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Matrix-assisted laser desorption ionization-time of flight Mass spectrometry can detect *Staphylococcus aureus* clonal complex 398

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ABSTRACT

Within the last decade methicillin-resistant *Staphylococcus aureus* belonging to CC398 has become a worldwide threat associated with livestock. More recently, methicillin-susceptible *S. aureus* (MSSA) belonging to CC398 have been increasingly reported as a cause of invasive infections in patients without livestock contact. It appears therefore necessary to implement a convenient tool for the surveillance this emerging pathogen. We evaluated the MALDI-TOF MS as a tool for rapid detection of *S. aureus* CC398.

We used 626 *S. aureus* isolates characterized by a CC398-specific PCR, to constitute independent training (300 isolates including 60 isolates CC398) and validation sets (326 isolates including 82 isolates CC398). Fifteen peak biomarkers of CC398 were identified from the mass spectra of the training set. Ninety four % (307 of 326) of strains of the validation set were well assigned with an overall sensitivity of 93% and a specificity of 95%. Six CC398 and 13 non-CC398 isolates were misclassified.

With MALDI-TOF MS, clinical laboratories could rapidly detect *S. aureus* CC398 associated with a higher mortality in hospitalized patients.

1. INTRODUCTION

During the past decade, methicillin-resistant *Staphylococcus aureus* (MRSA) of sequence type 398 (ST398), which belongs to clonal complex (CC) 398, has gained much attention because it has been found in livestock, food products and causes infections in humans worldwide (Smith and Pearson, 2011; Witte et al., 2007). More recently, methicillin-susceptible *S. aureus* (MSSA) CC398 has been increasingly reported in Europe, the USA and China as a cause of invasive infections in patients without contact with livestock (Fan et al., 2009; Uhlemann et al., 2012; Valentin-Domelier et al., 2011; Verkade et al., 2012). Most notably, the incidence of CC398 MSSA bloodstream infections has been increasing since 2007 in France (Valentin-Domelier et al., 2011) and our group recently found that bloodstream infections due to CC398 MSSA were associated with higher 30-day mortality than those due to non-CC398 MSSA (Bouiller et al., 2016). It appears therefore necessary to implement a convenient tool for the surveillance this emerging pathogen. CC398 strains are identified by multilocus sequence typing (MLST) or *spa* typing (Enright et al., 2000). Since these typing methods are time-consuming, CC398-specific PCR that targets the sequence of the gene *sau1hsdS* was implemented (Stegger et al., 2011). This simple and reliable PCR technique is used in research laboratories but remains inappropriate for clinical laboratories.

Mass Spectrometry Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF MS) identifies microbial species by analysis of total protein (Bizzini and Greub, 2010). For bacteria, this technique is based on the generation of mass spectra from whole cells and their comparison to reference spectra (Fenselau and Demirev, 2001). Compared to traditional biochemical or molecular techniques, MALDI-TOF MS allows for a quick, automated, simple and relatively cheap bacterial identification. Additionally, sub-species groups such as *Listeria monocytogenes* serotypes, *Yersinia enterocolitica* biotypes, *Salmonella enterica* subspecies, *S. aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* sequence types can be further identified with MALDI-TOF MS (Barbuddhe et al., 2008; Boggs et al., 2012; Cabrolier et al., 2015; Dieckmann et al., 2008; Lafolie et al., 2015; Sauget et al., 2014; Stephan et al., 2011).

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We provided here a simple, rapid MALDI-TOF MS-based method that can be used in the routine clinical laboratories to identify *S. aureus* isolates of CC398.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A panel of 626 well-characterized clinical isolates of *S. aureus* were collected between 2007 and 2014 and stored at −80 °C until use at the Centre de Ressources Biologiques - Filière Microbiologie (Biobanque BB-0033-00090) at the University Hospital of Besançon (Table 1). All the isolates were identified as *S. aureus* by MALDI-TOF MS with a log value ≥2 according to the manufacturer's recommendations (Bruker Daltonik GmbH, Bremen, Germany). PCR method described by Stegger et al. was used to classify all the isolates into two groups: CC398 or non-CC398 (Stegger et al., 2011). We deliberately included as much as possible different STs in each categories to extend the genetic diversity of the tested panel (Table 1).

2.2. Samples preparation for MALDI-TOF MS

For experiments with high-quality spectra, we used the ethanol-formic acid extraction method recommended by Bruker Daltonics. After overnight culture on Mueller-Hinton agar (bioMérieux, Craponne, France), about half a loopful of fresh culture was suspended in 300 μl of demineralized water. Then 900 μl of absolute ethanol was added and the suspension was mixed for 15 s on a vortexer. The suspension was centrifuged at 18,000 ×*g* for 2 min. The supernatant was removed and the ethanol-killed bacterial pellet was used for protein extraction. Fifty μl of 70% formic acid (Sigma-Aldricht, Saint-Quentin Fallavier, France) was added before vortexing for 30 s for cell wall disruption. Then 50 μl of pure acetonitrile (Sigma-Aldricht) was added. The suspension was vortexed for 30 s and centrifuged at 18,000 ×*g* for 2 min. One μl of the supernatant was spotted on the target plate for MALDI-TOF MS experiments. The spots were overlaid with 1 μl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile with 2.5% trifluoroacetic acid, Bruker Daltonik) and air dried for 15 min.

