min after start of incubation. However, 130 min later, it is clearly shown, that strain 1 is resistant to the phage mixture whereas strain 2 is

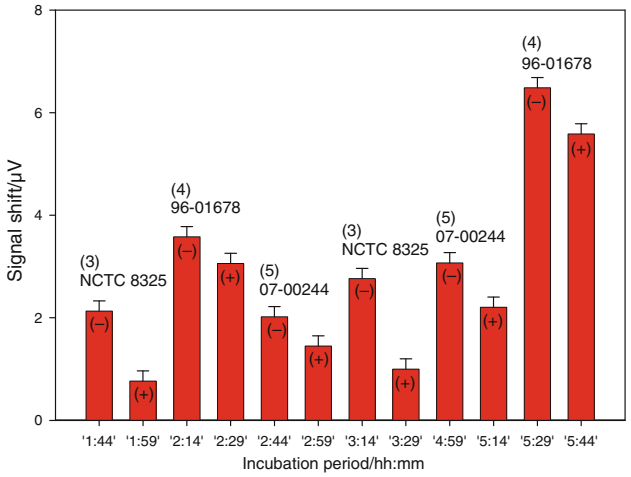

Fig. 2 Susceptibility of different *S. aureus* strains to cefoxitin grown in Tryptic Soy Broth (TSB). −, control (without cefoxitin); +, with cefoxitin; (3), *S. aureus* NCTC 8325 (cefoxitin-sensitive); (4), *S. aureus* 96-01678 (cefoxitin-resistant); (5), *S. aureus* 07-00244 (cefoxitin-borderline-resistant)

strongly inhibited by it. The measurements after 420 min depict the growth of the control samples.

Kinetics of phage infection

A phage-host system can be characterised by key figures for parameters such as the *latent period* which is the time until the first new phages are released, the *burst size* which determines the number of released phages, and the *burst time* which spans the time for a release of new phages. The pair of *S. aureus* DSM 18421 (strain 2) and mixture DSM 18723/DSM 18722 is characterised with a latent period of ~40 min, a burst time of ~20 min, and a burst size of ~45 phages/infected bacterium (data not shown). We tried to reproduce these parameters by analysing the progression of thermal output during phage infection with the chip calorimeter. *S. aureus* DSM 18421 was infected with bacteriophage mix DSM 18723/DSM 18722 at a MOI (number of phages per bacterial cell) of 1.4. Heat output increased until ~50 min, which approximately corresponds to the latent period as determined before (Fig. 5).

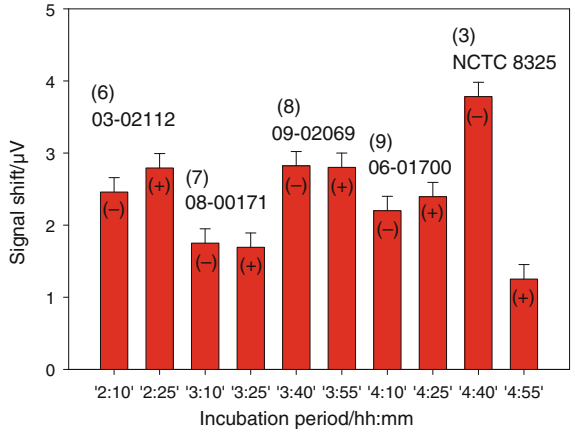

Fig. 3 Susceptibility of different *S. aureus* strains to cefoxitin grown in Tryptic Soy Broth (TSB). −, control (without cefoxitin); +, with cefoxitin; (3), *S. aureus* NCTC 8325 (cefoxitin-sensitive); (6), *S. aureus* 03-02112 (cefoxitin-resistant); (7), *S. aureus* 08-00171 (cefoxitin-resistant); (8), *S. aureus* 09-02069 (cefoxitin-resistant); (9), *S. aureus* 06-01700 (cefoxitin-resistant)

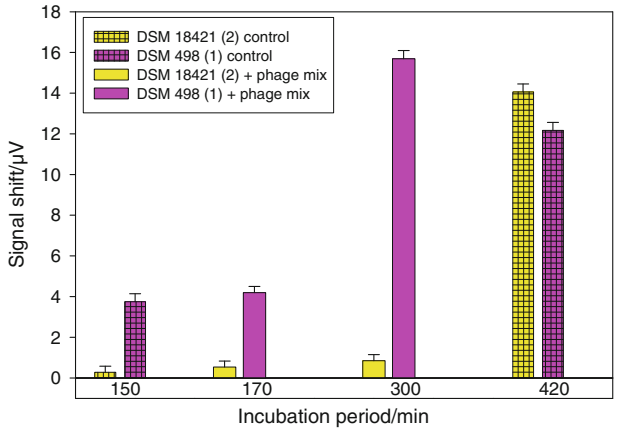

Fig. 4 Discrimination of the strains *S. aureus* DSM 18421 and *E. coli* DSM 498, which are sensitive and non-sensitive, respectively, to the bacteriophage mix DSM 18723/DSM 18722

Heat increased for 20 min, indicating a first burst, and then dropped to a plateau for 20 min before decreasing constantly. The plateau at 80 min can be explained by a second burst which we often observe at this point of time; however, due to the high MOI the peaks for bursts are not very distinct. After a second burst, the amount of produced phages exceeds remaining bacteria by a multiple so that the culture is rapidly degraded. Parallel analyses of OD_{600}, CFU and PFU support this interpretation. Due to the high inoculum density, OD_{600} rises only slowly until the second burst and then decreases. CFU and PFU were nearly constant until the beginning of the first burst and then CFU decreased drastically while PFU increased.

