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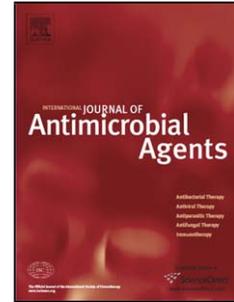
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MALDI-TOF-MS for rapid detection of staphylococcal Pantón–Valentine leukocidin

Fadi Bittar ^a, Zoulikha Ouchenane ^b, Farida Smati ^b, Didier Raoult ^a, Jean-Marc Rolain ^{a,*}

^a *Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes (URMITE), CNRS-IRD, UMR 6236, Faculté de Médecine et de Pharmacie, Université de la Méditerranée, 27 Bd Jean Moulin, 13385 Marseille Cedex 05, France*

^b *Laboratoire de Bacteriologie, Hôpital Militaire Universitaire de Constantine, Algeria*

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* Corresponding author. Tel.: +33 4 91 32 43 75; fax: +33 4 91 38 77 72.

E-mail address: jean-marc.rolain@univmed.fr (J.-M. Rolain).

Abstract

Toxin-producing Gram-positive bacteria are responsible for emerging and life-threatening infections in humans worldwide. Both rapid toxin detection and adapted therapy are essential to limit the morbidity due to such toxins, especially staphylococcal Pantón–Valentine leukocidin (PVL). Here we describe the use of a mass spectrometry profile generated by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) followed by ClinProTools™ 2.0 software analysis to find a reproducible model able to identify PVL in *Staphylococcus aureus* strains. Eighty-one *S. aureus* strains were used and tested for the presence of PVL, toxic shock syndrome toxin (TSST-1) and *mecA* genes. The peak at 4448 mass-to-charge ratio (m/z) was the most relevant peak to differentiate between PVL-producing and non-PVL-producing *S. aureus*. A model using only this peak had an overall recognition capability of 100% and an overall cross-validation of 77.07%. Prospective evaluation of the model allowed two cases of PVL-producing strains to be detected within a few minutes during the time of care and before polymerase chain reaction (PCR) results. Our study represents a proof of concept for the use of such rapid technology as a point-of-care method to identify potential lethal toxin quickly. We believe that such a rapid method will be timely to help change the therapeutic strategy and could be used in the future for other pathogens and infectious diseases.

1. Introduction

Serious complications caused by *Staphylococcus aureus*, one of the most significant and frequent human pathogens, are a worldwide phenomenon that concern both hospital and community settings. Toxin production contributes mainly to the pathogenicity of this bacterium. Pantón–Valentine leukocidin (PVL), a two-component, pore-forming, cytolytic toxin that targets polymorphonuclear cells and causes cell death by necrosis or apoptosis [1], is one of the most virulent staphylococcal toxins described. Necrotizing pneumonia [2] and necrotizing skin and soft tissue infection [3] are the predominant results due to infection by PVL-producing *S. aureus* strains. Because severe infection caused by strains producing PVL should benefit from a quickly adapted therapy [4] in order to decrease mortality, rapid detection of this toxin is critical.

Classical approaches for recognising PVL toxin are generally based on the immunological properties of the toxin, such as enzyme-linked immunosorbent assay (ELISA) [5] and latex agglutination [6], or on polymerase chain reaction (PCR)-based methods [7]. These assays are costly and time-consuming methods. Recently, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has played an interesting role in microbiological laboratories owing to its speed, ease of use and accuracy in the identification and classification of bacteria, including *S. aureus* [8], as well as its discriminatory power to differentiate between strains with different phenotypes, including the expression of antibiotic resistance [9,10].

This work provides a proof of concept that the spectra profiling generated by MALDI-TOF-MS could be used as a clinical microbiology strategy to identify quickly *S. aureus* isolates harbouring PVL toxin and thus to adapt therapy during the time of care of the patient.

