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Development of a magnetic separation method to capture sepsis associated bacteria in blood

Ana Luisa Kalb Lopes [a,*], Josiane Cardoso [a], Fernanda Roberta Correa Cleto dos Santos [a], Ana Claudia Graziani Silva [a], Maria Isabel Stets [a], Nilson Ivo Tonin Zanchin [b], Maurilio José Soares [c], Marco Aurélio Krieger [a]

[a] Instituto de Biologia Molecular do Paraná, Department of Research and Development, Prof. Algacyr Munhoz Mader Street 3775, 81350-010 Curitiba, PR, Brazil
[b] Laboratory of Proteomic and Protein Engineering, Carlos Chagas Institute, Fiocruz, Prof. Algacyr Munhoz Mader Street 3775, 81350-010 Curitiba, PR, Brazil
[c] Laboratory of Cell Biology, Carlos Chagas Institute, Fiocruz, Prof. Algacyr Munhoz Mader Street 3775, 81350-010 Curitiba, PR, Brazil

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ABSTRACT

Bloodstream infections are important public health problems, associated with high mortality due to the inability to detect the pathogen quickly in the early stages of infection. Such inability has led to a growing interest in the development of a rapid, sensitive, and specific assay to detect these pathogens. In an effort to improve diagnostic efficiency, we present here a magnetic separation method for bacteria that is based on mutated lysozyme (LysE35A) to capture *S. aureus* from whole blood. LysE35A-coated beads were able to bind different MSSA and MRSA isolates in the blood and also other six Gram-positive and two Gram-negative species in whole blood. This system was capable to bind bacteria at low concentrations (10 CFU/ml) in spiked blood. Samples captured with the mutated lysozyme showed more responsive amplification of the 16S gene than whole blood at concentrations of 10^3–10^5 CFU. These data demonstrate detection of *S. aureus* directly in blood samples, without in vitro cultivation. Our results show that capture with LysE35A-coated beads can be useful to develop a point of care diagnostic system for rapid and sensitive detection of pathogens in clinical settings.

1. Introduction

Bacterial pathogens have a significant impact on human health, especially those causing bloodstream infection, a relevant cause of sepsis. This condition usually requires at least one day or more for precise diagnosis, increasing the chances of patient mortality (Liesenfeld et al., 2014). The extremely high mortality by blood infections is due, in part, to the inability to rapidly detect, identify and thus treat patients with appropriate antibiotics in the early stages of infection (Carrigan et al., 2004). Consequently, considerable effort has been devoted to the development of rapid, sensitive, and specific assays to detect these pathogens.

Amplification-based molecular diagnosis methods such as PCR can reduce the assay time to hours. However, this methodology is often not sensitive enough to detect low concentrations of bacteria, thus needing additional steps such as initial enrichment (D.K. Kang et al., 2014), as well as dilution, inactivation or removal of inhibitors from the sample (Fredricks and Relman, 1998; Yamamoto, 2002). Magnetic separation is an alternative for the isolation of target cells directly from samples, by eliminating the components that interfere with the PCR and related techniques (Olsvik et al., 1994; Šafařík and Šafaříková, 1999). Magnetic separation has several applications for the detection of pathogenic microorganisms, especially in food (Tomoyasu, 1992; Opsteegh et al., 2010), clinical (Kassimi et al., 2002; Nam et al., 2013), veterinary (Coklin et al., 2011; Isaksson et al., 2014) and environmental microbiology (Yakub and Stadterman-Knauer, 2004; Sierra et al., 2014). This methodology uses small magnetic particles coated with antibodies, peptides or oligonucleotides to bind cell surfaces and has been shown to be efficient for the isolation of different organisms (Šafařík and Šafaříková, 1999).

Magnetic separation of bacteria using mutated lysozyme coupled to magnetic beads allows specific capture from cell suspension, by recognition of bacterial cell wall peptidoglycans by the lysozyme-beads complex (Diler et al., 2011). Lysozyme catalyses the breakdown of peptidoglycans of bacterial cell wall, lysing sensitive bacteria (Benkerroum, 2008). Two amino acids are essential for this lytic activity: glutamate at position 35 (E35) and aspartate at position 52 (D52) (Malcolm et al., 1989). Mutation of glutamate-35 to its corresponding amide alanine (LysE35A) completely abolished bacteriolytic activity, whereas the affinity for target structures is maintained (Diler et al., 2011).