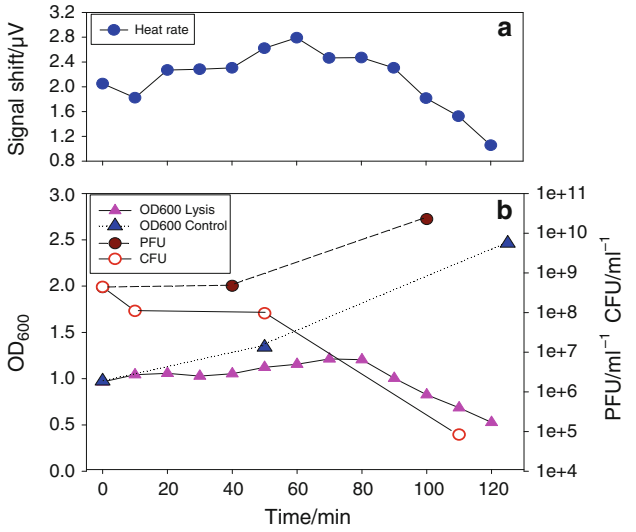

Fig. 5 Progression of the heat rate of *S. aureus* DSM 18421 during infection by the phage mix DSM 18723/DSM 18722 (**a**). For comparison, the optical density as well as PFU and CFU numbers is depicted (**b**)

Discussion

Our results confirm the high potential of calorimetry for the detection of susceptibility of bacteria to antibiotics and lytic bacteriophages. Because the heat rate is a direct measure of the metabolic activity, inhibition effects can be detected in real-time. Chip-calorimetry is an excellent precondition for kinetic investigations as demonstrated by the phage infection study. The response time (delay between sampling and signal provision) is only depending on the thermal time constants of the calorimeter. Furthermore, amplification processes such as PCR or enzyme catalysed reactions are not necessary. This essentially simplifies the assay, reduces the operation time, and hence it excludes the need of expensive consumables. In comparison to molecular methods, calorimetry is such as "classical" culture methods applicable to all non-fastidious bacteria.

The used chip calorimeter is characterised by low thermal inertia and small sample volume. Both leads to a response time of less than 5 min (in the TAM calorimeter, 1 h is required for thermal equilibration). In the present paper, the lower values for the response time (1–2 h) had been due to the lag phase after suspension of the corresponding bacterial colony. The evaluation of the efficacy of inhibiting agents on a minute-timescale requires a heat production rate of more than 4 nW in a bacterial sample of 1 µL. If the bacteria are in the logarithmic growth phase a specific heat production rate of about 2 pW per cell can be expected, i.e. an amount of 1.2×10^4 bacteria is necessary

inside the measurement chamber of the calorimeter which is one order less than for the TAM calorimeter. Related to the sample volume of 6 μL, a cell titre of 10^6 cfu/mL results. Practically, cell titres of about 10^7 CFU/mL should be used to achieve a clear discrimination of antibiotic-resistant and antibiotic-non-resistant strains. Additionally, we could show that it is possible with our chip calorimeter to differentiate between cefoxitin-resistant and cefoxitin-susceptible *S. aureus* strains already by use of only *one single colony* of *S. aureus* resulting from a fresh overnight culture on a blood agar plate. Bacteria in the lag or stationary growth phase can only be detected with considerably lower sensitivity. At present time, the system used represents the proof of the principle (prototype). With an improved thermal management of the calorimeter, the cycle time for serial measurements could be reduced to about 5 min. Additionally, a parallelisation by factor four seems to be possible which would enable a throughput of about 500 measurements per day.

Acknowledgements We thank Dr. Valentina Margania for invaluable help in sample processing. The development of the chip calorimeter was funded by German Research Council (Deutsche Forschungsgemeinschaft, Le1128/1-1, Ma3746/2-1) and German Federation of Industrial Research Association (AiF BMWi, AiF-Nr. 244 ZBG).

References

1. Baldoni D, Hermann H, Frei R, Trampuz A, Steinhuber A. Performance of microcalorimetry for early detection of methicillin resistance in clinical isolates of *Staphylococcus aureus*. J Clin Microbiol. 2009;47:774–6.
2. Johnson G, Millar MR, Matthews S, Skyrme M, Marsh P, Barringer E, O'Hare S, Wilks M. Evaluation of BaLite Rapid MRSA, a rapid culture based screening test for the detection of ciprofloxacin- and methicillin-resistant *S. aureus* (MRSA) from screening swabs. BMC Microbiol. 2006;6:83.
3. Mardh PA, Ripa T, Andersson KE, Wadsö I. Kinetics of the action of tetracyclines on *Escherichia coli* as studied by microcalorimetry. J Antimicrob Chemother. 1976;10:604–9.
4. von Ah U, Wirz D, Daniels AU. Rapid differentiation of methicillin-susceptible *Staphylococcus aureus* from methicillin-resistant *S. aureus* and MIC determinations by isothermal microcalorimetry. J Clin Microbiol. 2008;46:2083–7.
5. Lerchner J, Wolf A, Schneider H-J, Mertens F, Kessler E, Baier V, Funfak A, Nietzsch M, Kruegel M. Nano-calorimetry of small-sized biological samples. Thermochim Acta. 2008;477:48–53.
6. Lerchner J, Wolf A, Buchholz F, Mertens F, Neu TR, Harms H, Maskow T. Miniaturized calorimetry—a new method for real-time biofilm activity analysis. J Microbiol Methods. 2008;74:74–81.
7. Deutsches Institut für Normung DIN 58940. Medical microbiology—susceptibility testing of pathogens to antimicrobial agents. Part 8: microdilution. General method specific-requirements. In: DIN Deutsches Institut für Normung e.V., editor. DIN-Taschenbuch 222: Medizinische Mikrobiologie und Immunologie—Diagnostische Verfahren. Berlin: Beuth-Verlag; 2004. p. 342–53.