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The differential detection of methicillin-resistant, methicillin-susceptible and borderline oxacillin-resistant *Staphylococcus aureus* by surface plasmon resonance

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ABSTRACT

Two hundred fifty *Staphylococcus aureus* clinical isolates were studied to determine their susceptibilities to β-lactam antibiotics. Among these isolates, 16 were methicillin-sensitive *S. aureus* (MSSA), 207 were methicillin-resistant *S. aureus* (MRSA) and 27 were borderline oxacillin-resistant *S. aureus* (BORSA). Currently, the reported mechanism of methicillin resistance in *S. aureus* is the production of a distinctive penicillin binding protein 2a (PBP2a), which exhibits low affinity toward β-lactams. A surface plasmon resonance biosensor was evaluated for its ability to identify MRSA and to distinguish these strains from MSSA and BORSA, by specifically detecting PBP2a. We found that the system permits label-free, real-time, specific detection of pathogens for concentrations as low as 10 colony forming units/milliliter (CFU/ml), in less than 20 min. This system promises to become a diagnostic tool for bacteria that cause major public concern in clinical settings.

1. Introduction

Nosocomial infections are the primary cause of mortality for hospitalized patients (Burke, 2003). Methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading cause of nosocomial and community-acquired infections worldwide with MRSA accounting for up to 40% of all *S. aureus* isolates (Panlilio et al., 1992; Louie et al., 2000). It has been estimated that nearly 50% of adults are carriers of *S. aureus* (Wertheim et al., 2005). Thus, *S. aureus* ranks amongst the primary causes of skin (Stefani et al., 2012; Tong et al., 2012; Krishna and Miller, 2012) and bloodstream bacterial infections (Biedenbach et al., 2004; Adam et al., 2011), as well as nosocomial pneumonia (Hoban et al., 2003; Ramirez et al., 2012; Parker and Prince, 2012). The development of resistance to methicillin and vancomycin has been a cause of concern among the medical community. MRSA infections have been associated with accelerated deterioration of pulmonary function, leading to increased hospitalization, and increased antibiotic usage and mortality (Leahy et al., 2011). The problem resides in the fact that therapeutic options to cure MRSA infections are scarce and that MRSA has the ability to acquire resistance to novel antibiotics.

Methicillin is a β-lactam, a member of a broad class of antibiotics characterized by the presence of a β-lactam ring in their chemical structures, which disrupts bacterial cell wall synthesis. They are the most prescribed antibiotics due to their specificity and non-toxicity to host cells. MRSA has acquired resistance to such antibiotics due to the production of altered penicillin binding proteins (PBPs) (Liu et al., 1990) that have low affinity for β-lactams, and are encoded by the chromosomal *mecA* gene (Leahy et al., 2011).

Substantial efforts have been directed at properly distinguishing this resistance pattern from those of susceptible strains. Current guidelines call for antibiotic susceptibility testing of *S. aureus* by agar dilution, disk diffusion tests, latex agglutination tests, and molecular methods, such as PCR and radio-labeled DNA probes (Louie et al., 2000; Hackbarth and Chambers, 1989). MRSA is

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defined as having an oxacillin minimum inhibitory concentration (MIC)≥4 μg/ml (CLSI, 2013). However, heterogeneous expression of oxacillin resistance in multiple strains of *S. aureus* (Louie et al., 2000; Chambers, 1997) has complicated the identification of MRSA via conventional microbiological procedures. Strains with extremely low-level methicillin resistance are susceptible to most of the non-β-lactam antibiotics (Liu et al., 1990), and routine tests may lead to false positive results. For instance, borderline oxacillin-resistant *S. aureus* (BORSA) strains exhibit oxacillin MICs at, or just above, the susceptibility breakpoint (≥2 μg/ml) (Chambers, 1997), but lack the *mecA* gene. Optimal conditions for the identification of BORSA and their differentiation from MRSA have not been determined (Hiramatsu et al., 1992). Moreover, a novel gene coding for methicillin resistance (*mecC*) has recently been identified in *S. aureus* clinical isolates from humans and animals. These strains, which have a variable resistance profile to oxacillin, cannot be detected by standard molecular techniques, such as PCR and slide agglutination tests, and are thus falsely identified as BORSA (Garcia-Alvarez et al., 2011).