2. Material and methods

2.1. Bacterial strains

Study strains comprised 81 *S. aureus* isolated from patients either at Hôpital Militaire Universitaire de Constantine, Algeria, between January 2005 and March 2007 (64 strains) or at Timone Hospital in Marseille, France (17 strains). An additional 34 *S. aureus* strains from Timone Hospital were also used prospectively for external validation. The isolates were recovered from different pathology samples. The clinical sources of the different strains are summarised in Table 1.

2.2. Identification of *Staphylococcus aureus*

The isolates were first identified by conventional methods including Gram staining, catalase activity, mannitol fermentation, ability to coagulate rabbit plasma (bioMérieux, Marcy l'Etoile, France) and Pastorex Staph-Plus agglutination (bioMérieux). The identification was confirmed using the VITEK 2 Auto System (bioMérieux). Antimicrobial susceptibility testing was performed by the disk diffusion method according to the Clinical and Laboratory Standards Institute and also using VITEK 2.

2.3. Detection of the *mecA* gene PVL toxin and SCCmec typing

To confirm resistance to meticillin in *S. aureus* isolates, the primers *mecAF* (5'-GTTAGATTGGGATCATAGCGTCATT) and *mecAR* (5'-TGCTAATCTCATATGTGTTCTGTAT) and the TaqMan probe *mecAProbe* (6FAM-TTCCAGGAATGCAGAAAGACCAAAGCAT-TAMRA) were used in this study.

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing was performed as described previously [7]. For SCC*mec* type V, the following primers and TaqMan probe were used: *cassMecVF* (5'-TCTGGGAGTTCTGCCTGTCA); *cassMecVR* (5'-TCACATTTGACGCAATCTGCT); and *cassMecVP* (6FAM-TGCTGAAGTCGTCGAACGCGTAATCA-TAMRA).

All isolates were also screened by real-time PCR for the presence of PVL toxin and toxic shock syndrome toxin (TSST-1) using the primers and TaqMan probe as described previously [7,11].

2.4. Bacterial analysis using MALDI-TOF-MS

Strains were plated on Columbia agar with 5% sheep blood (bioMérieux) and incubated for 24 h at 37 °C. One colony from each strain was harvested and deposited on a target plate (Bruker Daltonics, Bremen, Germany) in three or four replicates to minimise random effect. Two microlitres of matrix solution (saturated α cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% trifluoroacetic acid) was then added and allowed to co-crystallise with the sample. Analysis was performed in an MALDI-TOF-MS spectrometer (337 nm) (Autoflex; Bruker Daltonics) with the FLEX control software (Bruker Daltonics). Positive ions were extracted with an

accelerating voltage of 20 kV in linear mode. The spectra were analysed in a mass-to-charge ratio (m/z) range of 2000 to 20 000. The profiles were compared and analysed using BioTyper 2.0 software and finally mass spectrometric data were analysed using ClinProTools™ 2.0 to find PVL biomarker candidates. Generating the classification model in ClinProTools™ 2.0 was performed using Genetic Algorithm. Recognition capability and cross-validation (i.e. the measure of the reliability used to predict the accuracy of the model) were calculated by the software with percentage values. External validation was carried out using 34 strains collected at Timone Hospital in Marseille in a blinded fashion. A P -value of <0.05 was considered statistically significant.

3. Results

All the isolates included in the study were first tested for the presence of PVL, TSST-1 and *mecA* genes as well as determination of SCC*mec* type. Table 1 presents the results of the different PCRs obtained from the 81 *S. aureus* strains. The following different isolate types were found in the collection: PVL⁺ *mecA*⁺ *S. aureus* ($n = 20$); PVL⁺ *mecA*⁻ *S. aureus* ($n = 4$); TSST-1⁺ *mecA*⁺ *S. aureus* ($n = 1$); TSST-1⁺ *mecA*⁻ *S. aureus* ($n = 4$); *mecA*⁺ *S. aureus* ($n = 47$); and *mecA*⁻ *S. aureus* ($n = 5$).

