RAPID IDENTIFICATION OF BACTERIA BY BIOBARCODE ASSAY

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Abstract: Presence of bacteria with antibiotic resistance is becoming a very large problem throughout the world. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a dangerous pathogen resistant to β-lactam antibiotics with biofilm-formation ability. Because of an increasing resistance of bacterial species to ATBs, it is necessary to develop new methods for rapid identification of bacteria. Biobarcode assay provides a rapid detection of the antigen presence in the sample. Detection is based on the antibody-antigen interaction. The antibodies were bound to magnetic and non-magnetic particles. Next, immunoglobulin G (IgG) was bounded to magnetic particles. The non-magnetic particles were bound with anti-plasminogen antibody that is a specific antigen for MRSA as well as 20 bp oligonucleotides for detection. The first step involved determination of binding capacity of antibodies for different bacteria by ELISA. The IgG were able to bind $4.6 \cdot 10^4 \pm 8\%$ CFU · ml^{-1} of MRSA, *Escherichia coli* and *Proteus mirabilis* and the anti-plasminogen antibody (anti-Pls) was specific for MRSA only with the binding capacity of $5 \cdot 10^3$ CFU · ml^{-1}. After binding of antibodies to particles, the bacterial strain MRSA was captured by these antibody-modified particles and the detection oligonucleotide was released and determined by electrochemical method. The results suggest that the IgG is non-specific for MRSA while specificity of the anti-plasmin antibody for MRSA was confirmed. In this study, we developed a method for rapid detection of MRSA in the pooled sample.

Key Words: antibodies; detection; magnetic and non-magnetic particles; methicillin-resistant *Staphylococcus aureus*

INTRODUCTION

For identification, an assessment of bacterial resistance to antibiotics is inherent (Jia et al. 2014). Currently, the most commonly used microbiological methods are classical selective incubation with biochemical confirmation (Jia et al. 2014), polymerase chain reaction (PCR) using specific gene of bacteria (Garrido-Maestu et al. 2014), identification by mass spectrometric MALDI-TOF/TOF or immunochemical detection such as an ELISA (Kopcakova et al. 2014). Cultivation methods are time consuming and MALDI-TOF/TOF has the need of expensive instrumentation. For the identification of bacteria by PCR method, the DNA isolation is necessary as well as the knowledge of the specific DNA sequence for finding bacterium and specific primers must be designed. Moreover, the results may be negatively affected by low amounts of target DNA or by composition and type of the sample (Li et al. 2013). These methods are also very time-consuming since they take days and the assays are necessary to carry out in laboratory with special equipment. It is therefore necessary to focus on the development of modern, fast, reliable and inexpensive techniques for identifying bacteria. The biobarcode method is based on immunomagnetic separation of the analyte with the detection by short oligonucleotides (20 bp) (Mirkin 2005). The method can detect a wide range of substances such as proteins, nucleic acids, bacteria or viruses (Cho et al. 2014). The advantages of this method is the omission of the lengthy steps during the sample preparation for routine screening methods such as removal of the matrix from the sample and signal amplification (Duan a Zhou 2012). Other advantage of this method is the multiplex determination. For multiplex analysis, nanoparticles with different antibody labelled by oligonucleotides with different lengths are used. Therefore, this technique has

the potential for the development of ultrasensitive sensors with fast detection and it is possible to use outside of a specialized workplace (Xiang et al. 2011). For detecting microorganisms, biobarcode system is able to both capture and separate the target group in organism using affinity of the group to antibodies captured on magnetic particles (Anderson et al. 2013, Yoo et al. 2006). Determination is concluded using barcode label to genus, species and serotypes levels of bacteria (Anderson et al. 2013). The antibody selection plays a very important role in biobarcode assay. These substances must have a specific affinity to bacterial surface antigens (Liu et al. 2013). Barcode method can be used to determine all species of bacteria and viruses with known specific antigen for establishing species. It can be also be used for determination of molecules (peptides, proteins) or nucleotides (gene-specific) in the samples (Araz et al. 2013, Yin et al. 2011).

MATERIAL AND METHODS

Selection of magnetic particles (MPs) and their IgG modification

For magnetic separation of bacteria, the MPs Dynabeads® M-270 Streptavidin, (Invitrogen, Norway) were used. Modification of particles was performed according to manufacturer's instructions (Invitrogen, Norway).

