Biosensors and Bioelectronics 49 (2013) 334–340

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

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

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ARTICLE INFO

Article history:
Received 19 March 2013
Received in revised form
10 May 2013
Accepted 20 May 2013
Available online 4 June 2013

Keywords:
Penicillin-binding protein (PBP)
Biosensor
Methicillin-resistant *Staphylococcus aureus* (MRSA)
Methicillin-sensitive *Staphylococcus aureus* (MSSA)
Borderline oxacillin-resistant *Staphylococcus aureus* (BORSA)
Surface plasmon resonance (SPR)

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.

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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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http://dx.doi.org/10.1016/j.bios.2013.05.031

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

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