First we compared the MALDI-TOF spectra obtained from strains harbouring the PVL gene (24 isolates) with the spectra obtained from the other *S. aureus* (57 isolates) using ClinProTools™ 2.0. Using Genetic Algorithm analysis, several models with a different number of peaks have been generated. Considering the P -value and standard deviation given by the software, many peaks were differentially expressed between the two groups. Two interesting peaks were 4448 m/z and 5302 m/z , which

were found repeatedly to be used by the software. Forcing these two selected peaks into a model of two peaks allowed PVL⁺ and PVL⁻ strains to be distinguished with a recognition capability of 100% (100% for PVL⁺ and 100% for PVL⁻) and cross-validation of 83.51% (77.91% for PVL⁺ and 89.1% for PVL⁻). We then searched for the presence or absence of these two selected peaks in the spectra of the different studied strains. Fig. 1 presents an example of the presence and absence of the 4448 m/z peak in a PVL⁺ and a PVL⁻ strain, respectively. The 4448 m/z peak exhibited a sensitivity of 83.33%, a specificity of 98.25%, a positive predictive value (PPV) of 95.24% and a negative predictive value (NPV) of 93.33%. Only one of 57 PVL⁻ strains had this peak, whereas 4 of 24 PVL⁺ strains did not. The 5302 m/z peak yielded a sensitivity of 100%, a specificity of 56.14%, a PPV of 48.98% and NPV of 100%. Thus, the 4448 m/z peak was the most relevant peak to separate the two groups (Fig. 2). Interestingly, a model using only the 4448 m/z peak had a recognition capability of 100% (100% for PVL⁺ and 100% for PVL⁻) and cross-validation of 77.07% (67.48% for PVL⁺ and 86.65% for PVL⁻).

Finally, the latest model (i.e. the model with the 4448 m/z peak only) was evaluated blindly using 34 clinical *S. aureus* strains (Table 1). Four spectra from each strain were generated by MALDI-TOF-MS and screened for the presence of PVL. Testing these additional strains in our model, five isolates were classified as positive strains and only two of them were later confirmed using molecular methods. Thus, this model exhibited a sensitivity of 100%, a specificity of 90.6%, a PPV of 40% and a NPV of 100%.

4. Discussion

In the present study, we describe a novel, fast and inexpensive tool for discrimination of *S. aureus* isolates that do or do not produce PVL toxin compared with the actual methods used. Our promising method is based mainly on screening the spectrum profile of *S. aureus* obtained from MALDI-TOF-MS by a model generated using ClinProTools™ 2.0 software. Whilst PCR requires time, technical experience and equipped laboratories, the method described here is very easy and cheap and the analysis can be done within a few minutes.

Our results demonstrate that the peak at 4448 m/z was the most relevant peak as it was differentially expressed between PVL⁺ and PVL⁻ strains ($P < 0.05$). Interestingly, the model with only this peak had an overall recognition capability of 100% and an overall cross-validation of 77.07%. Through this model, two additional cases were detected in 3 min and before the results of PCR that were obtained during the following 24 h. It is interesting to note that four spectra for each isolate were analysed to confirm the results. The 4448 m/z peak was not found in TSST-1-producing *S. aureus*. It will be interesting in the future to see whether other exotoxin-producing *S. aureus* strains share this peak with PVL-associated ones

The predacious infection caused by strains producing PVL and the high mortality (ca. 75% in the case of necrotizing pneumonia [12]) increase the need for quick detection of such strains. In fact, an appropriate antimicrobial treatment should be administered as soon as possible if a patient is likely to be infected by PVL-associated *S. aureus*. For example, it has been demonstrated that subinhibitory concentrations of oxacillin, an antibiotic frequently used in the treatment of methicillin-sensitive *S. aureus*, can