Selection and preparation of non-magnetic particles

For specific capture of magnetic particle with bacteria complex gold nanoparticles (AuNPs) were selected. The gold nanoparticles were prepared from 0.0197 g $HAuCl_4 \cdot 3H_2O$ (Mr=393.84) was dissolved in 50 ml of water (1 mM). Into 10 ml of 1 mM $HAuCl_4 \cdot 3H_2O$ was added 0.25 ml of trisodium citrate (0.265 g $\cdot 10$ ml^{-1}). After an hour of stirring, the colour changed to purple.

Cultivation of methicillin-resistant *S. aureus* and *E. coli*

Methicillin-resistant *Staphylococcus aureus* (ST239) and *Escherichia coli* (NCTC 13216) and *Proteus mirabilis* (ATCC 29906) as a negative control were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University in Brno, Czech Republic. Cultivation media (LB = Luria Bertani) were inoculated with bacterial culture and were cultivated for 24 hours on a shaker at 130 rpm and 37°C. For cultivation of MRSA 3 $\mu g \cdot ml^{-1}$ of oxacillin was used. Bacterial culture was diluted using the cultivation medium to OD_{600} = 0.1 for the following experiments.

Optimization of detection limits by ELISA

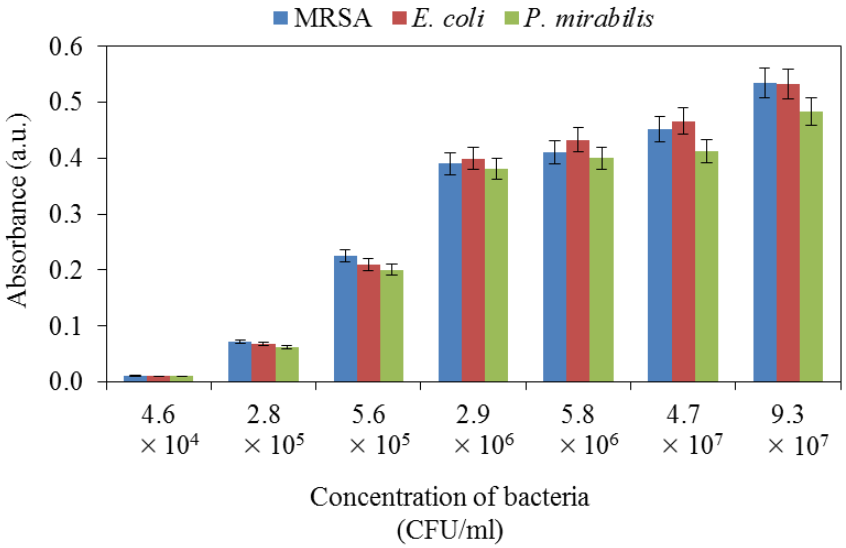
Dilution of the coating, primary and secondary antibodies for MRSA immunodetection was tested by ELISA. Microtitration plate was coated with 100 µl of polyclonal human immunoglubolin G (IgG) per well (SantaCruz Biotechnology, USA) and rabbit anti-plasminogen (anti-Pls) antibody (Baria, s.r.o., Czech republic) diluted 1:5000 in 0.05 M carbonate buffer (0.032 M Na_2CO_3 and 0.068 M $NaHCO_3$, pH 9.6) at 4°C for 16 hours. After coating, free surface of the wells was blocked with 150 µl of 1% BSA (*w*/*v*) in PBS per well (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 4.3 mM Na_2HPO_4, pH 7.4) for 30 min at 37°C, then the wells were washed 5 × with 350 µl of 0.05% (*v*/*v*) PBS-T (Hydroflex, TECAN, USA). Then, 100 µl of the sample of MRSA, *E. coli* or *P. mirabilis* were added and the microplate was incubated at 37°C for 1 hour. After washing with PBS-T, 100 µl of polyclonal human IgG and goat Pls antibody (SantaCruz Biotechnology, USA) in dilution 1:5000 or 1:10000 in PBS was added and the plate was incubated for 60 min at 37°C. After washing with PBS-T, 100 µl of different concentrations of bacteria were added and after washing with PBS-T, 100 µl of chicken anti-mouse-HRP (horseradish peroxidase) conjugate (SantaCruz Biotechnology, USA) in dilution of 1:1500 or 1:2000 was added and the plate was incubated for 60 min at 37°C. After incubation and washing 100 µl of 0.001% (*w*/*v*) TMB in 0.2 M sodium acetate adjusted to pH 5.8 with citric acid with 0.037% (v/v) of H_2O_2 was added. After 30 min, the reaction was stopped with 50 µl of H_2SO_4 and after additional 5 min the absorbance was read at 450 nm (Infinite M200 Pro, TECAN, USA).