Here we used *Staphylococcus aureus* as a model pathogen to demonstrate the feasibility of magnetic separation for bacteria in whole blood.

* Corresponding author at: Instituto de Biologia Molecular do Paraná, Department of Research and Development, 3375 Professor Algacyr Munhoz Mader Street, Curitiba, Paraná, Brazil.
E-mail addresses: analuisa_kalb@hotmail.com, alkalb@tecpar.br (A.L.K. Lopes).

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This Gram-positive bacterium is a common causative pathogen of bloodstream infections and is associated with high morbidity and mortality (del Rio et al., 2009; Corey, 2009). The occurrence of drug-resistant and/or highly virulent strains reinforces their public health threat. Methicillin-resistant *Staphylococcus aureus* (MRSA) is resistant to all existing penicillin and lactam-based antimicrobial drugs and has become one of the most prevalent antibiotic-resistant pathogens in hospitals (Pray, 2008), which emphasizes the importance of rapid initiation of appropriate treatment. Thus, we present here a magnetic separation method for bacteria that uses LysE35A to capture *S. aureus* from whole blood that can be used as an enrichment step for PCR. The mutated lysozyme was expressed in *Pichia pastoris* and immobilized in magnetic beads after purification by cation-exchange chromatography. Our data demonstrate detection of *S. aureus* directly in blood samples, without in vitro cultivation.

2. Material and methods

2.1. Microorganisms

The methylotrophic *Pichia pastoris* strain GS115 (his4, mut+) and the control strain for secreted expression GS115 albumin (HIS4, mur^S) were used for protein expression. The DH5α strain of *Escherichia coli* was used for plasmid selection/propagation. Both strains were purchased from Invitrogen (Karlsruhe, Germany). pPicZα vector (Invitrogen, Carlsbad, CA, USA) was used for secreted expression.

Pichia pastoris was cryopreserved at −80 °C in 15% (v/v) glycerol and cultivated on YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar]. For protein expression we used medium BMGY [1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) YNB (Yeast Nitrogen Base, Invitrogen, USA), 4×10^{-5}% (w/v) biotin and 1% (v/v) glycerol]. Alternatively, BMMY medium was used (the same as BMGY, except that glycerol was replaced by 1% methanol).

Staphylococcus aureus, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, *Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella pneumoniae* were isolated from various clinical samples (such as blood, cerebrospinal fluid, urine and pus) collected in Hospital de Clínicas-UFPR, Curitiba, Brazil. Six *S. aureus* clinical isolates were obtained from blood cultures, being 3 susceptible (MSSA) and 3 resistant (MRSA) to methicillin. *S. aureus* ATCC 29213 was used as a reference strain in all capture studies.

2.2. Plasmid construction

A synthetic lysozyme gene containing a mutation at position 35 (LysE35A) was synthesized using the services of a commercial provider (GenScript, Piscataway, NJ, USA). This gene and the pPicZα vector were digested by *Xho*I and *Not*I and then ligated by T4 DNA ligase. The resulting plasmid was transformed into DH5α cells by heat shock. After confirmation by sequencing (Macrogen Inc. Seoul, Korea), the pPicZα vector containing LysE35A was transformed to GS115 yeast cells by the lithium chloride method (Mount et al., 1996). For PCR control of gene insertion into the AOX1 locus of the host genome, colonies were picked from YPD agar plates and suspended in 10 μl H_2O. After addition of 25 U lyticase (Sigma-Aldrich, St. Louis, MO, USA), the suspension was incubated for 10 min at 30 °C, 10 min at −80 °C and then used in PCR.