Rapid assays for detecting methicillin resistance in Staphylococci, such as BD geneOhm MRSA (Kelley et al., 2009), Xpert MRSA (Kelley et al., 2009), BBL Crystal MRSA ID (Knapp et al., 1994), Velogene Rapid MRSA Identification (Bekkaoui et al., 1999) and MRSA-Screen (Cavassini et al., 1999), are commercially available. However, these methods lack accuracy for MRSA identification and differentiation from BORSA. For these methods, heavy inoculums are still needed, false negative results can still occur (Louie et al., 2000), and they are inappropriate for *mecC* gene detection. Other DNA-based genotyping techniques, such as polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), are time-consuming (6 h to 3 days) and require highly trained personnel. A bioluminescent assay was recently developed, using biotinylated firefly luciferase to detect PBP2a (Shiga et al., 2013). However, the assay suffered from requiring an extraction step, as well as having a low sensitivity (0.5×10^6 CFU/ml). Chen et al. recently developed an integrated microfluidic system that could effectively differentiate community-acquired MRSA (CA-MRSA) from hospital-acquired MRSA (HA-MRSA) in less than 40 min (Chen et al., 2013). However, this system requires PCR amplification and a labeling step. Similarly, another study demonstrated the possibility of detecting the *mecA* gene by EIS, after PCR has been carried out on genomic DNA from bacterial isolates (Corrigan et al., 2012).

Thus, it is our aim to respond to the pressing need for the development of a rapid and sensitive biosensing method to specifically detect and differentiate MRSA from both BORSA and methicillin susceptible *S. aureus* (MSSA), allowing for a better management of the bacterial infection. Furthermore, we aim to reduce the time of diagnosis, which is crucial for effective treatment and the prevention of disease spread (Chen et al., 2013), and will result in reduced morbidity and mortality rates (Lindsey et al., 2008). Here, we studied two hundred fifty *S. aureus* clinical isolates to determine their susceptibilities to β-lactam antibiotics. A surface plasmon resonance (SPR) biosensor was used to differentiate among CA-MRSA, HA-MRSA, BORSA and MSSA strains by specifically detecting PBP2a on whole bacterial cells, without labeling, without recourse to PCR or enrichment steps.

2. Materials and methods

2.1. Bacterial cultures

Bacteria were either isolated by Biophage Pharma (Saa1 to Saa 29) or provided by the Laboratoire National de Santé Publique du Québec (LSPQ) (Saa 30 to Saa 250). At LSPQ, the presence of *nuc* gene to confirm *S. aureus* was detected using PCR with the following primers: SN1 5′-CGAAAGGGCAATACGCAAAG-3′ and SN2 5′-ATCAGCGTTGTCTTCGCTCC-3′. The presence of *mecA* genes was detected using PCR as previously described. Susceptibility confirmation to oxacillin was done at LSPQ according to the CLSI standards (CLSI, 2013). The presence of PVL genes, lukS-PV and lukF-PV, were assessed by PCR amplification as previously described (Lina et al., 1999). Molecular typing was done by spa typing (Harmsen et al., 2003) and epidemic type determination was determined according to the guideline for Canadian epidemic type (Golding et al., 2008). MRSA, BORSA and MSSA bacteria were grown in 4 mL of Luria-Bertani (LB) medium in an incubator-shaker, for 3 h at 37 °C. The bacteria were then centrifuged at 2500*g* (Sorvall RT7, 3500 rpm) for 20 min. The supernatant was discarded and the bacteria were resuspended in phosphate-buffered saline (PBS). This was repeated twice. The concentration of bacteria was determined by plate count technique, and expressed as colony forming units per milliliter (CFU/ml).

2.2. Chemicals

L-cysteine, 1-(3-imethylaminopropyl) ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 11-mercaptoundecanoic acid (MUA), bovine serum albumin (BSA), sodium chloride, magnesium sulfate, and gelatin were purchased from Sigma-Aldrich. LB medium was purchased from Quelabs (Montréal, Québec, Canada); 25 g of LB powder were dissolved in 1 L of distilled water and autoclaved. LB-agar plates were prepared by adding 6 g of granulated agar to 400 mL of LB media. The LB-agar was autoclaved, melted and placed in Petri dishes. Phosphate buffered saline (PBS) was purchased from Fisher Scientific (Nepéan, Ontario, Canada).

2.3. Extraction of genomic DNA from S. aureus isolates

After centrifugation of 5 ml of cultured bacteria (4000 rpm, 15 min), pellets were resuspended in 564 μl of TE buffer (10 mM Tric-HCl pH8/1 mM EDTA pH8), frozen at −80 °C and thawed at 45 °C. After addition of 6 μl of Proteinase K in 30 μl of 10% of sodium dodecyl sulfate (SDS) (2 h, 45 °C), 80 μl of CTAB/NaCl (10% hexadecyltrimethyl ammonium bromide/0.7 M NaCl) (30 min, 65 °C), and 750 μl chloroform/iso-amyl alcohol (ratio of 24:1), the mixed solution was centrifuged at 15000*g* for 15 min. The supernatant was then subjected to the addition of 600 μl phenol/chloroform/iso-amyl alcohol (25:24:1) and centrifugation (15000*g*, 15 min). DNA was precipitated, using 600 μl of isopropyl alcohol. After centrifugation, the pellet was allowed to dry and, then, resuspended in 20 μl of TE buffer.