RESULTS AND DISCUSSION

Optimization of detection limit of IgG

The binding capacity and specifity of antibody IgG for detection of MRSA by biobarcode assay was determined using the ELISA method. For this determination 500 ng of IgG or anti-Pls was used. In the Figure 1 we can see an increasing absorbance of HRP, which corresponds to the concentration of bacteria. Limit of bacteria detection was determined as $4.6 \cdot 10^4$ CFU $.ml^{-1}$. It was demonstrated that the IgG is specific for the tested bacteria. For our experiment, IgG can be used to bind bacteria in the first non-selective step.

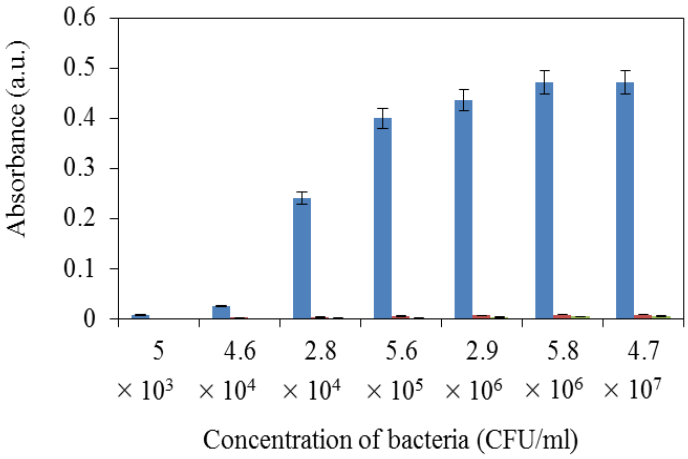
Figure 1 Determination of binding capacity of antibody IgG in dependence on the bacteria concentration



Optimization of detection limit of anti-Pls

The binding capacity and specifity of antibody IgG for detection of MRSA by biobarcode assay was determined using ELISA. For this determination 500 ng of IgG or anti-Pls was used. In the Figure 2 we can see increasing absorbance of HRP, which corresponds to the concentration of bacteria. It was confirmed that the anti-Pls is selective for MRSA. The anti-Pls limit of detection of MRSA was determined as $5 \cdot 10^3$ CFU $\cdot$ ml^{-1}.

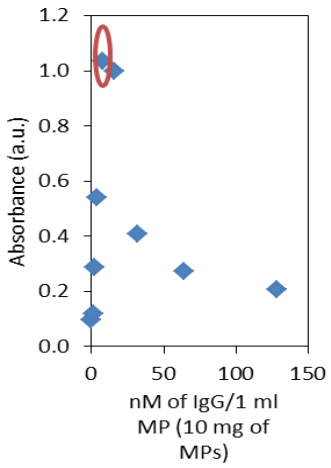
Figure 2 Determination of binding capacity of anti-Pls antibody in dependence of bacteria concentration



Optimization of binding capacity of MPs

The binding capacity of MPs was determined. For this measurement, 9 different concentrations were used in the range from 0 to 128 ng of IgG in 1 ml of MPs (10 mg of MPs in 1 ml of PBS). (The absorbance of IgG after binding was measured by the absorbance of bound antibody to MPs. From the results it can be determined that the optimal concentration of IgG for binding to the commercial MPs is about 10 ng of antibody per 1 ml of the magnetic particles at a concentration of 10 mg · ml^{-1} (Figure 3). This concentration is used in other assays of this experiment.

Figure 3 Optimization of binding capacity of MPs for IgG



CONCLUSION

In conclusion, the detection limit of IgG for determination of presence of different bacterial strains was determined. For MRSA, *E. coli* and *P. mirabilis* the values of HRP absorbance intensity were comparable. The limit of detection was determined as 4.6 ·10^6 CFU · ml^{-1} of used bacterial strains. These data suggest that IgG is not selective for any group of selected bacteria. Next measurement determined the limit of detection for anti-Pls, which was measured as 5 ·10^3 CFU · ml^{-1} but for MRSA only. In this assay, we found that anti-Pls is selective for MRSA and thus can be used as a specific antibody for the determination of MRSA in a sample. This antibody is bound to a non-magnetic particle when the positive reaction is detected by denatured oligonucleotide. The binding capacity of Dynabeads® M-270 Streptavidin was determined for IgG as 10 nM of IgG per 10 mg of magnetic particles.

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