



Evaluation of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for identifying *Burkholderia pseudomallei* and *Burkholderia thailandensis* isolates

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Abstract

Since *Burkholderia thailandensis* is included in the reference spectra of the VITEK MS libraries rather than *Burkholderia pseudomallei*, *B. pseudomallei* cannot be correctly identified in the current version of VITEK MS. This study was undertaken to evaluate the utility of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with the VITEK MS plus system in the detection of *B. pseudomallei* and *B. thailandensis* isolates. For each species, we increased the reference spectra, and then, a SuperSpectrum was created based on the selection of 39 specific masses. In a second step, we validated the SuperSpectra with 106 isolates identified by 16S rRNA gene sequencing. The results showed that there was 100% agreement between the validation strains analyzed by MALDI-TOF MS and those evaluated using 16S rRNA gene sequencing analysis methods. Therefore, MALDI-TOF MS is a promising, rapid, and economical method to monitor the outbreaks and spread of *B. pseudomallei* and *B. thailandensis* isolates.

Keywords *Burkholderia pseudomallei* · *Burkholderia thailandensis* · Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry · 16S rRNA gene sequencing analysis · SuperSpectra

Introduction

Burkholderia pseudomallei is a causative agent of melioidosis, an infectious disease endemic to Southeast Asia, northern Australia, and other tropical regions [1–4]. In China, the first case of melioidosis was reported in 1990 [5]. More sporadic and epidemic cases have been reported in

several tropical provinces, including Hainan, Guangdong, and Guangxi since then [6–10]. In the genus *Burkholderia*, the morphology and immunogenicity of colonies of *B. pseudomallei* and *B. thailandensis* are very similar and difficult to distinguish. Therefore, the identification of *B. pseudomallei* and *B. thailandensis* presents a considerable challenge for clinical microbiology laboratories [11–15].

B. pseudomallei is included in the API 20NE and VITEK-2 Compact databases, but the accuracy of identification by these systems varies [12, 14–17]. *B. pseudomallei* can be distinguished from *B. thailandensis* by arabinose assimilation; *B. thailandensis*, but not *B. pseudomallei*, is able to utilize L-arabinose as its sole carbon source [17]. Misidentification of *B. pseudomallei* as other *Burkholderia* species, such as *B. cepacia* and *B. putida* as well as *Pseudomonas aeruginosa*, is common when using these commercial systems [12, 14, 15]. Genotypic differentiation between *B. pseudomallei* and *B. thailandensis* can be achieved by specific PCR-based identification using *B. pseudomallei*-specific gene targets [18, 19]. As these methods are time consuming and complex, they are only suitable for retrospective studies and not rapid clinical detection.

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a revolutionary technique for pathogen identification, has been shown to be a potentially useful tool for the rapid identification of suspicious isolates [11, 16, 20, 21]. Since *B. thailandensis* was included in the reference spectra of the VITEK MS libraries (Saramis database) rather than *B. pseudomallei*, *B. pseudomallei* cannot be correctly identified in the current version of VITEK MS [12]. Therefore, the existing database requires optimization by adding reference spectra for *B. pseudomallei* and creating the SuperSpectra for *B. pseudomallei* and *B. thailandensis*. In this study, we evaluated the utility of MALDI-TOF MS with the VITEK MS plus system for detecting *B. pseudomallei* and *B. thailandensis* isolates.

Materials and methods

Bacterial isolates

A total of 26 *B. pseudomallei*, 20 *B. thailandensis*, 20 *B. cepacia*, 20 *B. multivorans*, and 20 *B. cenocepacia* isolates were collected from the Hainan Medical College Hospital, Daping Hospital, Southwest Hospital, and Children's Hospital of Chongqing Medical University and used as validation strains. In addition, 10 strains of *B. pseudomallei* and 10 strains of *B. thailandensis*, including *B. pseudomallei* C006 (BPC006) and *B. thailandensis* E264 (ATCC700388), were collected from the above-mentioned hospitals and selected as the established strains. As *B. pseudomallei* could be used in a bioterrorism attack, access to the international standard strain of *B. pseudomallei* is limited, and we therefore selected *B. pseudomallei* C006 (BPC006) and *B. thailandensis* E264 (ATCC700388) as the reference strains. The arabinose assimilation test was performed for all genetically confirmed isolates as reported previously [12]. Culturing and analysis of all 126 strains were performed in a biosafety level two-plus mycobacteriology laboratory at the Daping Hospital following biosafety level two-plus precautions.