2.4. Polymerase chain reaction (PCR)

Total *S. aureus* and MRSA DNAs were prepared, as previously described (Lévesque, 2011–2012). The primer mixes used for the multiplex PCR for SCC*mec* I, II, II, and IV are listed in Table 1. The monoplex PCR primers used for SCC*mec* IV detection are listed in Table 2. The PCR was carried out with Taq DNA Polymerase (New England Biolabs, Ipswich, MA), using 30 cycles, under the following conditions: 30 s at 95 °C, 30 s at 57 °C, and 90 s at 70 °C.

2.5. Extraction and isolation of S. aureus PBP2a membrane protein

A total of 2.0×10^{10} CFU/ml of cultured bacteria (Saa 4, 5, 30, and 226) were used. Membranous protein extraction was performed, using a ReadyPrep Protein Extraction kit (Membrane I) (BioRad), according to the manufacturer's protocol. After centrifugation (16000 *g*, 5 min at room temperature), hydrophobic and hydrophilic phases were separated, and the hydrophobic protein

Table 1
Multiplex PCR mixes for SCC*mec* I, II, III, V and *mecA* typing.

	Primer	Length (bp)	Forward	Reverse
1	SCC*mec* I	613	GCTTTAAAGAGTGTCGTTACAGG	GTTCTCTCATAGTATGACGTCC
	SCC*mec* II	287	CGTTGAAGATGATGAAGCG	CGAAATCAATGGTTAATGGACC
	Sa442	108	AATCTTTGTCGGTACACGATATTCTTCACG	CGTAATGAGATTTCAGTAGATAATACAACA
2	SCC*mec* V	325	GAACATTGTTACTTAAATGAGCG	TGAAAGTTGTACCCTTGACACC
	*Mec*A	162	TCCAGATTACAACTTCACCAGG	CCACTTCATATCTTGTAACG
3	SCC*mec*III	243	CATTTGTGAAACACAGTACG	GTTATTGAGACTCCTAAAGC
	Sa442	108	AATCTTTGTCGGTACACGATATTCTTCACG	CGTAATGAGATTTCAGTAGATAATACAACA

Table 2
Monoplex PCR primers for SCC*mec* IV typing.

SCC*mec* IV type	Length (bp)	Forward	Reverse
IVa	776	GCCTTATTCGAAGAAACCG	CTACTCTTCTGAAAAGCGTCG
IVb	1000	AGTACATTTTATCTTTGCGTA	AGTCATCTTCAATATGGAGAAAGTA
IVc	677	TCTATTCAATCGTTCTCGTATT	TCGTTGTCATTTAATTCTGAACT
IVd	1242	AATTCACCCGTACCTGAGAA	AGAATGTGGTTATAAGATAGCTA

concentration was determined, using RC DC protein assays (Bio-Rad). Proteins in each sample were purified, using ReadyPrep 2-D Cleanup kits (BioRad), following the manufacturer's protocol. Equal amounts of proteins were separated by SDS-PAGE, under reducing conditions, and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA and incubated with primary antibody (monoclonal PBP2a antibodies, Fitzgerald) overnight at 4 °C. After five washes with PBS Tween-20 (PBST), membranes were incubated overnight, at 4 °C, with secondary antibodies (Cell Signaling, Beverly, MA), conjugated with horseradish peroxidase. Immunoreactive proteins were then detected by an Opti-4CN detection kit (BioRad). Dried membranes were scanned and densitometric analyses were carried, using the Image J software. The following antibody dilutions were used: anti-PBP2a, 1:10 000, anti-lgG HRP, 1:3000.

2.6. Au surface preparation

Biacore Platypus biosensor chips, to be described below, were used as the gold surface. Prior to modification, they were placed in freshly prepared "Piranha" solution, a 7:3 (v/v) mixture of concentrated H_2SO_4 and 30% H_2O_2 (caution: piranha solution reacts vigorously with organics, and can splatter), at room temperature, for 30 min, then rinsed thoroughly with MilliQ water (Millipore, Mississauga, Ont.), ultrasonicated in water for 15 min and dried in a nitrogen flow. The gold substrate was coated with a self-assembled monolayer (SAM) of L-cysteine overnight at 37 °C, washed with PBS and subsequently coated with mercaptoundecanoic acid (MUA) to create mixed self-assembled monolayers[19]; these were treated with 1-(3-dimethylaminopropyl)ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Anti-PBP2a antibodies were then exposed to the surface for an hour, followed by BSA for 15 min, to prevent non-specific adhesion.