2.3. Protein expression, purification and analysis

Expression of LysE35A was obtained based on the procedure outlined in Invitrogen's EasySelect *Pichia* Expression Kit manual, using the Mut^S strain. A single colony of transformed yeast GS115 cells was inoculated into 40 ml of BMGY medium and incubated at 28–30 °C in a shaking incubator (250 rpm) until the culture reached OD600 ranging from 2 to 6. The cells were harvested by centrifugation and the pellet was resuspended in four liters of BMGY medium, with incubation at 28–30 °C (shaking at 250–300 rpm) until the culture reached OD600 ranging from 2 to 6. Protein expression was induced by resuspending the cells in one liter of BMMY medium, followed by incubation at 28–30 °C for 120 h in a shaking incubator (250 rpm). To induce and maintain the recombinant protein expression, methanol was added daily to a final concentration of 1%. One sample per day was collected to analyze expression levels by Silver-stained SDS-PAGE and immunoblotting.

The recombinant protein was concentrated with 2.6 M ammonium sulfate and the precipitate was dissolved in Tris-HCl buffer pH 8/50 mM NaCl/7 mM β-mercaptoethanol. Purification of LysE35A was performed by ion exchange chromatography in AKTA system (GE Healthcare Life Sciences). The resin used was SP Sepharose and the bound protein was eluted with a gradient of 0–1 M NaCl in Tris-HCl buffer pH 8.

Analysis of LysE35A expression levels was performed with 17% polyacrylamide-containing SDS gels. For immunoblotting analysis, protein was separated by SDS-PAGE using 17% polyacrylamide gels and the protein bands transferred onto a nitrocellulose membrane (HybondC, Amersham Biosciences, England) according to standard protocols (Sambrook et al., 1989). Nonspecific binding sites were blocked by incubating the membrane for 1 h with 5% nonfat milk and 0.05% Tween-20 in PBS, pH 8.0. The membrane was then incubated for 1 h with polyclonal commercial hen egg white lysozyme (HEWL) antibody (Thermo Scientific, Rockford, IL, USA) (1:10,000 dilution), washed three times with 0.05% Tween-20 in PBS and then incubated for 45 min with anti-rabbit horseradish peroxidase-conjugated IgG (Amersham Biosciences, England). Bound antibodies were detected with the ECL Western blotting analysis system (Amersham Biosciences, England), according to the manufacturer's instructions.

The activity of the recombinant proteins was determined by lysoplate assay, as previously described (Maeda et al., 1980). The method was carried out with 250 μg of commercial (positive control) and mutated lysozyme. The plates containing *Micrococcus luteus* and lysozyme were incubated and examined for clear zones surrounding the disc.

2.4. Cell capture

Purified recombinant lysozyme molecules were coupled to Dynabeads M-280 Tosylactivated (Invitrogen) according to the manufacturer's instructions. EDTA treated blood from human volunteers was obtained from the Biobanco of Hospital de Clínicas-UFPR after approval by the Ethics Committee (Protocol number #42012).

Fifty microliter of coated or uncoated beads (negative control) were added to 1 ml of a desired concentration (CFU/ml) of bacteria in PBS. Alternatively, whole blood was used. In this case, prior to capture the red cells were removed from the whole blood. Two lysing buffers were tested: Triton/Tris-EDTA (TTE: 20 mM Tris-HCl, pH 8.3, 1 mM EDTA, 1% Triton X-100) and ammonium chloride (NH_4CL:155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.3). The whole blood was diluted at 1:5 in TTE buffer or 1:1 in NH_4Cl buffer. Each tube was vortexed immediately after adding the lysing solution and incubated at room temperature for 10 min.

PBS and blood samples were incubated at room temperature for 90 min under stirring. Thereafter, they were placed in a magnetic separator rack and resuspended in 100 μl PBS, plated on PCA medium and incubated for 24 h. Colony counts (CFU per milliliter) were determined for each bacterium after magnetic separation and the capture efficiency was calculated. For qPCR detection, samples were placed in a magnetic separator rack and resuspended in 1 ml PBS, from which 100 μl was plated on PCA medium for quality control and the remaining was used for DNA extraction and PCR. All experiments were performed in triplicate.