Identification of isolates by gene sequencing analysis

The identification of all 126 strains used in this study was confirmed at the species level by 16S rRNA gene sequencing by using the primers described by Chang et al. [22]. The PCR products (1397 bp) were purified using a 3S spin PCR product purification kit (Shenergy Bicolor, China), and the sequences were searched against the GenBank database using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast>).

MALDI-TOF MS

Sample preparation All isolates were inoculated onto sheep blood agar and incubated in 5% CO₂ at 37 °C for 24 h. The sample preparation methods, interpretation of the results, and quality control strains have been previously described [23]. Briefly, two or three colonies were transferred to a 1.5-mL Eppendorf tube containing 300 µL of water and 900 µL of ethanol. The microcentrifuge tube was vortexed for 5 min and then incubated at room temperature for 10 min, the supernatant was discarded, and the bacterial pellet was resuspended in 50 µL of 70% formic acid and 50 µL of acetonitrile. The suspensions were centrifuged at 13,000 rpm for 2 min, after which 1.0 µL of each supernatant was applied to a 48-spot polished-steel target plate (bioMerieux, France) and dried. One microliter of a saturated α-cyano-4-hydroxycinnamic acid matrix solution (bioMerieux, France) was applied to each sample and allowed to dry before the plate was loaded into MALDI-TOF MS.

MALDI-TOF MS acquisition Protein mass fingerprints were obtained using a MALDI-TOF MS plus mass spectrometer and were within a mass range of 2000 to 20,000 Da with a tolerance of 0.08%. For each target slide, *Escherichia coli* ATCC8739 was used for instrument calibration according to the manufacturer's protocol. After the spectrum acquisition, the data were transferred from a VITEK MS acquisition station to the Saramis analysis server. The data were reported as the number of peaks and the highest-level matches compared to those for the Saramis 4.14 research-use-only (RUO) database.

Selection of reference spectra Because *B. pseudomallei* was not listed in the current RUO Saramis database, we created new reference spectra for *B. pseudomallei*. A new folder for *B. pseudomallei* was added to the spectral taxonomy tree, and then the imported spectra were pasted into the *B. pseudomallei* folder. In addition, we tested the *B. pseudomallei* C006 (BPC006) and *B. thailandensis* E264 (ATCC700388) using the VITEK MS plus system and determined the different mass spectrum peaks by Launchpad software and cluster analysis. Then, all of the established strains were repeatedly tested by the VITEK MS plus system eight times each. To be selected, a reference spectrum needed to have a mass number between 100 and 200 and have more than 65% of common masses with other spectra from the same species. Moreover, to be chosen, spectra from the same considered isolate needed to have more than 65% homology. These criteria were recommended by the manufacturer (bioMerieux, France).

Creation of SuperSpectra To create the SuperSpectra, we first imported the reference spectra data. Next, the SuperSpectra were created on the basis of the selection of protein masses specific to *B. pseudomallei* and *B. thailandensis*. Finally, we