2.7. Surface plasmon resonance apparatus

We used a homemade highly sensitive surface plasmon resonance (SPR) system. Briefly, a superluminescent light-emitting diode (SLED), emitting at 650 nm, was used as the light source. An achromatic lens produced a collimated beam, which passed through a polarizer. The linearly *p*-polarized light was focused by a lens, and then used to excite surface plasmons on the sensing surface, situated on top of a coupling prism. A commercial sensing surface (Platypus Technologies), consisting of a glass microscope slide (BK7), covered by an adhesion layer of 5 nm of titanium and a sensing layer of 50 nm of gold, was placed in oil immersion contact (Cargille Labs) on the top of the coupling prism. For the real-time tests, a flow injection double channel-measuring cell, with a 12 μl volume, was developed. The entire system was placed on a goniometer stage, with 2-D linear translation for exact beam angle and position, permitting surface plasmon excitation at the gold/adjacent medium interface. The spatial distribution of the reflected light intensity was recorded by a CCD camera (Hamamatsu C4742-95), and examined by appropriate software image treatment. A more detailed description of the system can be found in references (Tawil et al., 2012; Dallaire et al., 2012).

2.8. DNA sequence analysis and bioinformatics

Open reading frame identification was performed, using GeneMark.hmm (Lukashin and Borodovsky, 1998). Similarity searches, for nucleotide sequences and for the deduced amino acid sequences, were performed using the FASTA (Pearson, 1990), BLAST (Altschul et al., 1990), and PARALIGN (Rognes and Seeberg, 2000) programs available on the Online Analysis Tools website (http://molbio-tools.ca/).

3. Results and discussion

3.1. Characterization of staphylococcus aureus resistance cassettes

Current methods employed to type bacteria are based on genotypic characteristics of the strains. The staphylococcal cassette chromosome, *mec* (SCC*mec*), is a mobile genetic element that contains the *mecA* gene, which encodes methicillin resistance in *S. aureus*. SCC*mec* typing is used to differentiate strains or lineages within the MRSA population. Eleven SCC*mec* types and several variants have been described, with types I–V being the most common (Milheirico et al., 2007).

From a bacteriological standpoint, CA-MRSA differs from HA-MRSA owing to various characteristics. CA-MRSA is typically susceptible to most non-β-lactam antibiotics and comprises a SCC*mec* element of type IV, type V or of the newly described type VII. On the other hand, HA-MRSA is usually multidrug-resistant and contains a SCC*mec* element of type I, II or III (Milheirico et al., 2007).

A study by the Society for Healthcare Epidemiology of America, which surveyed MRSA colonization in Canada (Simor et al., 2010), surveying both community and hospitalized patients in Canada. This study demonstrated the incidence and describes the changing epidemiology of *S. aureus* infections in Canada. This study demonstrates the emergence of CA-MRSA strains in Canada and, in particular, the presence of CMRSA-10 (USA300) and CMRSA-7 (USA400) in the province of Quebec (Simor et al., 2010).

MRSA infections are endemic in hospitals worldwide. In recent years, infections have emerged in the community and livestock, and thus, MRSA is no longer exclusively considered as a healthcare associated problem. Validation of the current SPR platform was carried out on 250 well-characterized *S. aureus* strains (82.8% MRSA, 10.8% BORSA, and 6.4% MSSA). *S. aureus* isolates were characterized by multiplex PCR amplification of the Panton-Valentine leukocidin (PVL) and *mecA* genes, staphylococcal cassette chromosome *mec* (SCC*mec*) typing and oxacillin susceptibility. The results are summarized in Table 3. Amongst these isolates, 28.9% were HA-MRSA, and 71.1% were CA-MRSA. Further analysis of the LSPQ isolates determined that the strains belonged to six epidemiologic types, with CMRSA-10 (USA300) accounting for 65.7% of isolates, CMRSA-2 (USA100/800) for 23.4%, CMRSA-7 (USA400) for 5.4%, USA700 for 2.2%, USA1100 for 2.2% and CMRSA-1 (USA600) for 1.1%.

As previously noted, *mecA* resides in the large mobile genetic element, SCC*mec*. Methicillin resistance is known to be inducible, and the *mecA* gene, which codes for PBP2a, is under the control of the *mecI* and *mecR*1 regulatory genes. These genes encode, respectively, for a repressor and for a β-lactam sensing signal transducer. In an antibiotic-free environment, *mec*I represses the transcription of *mecA* and *mec*R1. However, when β-lactams are introduced, *mec*R1 is cleaved, thus freeing the metalloprotease domain and inducing *mecA* transcription (Berger-Bachi and Rohrer, 2002). The presence of *mecA* and the production of PBP2a provide methicillin resistance in *S. aureus*. Thus, testing with both SCC*mec* and *mecA* gene markers provides conclusive proof of resistance in MRSA.