2.3. MALDI-TOF MS measurements

Spectra were obtained with a Microflex LT mass spectrometer (Bruker Daltonik GmbH) in the linear positive ion mode, with a laser frequency of 60 Hz. The mass range was *m/z* 2000 to 20,000. Parameter settings for Microflex LT were: ion source 1: 20 kV, ion source 2: 18.5 kV, lens: 6 kV, pulsed ion extraction: 100 ns. Each series of measurements was preceded by a calibration with a Bacterial Test Standard (Bruker Daltonik) that contains an *E. coli* reference strain along with RNase A and myoglobin. For each sample, the sum spectra of 240 single spectra were acquired, in portions of 40 single spectra from 6 different positions on a spot.

2.4. Identification of peak biomarkers for the S. aureus CC398

Automated analysis of the data was performed using Clin-ProTools 3.0 (Bruker Daltonik) for peaks ranging from *m/z* 2000 to 20,000. This software uses a standard data preparation workflow that includes spectra pretreatment and peak calculation operation. We used a training set of 300 isolates to calculate models that distinguish CC398 isolates. Two groups were defined, the CC398 isolates ($n = 60$) and the non-CC398 isolates ($n = 240$). The reliability and the accuracy of the models were assessed through recognition capability and cross-validation values. The pertinence of the generated models was verified with an independent validation set of 326 isolates (including 82 CC398 isolates and 244 non-CC398 isolates) (Table 1). The sensitivity, specificity, positive and negative predictive values of the method were calculated with the Wilson score method using OpenEpi 3.03a software (www.OpenEpi.com).

3. Results and discussion

We defined here a method for the detection of *S. aureus* CC398 by the analysis of MALDI-TOF MS spectra with the ClinProTools 3.0 (Bruker Daltonik) software. The great majority of the *m/z* peaks were shared by all the CCs in which highly abundant proteins such as ribosomal and periplasmic proteins are constantly expressed (Holland et al., 1999). However, minimal differences in mass spectrum (*i.e.* mass, signal-to-noise, intensity, peak heights, and peak areas) were detected by the ClinProTools software and allowed us to create a model of detection of CC398 *S. aureus*.

3.1. Creation and validation of a S. aureus CC398 recognition model

In agreement with the good practice guidelines for biomarker discovery, the predictive biomarkers were first developed with a training set of data and then validated on an independent set of data (25). With a training set of spectra from 300 *S. aureus* isolates, we created a model that was able to distinguish CC398 isolates from non-CC398 isolates. This model was based on a Support Vector Machine algorithm that used 15 peak markers (Table 2). It has a recognition capability of 96% and a cross-validation of 89%. We further tested the model with spectra from 326 *S. aureus* isolates independent from those used in the training set (Table 1). The test has a good positive predictive value (85%) and high values of sensitivity (93%), specificity (95%), and negative predictive value (97%) (Table 4). Overall, 94% of strains (307 of 326) were well classified. The test assigned 76 of the 82 CC398 isolates and 231 of the 244 non-CC398 isolates correctly (Table 3). Six CC398 strains were misclassified. Thirteen non-CC398 strains were misclassified, 2 of which belonged to ST30, 2 other ST30 having been correctly classified.

3.2. Discussion of the model

All the isolates were classified into two groups (CC398 or non-CC398) using the PCR method described by Stegger et al. in 2011 (Stegger et al., 2011). PCR specifications were evaluated on a large collection ($n = 1307$) and showed high sensitivity (100%) and specificity (100%). Later, non-CC398 sequence types that had not been tested by

Table 1
Complex clonal and sequence type of the 626 strains of *S. aureus* used in the study.

Clonal complex[a]	Creation of the model		Validation of the model	
	n	Sequence type (*n*)	*n*	Sequence type (*n*)
CC398	60	ST398 (*31*)	82	ST398 (*6*), ST3151 (*1*), ST3152 (*1*)
Non-CC398	240	ST1 (*2*), ST5 (*5*), ST6 (*2*), ST8 (*8*), ST9 (*1*), ST15 (*2*), ST22 (*3*), ST25 (*1*), ST30 (*3*), ST45 (*3*), ST63 (*1*), ST78 (*1*), ST80 (*2*), ST81(*2*), ST88 (*2*), ST121 (*2*), ST247 (*1*), ST1083 (*1*), ST3233 (*1*)	244	ST7 (*1*), ST10 (*1*), ST30 (*4*), ST34 (*1*), ST88 (*1*), ST199 (*1*), ST3231 (*1*), ST3232 (*1*)
Total	300		326	

[a] As defined by the CC398-specific PCR described by Stegger et al. (Stegger et al., 2011).