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An immunochemical strategy based on peptidoglycan synthetic peptide epitopes to diagnose *Staphylococcus aureus* infections

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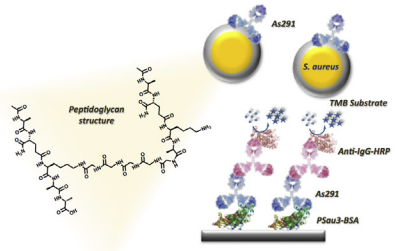
HIGHLIGHTS

- Antibodies towards a specific *S. aureus* peptidogycan epitope have been developed.
- The developed ELISA is able to detect *S. aureus* down to 10^4 CFU mL^{-1} in 2.5 h.
- Deglycosylation of the peptidoglycan increases the limit of detection of the assay.
- The assay is able to detect specifically *S. aureus* in respiratory samples.

GRAPHICAL ABSTRACT



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ABSTRACT

The characteristic pentaglycyl cross-bridge of the *Staphylococcus aureus* peptidoglycan (PG) cell wall component is an attractive epitope to raise specific antibodies against this microorganism. Based on this approach, we report here for the first time a competitive ELISA able to detect *S. aureus* down to 10^4 CFU mL^{-1}, without pre-enrichment on cell culture. The antibodies were raised against peptide-protein bioconjugates prepared by covalently coupling peptide haptens (PSau6 and PSau8) designed and synthesized taking into consideration the complex tridimensional structure in the PG polymer. Deglycosylation of the PG under acidic conditions has found to increase assay detectability. Assay performance has been evaluated in clinical samples such as bronchoalveolar lavage (BAL) and bronchoalveolar endotracheal aspirates (BAS) showing promising results for further implementation of this immunoassay as a daily routine diagnostic tool. Cross-reactivity studies have demonstrated that the immunoassay is specific for *S. aureus*.

1. Introduction

Staphylococcus aureus is commonly found on the skin and in the nose of about 30% of the individuals without causing any harm in most of the cases. However, eventually *S. aureus* may cause

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infections leading to pneumonia (patients underlying other lung diseases), endocarditis (infection of the heart valves), osteomyelitis or even sepsis. At the health care settings, the risk for a more serious infection is higher because patients often have a weakened immune system, have undergone procedures involving surgery or use of intravenous catheters. *S. aureus* is the second most frequently isolated microorganism, after *Escherichia coli*, in health care associated infections (HAI) in acute care hospitals in the EU. Moreover, there is the risk of antibiotic resistance strains, being the methicillin-resistant *S. aureus* (MRSA), one of the most important global concerns regarding these infections [1–5].

Fast and accurate diagnostic, identifying the pathogen causing the infection, is crucial for an appropriate treatment of the patient and its survival, particularly when the infection passes at the blood stream in which case, the fatally rate increases significantly. The actual methods used in microbiological laboratories are still mainly based on culture plates [4]. Unfortunately, these methods require enrichment steps on specific media to reach the necessary detectability. This causes an unaffordable delay until the time when the results are provided to the doctor in order to start treatment. Many efforts are being invested to develop more efficient techniques to diagnose infections, including PCR approaches or serological methods. Quantitative real-time PCR (qPCR) reaches very good concordances with the culture (100% sensitivity and 92–99% selectivity) significantly decreasing the analysis time [6,7]; however, requires expensive equipment, highly trained personnel and extensive validation for clinical interpretation of the results on routine clinical analyses. Serological assays have been used to diagnose endocarditis [8,9], to distinguish between deep or superficial infections [10], or to identify the organ infected, by analyzing the IgG profile against several *S. aureus* antigens [11]; but, the specificity of these assays in respect to other microorganisms is low [12], and do not provide sufficient detectability to diagnose the disease at early stages. However, these serological studies have consistently pointed at the high antigenicity of wall components such as the peptidoglycan (PG) and the teichoic acid (TA) [13–15]. At the light of these evidences, several authors have attempted to set-up immunochemical assays by raising antibodies against UV inactivated bacteria [16,17] or peptidoglycan fragments [18–21].