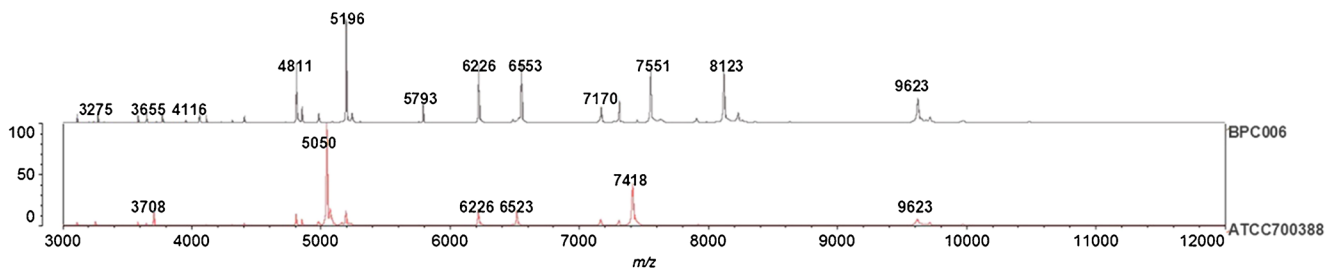


Fig. 1 Peak profiles of the spectra from *B. pseudomallei* C006 (BPC006) and *B. thailandensis* E264 (ATCC700388). Characteristic spectra of *B. pseudomallei* C006 (BPC006) and *B. thailandensis* E264 (ATCC700388) were generated using Launchpad software. The absolute intensities of the ions are shown on the y-axis, and the masses (m/z) of the ions are shown on the x-axis. The m/z values represent the

mass-to-charge ratios. For *B. pseudomallei* C006 (BPC006), 12 peaks were regarded as highly specific signals (3275 m/z, 3655 m/z, 4116 m/z, 4811 m/z, 5196 m/z, 5793 m/z, 6226 m/z, 6553 m/z, 7170 m/z, 7551 m/z, 8123 m/z, and 9623 m/z). For *B. thailandensis* E264 (ATCC700388), six peaks were regarded as highly specific signals (3708 m/z, 5050 m/z, 6226 m/z, 6523 m/z, 7418 m/z, and 9623 m/z)

chose 39 specific masses to create SuperSpectra based on comprehensive factors, such as the number of consensus peaks and peak intensity. For each species, a SuperSpectrum was created on the basis of the selection of the protein masses specific to the considered species, which were compared with all the peaks in the Saramis database. A greater or lesser weight was assigned to each of the selected masses depending on the number of matches for the first nontarget species. Finally, the SuperSpectra were activated for subsequent automated identification at the species level.

Identification of isolates with the VITEK MS plus system

To assess the capability and stability of the newly created SuperSpectra, external validation was performed for the

remaining 106 validation isolates. Each MALDI-TOF MS spectrum was first compared with the SuperSpectra in the Saramis database provided by the manufacturer. When a spectrum matched the SuperSpectra, a confidence value for the best match was given. Identification was valid when the confidence value was higher than 75% compared with the SuperSpectra as defined by the manufacturer (Saramis Premium User Manual, VITEK MS Plus; bioMerieux).

Results

Bacterial identity

A complete VITEK-2 Compact GN card test (Supplementary Fig. S1), an arabinose assimilation test (Supplementary Fig.

Fig. 2 Clustering of mass spectra of *B. pseudomallei* and *B. thailandensis*. Based on the Saramis dendrogram threshold of 65%, the mass spectrum peaks of 20 established strains (10 strains of *B. pseudomallei* and 10 strains of *B. thailandensis*) were divided into two groups by the VITEK MS plus system. For details, see text