We arbitrarily selected Saa 4 as a representative strain for HA-MRSA, Saa 5 for MSSA, Saa 30 for CA-MRSA and Saa 226 for BORSA to carry on biosensing studies. Amplification of the *mecA* gene and SCC*mec* types for selective *S. aureus* isolates included in Table 2 are shown in Fig. 1. MSSA and BORSA strains (Saa 5 and 226) were negative for the 162-bp internal fragment of the *mecA* gene, while (Saa 4 and Saa 30) were positive, confirming that these strains are MRSA. The Saa 4 strain showed resistance to oxacillin, cefazolin, ceftriaxone, ciprofloxacin, and ampicillin (MIC > 32 μg/ml for all antibiotics tested), but was susceptible to vancomycin (MIC= 2 μg/ml). PCR SCC*mec* typing showed that it was type II, making it a HA-MRSA strain. Saa 5 was chosen as representative of MSSA strains, with susceptibility to all antibiotics tested (MIC≤1 μg/ml); it was also negative for all SCC*mecs* and *mecA* genes tested. Saa 30 was chosen as representative of CA-MRSA, as it was resistant to β-lactams, positive for *mecA*, positive for PVL, and was a SCC*mec* IV. For BORSA, Saa 226 was chosen, as it was negative for *mecA* and SCC*mecs* but showed borderline susceptibility to oxacillin (MIC=8 μg/ml).

Although PCR, spa typing, and SCCmec subtyping are suitable for epidemiological studies, risk assessment and surveillance, there is still a paramount need for a highly discriminatory, inexpensive, and rapid technology for point-of-care diagnosis.

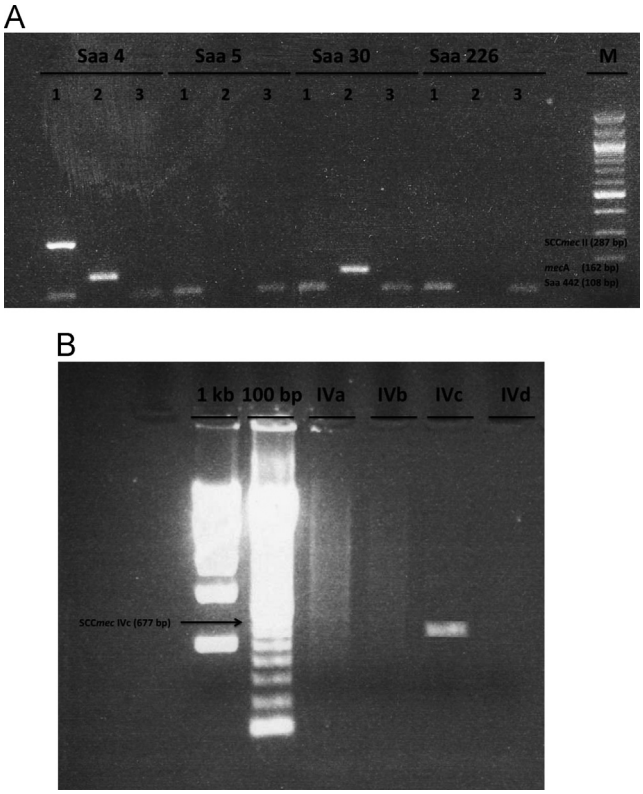

Fig. 1. (a) Multiplexed PCR SCC*mec* and *mecA* typing. Representative Biophage Pharma isolates (Saa 4 and 5) and representative LSPQ isolates (Saa 30 and 226). First lanes in both gels are molecular weight markers. Lanes marked with the number 1 contain primers for SCC*mec* I, II and Sa442, a positive control to confirm *S. aureus* strain. Lanes marked with the number 2 contain primers for SCC*mec* V, and *mecA*. Lanes marked with the number 3 contain primers for SCC*mec* III, and Sa442. Lane marked with the letter M contains the molecular marker. (b) Monoplex PCR for SCC*mec* IV typing for the Saa 30 strain. Lanes marked with 1 kb and 100 bp are molecular markers, while lanes marked with IVa, IVb, IVc and IVd contain primers for SCC*mec* IVa, IVb, IVc and IVd, respectively.

Table 3
Microbiological characteristics of *S. aureus* isolates.