enhance PVL production [13], whilst PVL induction is suppressed with a combination of oxacillin and another antibiotic such as clindamycin, rifampicin or linezolid [13]. Recently it has been demonstrated that protein synthesis inhibitors such as clindamycin can suppress the translation of toxin genes and may have a favourable effect on infections caused by exotoxin-producing *S. aureus* [14]. On the other hand, intravenous immunoglobulin administration, as an adjunct to antimicrobials, has been found to improve clinically patients with PVL-associated pneumonia [4,15]. This latter essential therapy can neutralise pore formation and inhibit the cytopathic effect of PVL toxin [12] that is already produced and present in the lung substance with concomitant tissue damage [4]. Taken together, the high mortality rate of PVL-associated infections and the beneficial effect of early adapted therapy make the time to report results to physicians a critical point as they are likely to change the therapeutic strategy based on the knowledge of whether the strain produces PVL or not. Thus, a rapid method for predicting the presence of PVL toxin could be a critical tool even if the specificity is not 100%.

It is also important that these preliminary results should be expanded and confirmed with more strains obtained from different countries and/or using different conditions of culture and MS instruments.

In conclusion, we demonstrated as a proof of concept that MALDI-TOF-MS is a powerful tool to detect PVL-producing *S. aureus* rapidly. We believe that this methodology could replace conventional methods used in clinical microbiology for rapid identification of bacterial virulence factor and/or antibiotic resistance determinants, allowing changes in therapeutic strategies during time of care.

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Fig. 1. Example of matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra of (A) a Pantone–Valentine leukocidin (PVL)-positive strain and (B) a PVL-negative strain between 4000 mass-to-charge ratio (m/z) and 4800 m/z .

Fig. 2. (A) Average peaks of Pantone–Valentine leukocidin-positive (PVL⁺) strains (red) and PVL-negative (PVL⁻) strains (green) between 4000 Da and 5000 Da. (B) Calculated average of the peak areas/intensities in PVL⁺ strains (red) and PVL⁻ strains (green) between 4000 Da and 5000 Da.

Table 1

Strains used in the study

Clinical source	Country	No. of strains studied	<i>mecA</i> gene	SCC <i>mec</i> type	PVL gene	TSST-1 gene
Pus	Algeria	10	+	4	–	–
Pus	Algeria	22	+	5	–	–
Pus	Algeria	2	+	N/D	–	–
Pus	Algeria	13	+	4	+	–
Venous catheter	Algeria	2	+	4	–	–
Venous catheter	Algeria	4	+	4	–	–
Venous catheter	Algeria	1	+	4	+	–
Blood culture	Algeria	1	+	5	–	–
Blood culture	Algeria	1	+	N/D	–	–
Urine	Algeria	1	+	N/D	–	–
Puncture fluid	Algeria	2	+	4	–	–
Puncture fluid	Algeria	1	+	4	+	–
Tracheal aspirate	Algeria	1	+	4	–	–
Tracheal aspirate	Algeria	1	+	5	–	–
Tracheal aspirate	Algeria	2	+	4	+	–
Cystic fibrosis sputum	France	5	–	N/P	–	–
Cystic fibrosis sputum	France	4	–	N/P	–	+
Cystic fibrosis sputum	France	1	+	1	–	+
Pleural liquid	France	1	+	4	+	–
Skin biopsy	France	1	+	4	+	–
Bone biopsy	France	1	+	4	+	–
Pulmonary biopsy	France	2	–	N/P	+	–

Skin biopsy	France	2	–	N/P	+	–
Blood culture	France	22 ^a	N/P	N/P	–	N/P
Skin biopsy	France	4 ^a	N/P	N/P	–	N/P
Skin biopsy	France	2 ^a	N/P	N/P	+	N/P
Puncture fluid	France	3 ^a	N/P	N/P	–	N/P
Pulmonary biopsy	France	1 ^a	N/P	N/P	–	N/P
Venous catheter	France	1 ^a	N/P	N/P	–	N/P
Sinus sample	France	1 ^a	N/P	N/P	–	N/P

SCC*mec*, staphylococcal cassette chromosome *mec*; PVL, Panton–Valentine

leukocidin; TSST-1, toxic shock syndrome toxin; +, positive; –, negative; N/D, not determined (i.e. not SCC*mec* types 1, 2, 3, 4 or 5); N/P, not performed.

^a Strains used in the external validation.

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