The PG (also known as murein sacculus) is a complex non crystalline macromolecule whose three-dimensional structure is difficult to determine, although worth NMR [22], HPLC-MS [23,24], biochemical and electron microscopy experiments [25] have provided interesting insights about this heterogeneous biomolecule [26,27]. In general, PG consists of repeating disaccharides units, N-acetylglucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) cross-linked by peptide chains alternating D- and L-aminoacids. Regarding glycan strands, *S. aureus* has a short average chain length of about 18 disaccharides units (compared with some other species that can have a chain length up to 250 disaccharides [28] and the peptide units are highly cross-linked (up to 70% [22,29]). *S. aureus* PG presents a pentapeptide stem [Ala-DGlu(&1)-NH_2][(&1)-Lys-DAla-DAla] cross linked by a pentaglycyl bridge $(Gly)_5$ that links the ε-NH_2 group of the LLys on the third position of one stem to the carbonyl group of DAla on the fourth position of another stem peptide (see P*Sau*5 and P*Sau*7 in Fig. 1). This pentaglycyl bridge is quite unique for *S. aureus*, although certain strains, such as for example the wild type MRSA present *fem* factor mutations that lead to a certain proportion of co-existing triglycyl (FemB) and monoglycyl (FemA) [29–31] bridges. Other gram-positive bacteria contain dipeptides, a single aminoacid residue, or even no bridge [32].

Some authors in the past have addressed production of antibodies against *S. aureus* particular PG peptide sequences such as the Lys-DAla-DAla epitope [18] but this sequence is also present in other bacteria such as *Enterococcus lactobacillus* or *Streptococcus pneumonie*. In contrast, the $(Gly)_5$ bridge constitutes a very specific epitope of *S. aureus*. In fact, it has been reported the presence of anti$(Gly)_5$ antibodies in the serum of patients infected with *S. aureus* [33], indicating that it could be a good antigenic moiety. Previous attempts to produce antibodies against this particular specific moiety did not succeed on subsequently establishing improved reliable immunochemical diagnostic methods. Our hypothesis lies on the lack of an appropriate hapten design for this purpose. Thus, Wergeland et al. [19] raised monoclonal antibodies just using the oligopeptide $(Gly)_5$ in its free form, but because of its low immunogenicity, the antibodies produced were IgM which are much less robust and stable than IgG. Seidl and Schleifer [34] coupled the pentaglycan peptide to human serum albumin (HSA) to enhance the immune response, but the peptide sequence was still too small. More recently, Sandhu et al. [21] reported the preparation of antibodies against a semi-synthetic peptidoglycan precursor containing the stem peptide and the pentaglycine chain, but also the N-acetylmuramic acid, which is present in most of the PGs from either gram positive and gram negative bacteria. Although the authors show recognition of the *S. aureus* cell wall preparations do not go further into the potential use of these antibodies as diagnostic tools. As one step forward we report here the development of specific antibodies for *S. aureus*, using synthetic peptide haptens mimicking the PG peptide monomers containing the characteristic $(Gly)_5$ bridge avoiding the sugar moieties present in other bacteria. Based on the recognition of such epitope, we report for the first time a competitive microplate-based ELISA able to detect specifically *S. aureus* in clinical samples.

2. Materials and methods

2.1. Buffers

PBS is 10 mM phosphate buffer on a 140 mM NaCl solution, and the pH is 7.5. PBST is PBS with 0.05% Tween 20. PBT is PBST without NaCl. PBST-D is a blank aqueous phase extraction solution resulting from the chemical deglycosylation treatment, performed as described below, diluted 1/3.6 with PBT. Coating buffer is 50 mM carbonate-bicarbonate buffer pH 9.6. Citrate buffer is a 40 mM solution of sodium citrate pH 5.5. The substrate solution contains 0.01% TMB (3,3′,5,5′-tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer. Borate buffer is 0.2 M boric acid/sodium borate pH 8.7.

2.2. Reagents and immunoreagents

The peptides P*Sau*3, P*Sau*4, P*Sau*5, P*Sau*6, P*Sau*7 and P*Sau*8 were synthesized according to the scheme shown in Fig. S1 and as described in the Experimental Section of the Supplemental Material (SM) document. The peptidoglycan from *S. aureus* (77140) and the anti-rabbit IgG-Peroxidase (A8275) were purchased form Sigma–Aldrich (San Luis, Estados Unidos). *S. aureus* CECT 5190, *E. coli* K12 CECT 433 and *Pseudomonas aeruginosa* CECT 110 were supplied by the Protein Production Platform (PPP, Applied Microbiology group, Autonomous University of Barcelona, Spain) previously inactivated by heat at 100 °C. The samples were suspended in PBS and stored at −40 °C.

2.3. Synthesis of the bioconjugates

Peptide-protein bioconjugates of haptens with a Cys residue were synthesized using N-succinimidyl- 3-maleimidyl propanoate (N-SMP) or N-succinimidyl iodoacetate (N-SIA), as heterobifunctional cross-linkers, to obtain the corresponding peptide-