Identification (nb of isolates)	Oxacillin MIC μg/ml (nb of isolates)	*mecA* PCR	SCC*mec* type	Epidemiologic type	PVL (nb of isolates)
MRSA (207)	≥32 (207)	Positive	HA-MRSA (56/194)	CMRSA-1 (2/184) CMRSA-2/USA300 (43/184) CMRSA-7/USA400 (10/184)	Positive (134/196) Negative (62/196)
			CA-MRSA (138/194)	CMRSA-10 (121/184) USA700 (4/184) USA1100 (4/184)	
MSSA (16)	≤0.25 (6) ≤0.5 (7) ≤1 (3)	Negative	–	–	Negative
BORSA (27)	1–4 (16) 4–8 (11)	Negative	–	–	Negative

3.2. Expression of protein PBP2a

Currently, the reported mechanism of methicillin resistance in *S. aureus* is the production of a distinctive cell wall protein, PBP2a, which exhibits low affinity toward β-lactams (Pantosti and Venditti, 2009). In contrast, susceptible strains of *S. aureus* have normal PBPs that function as transpeptidases, endopepitdases or carboxypeptidases in the crosslinking of macromolecules in the cell wall during cell division. MRSA PBP2a has a slightly different molecular size than PBP, and is controlled by a repressor gene also confined within the *mec* gene. Various other PBPs can be found in both resistant and susceptible strains of *S.aureus*, but only PBP2a remains active in the presence of high concentrations of β-lactams. On the other hand, BORSA strains do not exhibit PBP2a in their cell wall membranes but appear to produce large amounts of staphylococcal penicillinase that slowly degrades methicillin and oxacillin (Jorgensen, 1991).

Due to the fact that multiple genes are involved in cell-wall precursor formation, regulation, transport and signal transduction, different MRSA strains appear to be heterogeneous in their level of resistance (Berger-Bachi and Rohrer, 2002). Thus, different strains of MRSA may not only manifest a wide range of resistance to β-lactams, but also different concentrations of PBP2a in the cell wall membranes. The inset of Fig. 2 shows SDS-PAGE of total cell lysate protein (10 μg) derived from Saa 4, 5, 30 and 226.

In this experiment, membranous proteins were extracted from 2.0×10^{10} CFU/ml of freshly cultured bacteria (Saa4, 5, 30, and 226) in order to determine the PBP2a concentration in different *S. aureus* strains. The normalized PBP2a concentrations were found to be $89.4 \pm 0.2\%$ for HA-MRSA Saa 4 strain, $6.9 \pm 1.7\%$ for MSSA Saa5, $17.9 \pm 1.3\%$ for CA-MRSA strain Saa 30, and $0.3 \pm 0.1\%$ for BORSA strain Saa 226, respectively. As expected, no significant PBP2a expression was found in the MSSA and BORSA strains, whereas MRSA strains (Saa 4 and Saa 30) expressed heterogeneous amounts of PBP2a ($P < 0.01$, N=3). In fact there is approximately five times more PBP2a in Saa 4 than in Saa 30.

Resistance levels in MRSA depend on PBP2a production, which is regulated by chromosomal factors and is dependent on controlled cell wall synthesis and chemical structure of the peptidoglycan precursor. The regulation of genes that govern resistant levels may, in turn, be contingent on control by global regulators whose fine-tuning may vary from strain to strain. Thus, resistance levels in different MRSA strains range from phenotypically susceptible to highly resistant. In the following section, we propose an SPR assay, which can prove efficient at determining the level of membranous PBP2a in different MRSA strains.

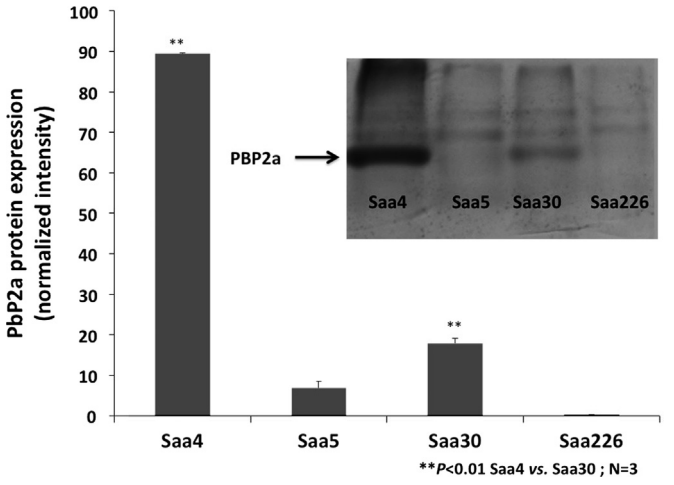

Fig. 2. PBP2a protein expression in *S. aureus*. Expression of PBP2a from membranes of Saa4 (HA-MRSA), Saa 5 (MSSA), Saa 30 (CA-MRSA) and Saa 226 (BORSA). Significant heterogeneous expression of PBP2a was found for Saa 4 MRSA strains compared to Saa 30 ($P < 0.01$, $N=3$). The inset shows SDS-PAGE of total membranous protein expression of *S. aureus* lysates. The arrow indicates PBP2a expression (82 kDa).

3.3. Differential detection of MRSA, MSSA and BORSA

One of the major objectives of our work is to develop a rapid and sensitive technique, using a SPR biosensor, to specifically detect and differentiate between MRSA, BORSA and MSSA strains. To meet this objective, we used a home-built biosensor, with anti-PBP2a antibodies as specific recognition elements, to detect low concentrations of methicillin-resistant bacteria. A PBS solution was injected onto the gold chip for 10 min, followed by a 20 min injection of a PBS solution containing different concentrations of *S. aureus* Saa 4 and Saa 5 strains. PBS was then injected to wash away the unbound bacteria (Fig. 3a). Results are shown in pixel units, with one pixel corresponding to 0.005 angle units. All experiments were carried out at room temperature and were performed in triplicates. The SPR responses for Saa 4 were 26.1 ± 1.2 pixels for 10^2 CFU/ml and 6.4 ± 0.9 pixels for 10 CFU/ml (inset of Fig. 3). Noticeably a small, but measurable, response was noted for 10^4 CFU/ml Saa 5 (1.1 ± 1.1 pixels). These results confirm the specificity of the antibody to PBP2a and the presence of the PBP2a protein on the membranes of MRSA strains (Saa 4) and not on the antibiotic susceptible strain (Saa 5).

The same experiment was repeated to establish the possibility of detecting CA-MRSA (Saa 30) bacteria using PBP2a antibody. All slides were previously coated with anti-PBP2a antibody and BSA, to prevent non-specific adhesion. The controls consisted of a solution of 10^6 CFU/ml of BORSA (Saa 226), injected on an anti-PBP2a-coated slide (Fig. 3b). SPR responses for predetermined concentrations of Saa 30 were 6.4 ± 1.0 pixels for 10^2 CFU/ml and 2.9 ± 0.3 for 10 CFU/ml. A concentration of 10^6 CFU/ml of Saa 226 produced a noticeably small, but measurable, response (0.4 ± 0.3 pixels). As anticipated, because MSSA and BORSA do not exhibit PBP2a on their cell wall membranes, we did not observe a significant change in SPR response. Any surface plasmon response difference between the PBS baseline and the non-specific adsorption of MSSA was determined to be due to noise. Using the criterion of S/N›3, the limit of detection is found to be 10 CFU/ml (inset of Fig. 3b). Moreover, the SPR responses for Saa 30 were 5 times lower than those of Saa 4, which is consistent with the SDS-PAGE results, where PBP2a expression was found to be approximately 5 times greater in Saa 4 than in Saa 30 strains. This shows that the SPR assay can effectively assess the amount of PBP2a in MRSA cell walls.

Our results demonstrate that, when the SPR biosensor assay was used to test *S. aureus* samples, it could effectively differentiate among MRSA, MSSA, and BORSA strains. The methicillin resistance measured was in total agreement with standard microbiological approaches. The chemical and optical components of the biosensing surface have excellent sensitivity, as evidenced by a detection limit of 10 CFU/ml, when MRSA strain response was compared to that of BORSA strains. This analytical limit of sensitivity is adequate to detect the numbers of organisms in positive isolates. Furthermore, the surface configuration is designed to be chemically inert, thereby limiting nonspecific interactions. The SPR assay was able to differentiate among *S. aureus* strains without the need for a culture step, target amplification, or PCR products. Thus, this approach has the advantages of being simple and rapid, allowing for identification of resistant strains of *S. aureus* up to 48 h earlier than conventional microbiological techniques. This method could have a significant impact on hospital costs, effective infection control, and patient mortality.

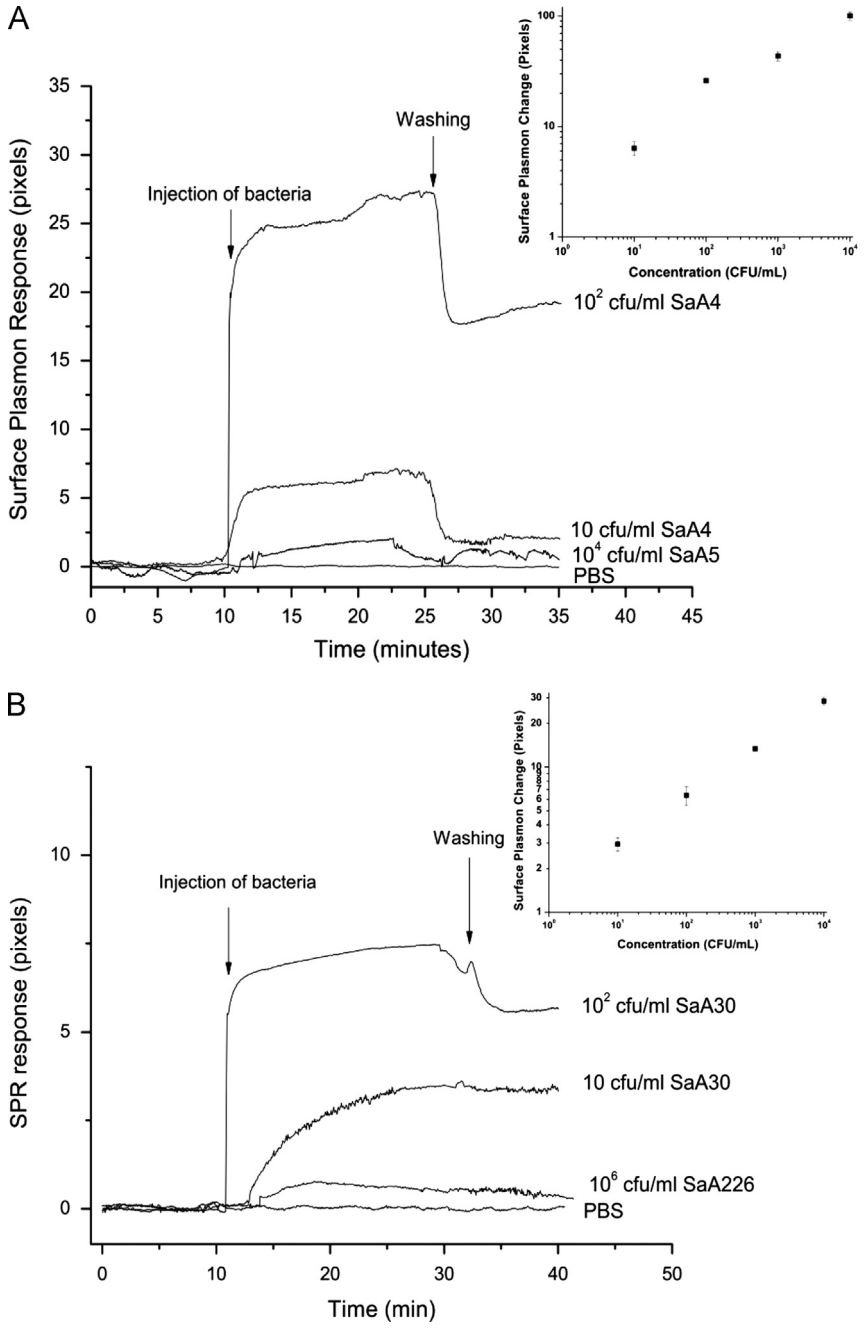

Fig. 3. Detection of MRSA, using anti-PBP2a antibodies, immobilized on the gold sensor chip. A baseline was created by injecting PBS buffer for 10 min. PBS was used as negative control. (A) Known concentrations of HA-MRSA (Saa 4) diluted in PBS were flowed onto the sensor surface. MSSA (Saa 5), at a concentration of 10^4 CFU/ml, was also tested, and manifested the high specificity of the anti-PBP2a antibody for MRSA detection. For detection of Saa4 MRSA strain, a dose-response curve is shown, in inset, for values taken after 20 min. (B) Known concentrations of CA-MRSA (Saa 30), diluted in PBS, were flowed onto the sensor surface. Surface plasmon responses for MRSA strains (Saa 30) showed the high specificity of the antibody for PBP2a. On the contrary, BORSA strains (Saa 226), at a concentration of 10^4 CFU/ml, did not show any significant SPR response. For detection of Saa 30 MRSA strain, a dose-response curve is shown, in inset.

4. Conclusions

Our proposed SPR assay allows rapid and accurate detection of MRSA, eliminating the necessity for specialized reference laboratories. This assay is easy to perform, can accurately differentiate MRSA from BORSA and MSSA isolates, and provides a rapid (less than 20 min) alternative for the detection of methicillin resistance in *S. aureus* in clinical laboratories, especially when *mecA* PCR gene detection is unavailable. This will permit clinicians to more effectively manage patients and to control the spread of MRSA. This new test shows great promise in providing rapid, sensitive and specific alternatives, when neither PCR nor DNA hybridization for the *mecA* gene is available.

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