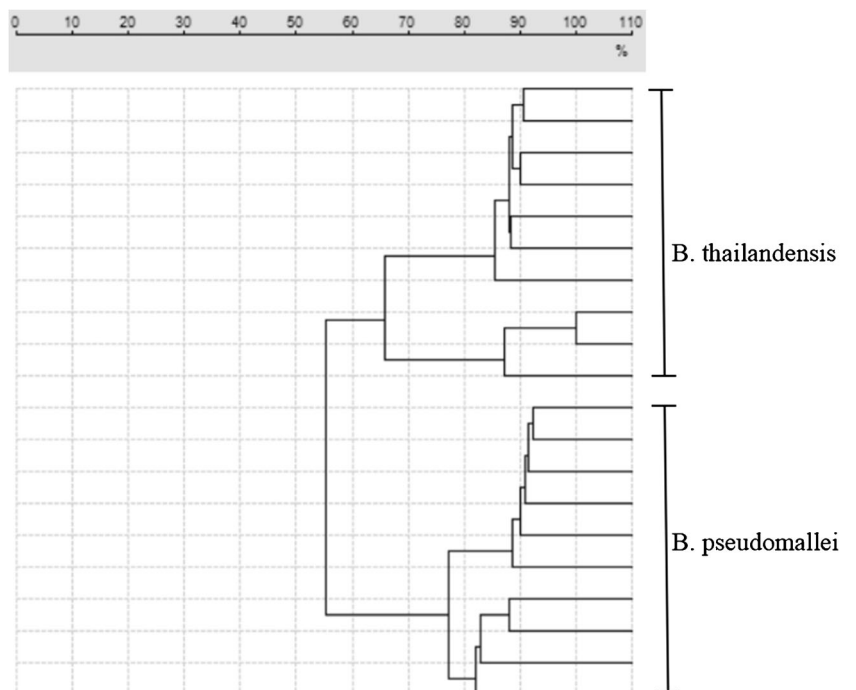


Table 1 Protein masses selected for the new SuperSpectra creation

Protein masses selected for the first SuperSpectra creation	
Species	Masses (Da) selected for the SuperSpectra creation
<i>B. pseudomallei</i>	3112; 3275; 3585; 3655; 3725; 3776; 3996; 4116; 4315; 4411; 4458; 4811; 4826; 4859; 4988; 5154; 5196; 5243; 5304; 5759; 5793; 6226; 6490; 6553; 6573; 7170; 7268; 7311; 7551; 7993; 8123; 8363; 8449; 8630; 8918; 9623; 9716; 9974; and 10,448
<i>B. thailandensis</i>	3239; 3261; 3655; 3708; 3756; 3839; 3961; 4043; 4063; 4210; 4277; 4290; 4315; 4327; 4411; 4811; 4859; 5050; 5073; 5196; 5223; 5243; 6226; 6523; 6842; 7170; 7311; 7418; 7455; 7923; 7986; 8087; 8125; 8233; 8554; 8654; 9623; 9718; and 10,486
Protein masses selected for the second SuperSpectra creation	
Species	Masses (Da) selected for the SuperSpectra creation
<i>B. pseudomallei</i>	3113; 3275; 3586; 3655; 3728; 3777; 3994; 4063; 4116; 4315; 4411; 4460; 4731; 4811; 4825; 4859; 4989; 5153; 5196; 5244; 5306; 5434; 5760; 5793; 6000; 6226; 6553; 7170; 7271; 7311; 7551; 7990; 8123; 8450; 8631; 9623; 9719; 9975; and 10,448
<i>B. thailandensis</i>	3239; 3261; 3708; 3962; 3994; 4043; 4063; 4209; 4277; 4290; 4315; 4327; 4411; 4811; 4859; 4886; 5076; 5156; 5196; 5220; 6226; 6523; 7170; 7311; 7418; 7457; 7925; 7986; 8088; 8128; 8235; 8408; 8556; 8635; 8656; 9463; 9623; 9722; and 10,486

A greater or lesser weight was assigned for each of the 39 selected masses, and we chose the two SuperSpectra to activate for subsequent automated identification of the *B. pseudomallei* and *B. thailandensis* isolates

S2), and 16S rRNA gene sequencing analysis (Supplementary Figs. S3 and S4) were performed for all 126 strains, and the results showed that all of the strains were correctly identified except *B. thailandensis* strains. Initially, *B. thailandensis* strains were misidentified as *B. pseudomallei* by the VITEK-2 compact GN card. Then, they were correctly confirmed as *B. thailandensis* by arabinose assimilation tests and 16S rRNA gene sequence analysis.

MALDI-TOF MS

MALDI-TOF MS characterization of bacteria is based on differences in the mass-to-charge ratio (m/z) fingerprints of the microorganisms' proteins, which are primarily ribosomal proteins that are most abundantly expressed under all growth conditions. For the first stage in this study, we found 12 specific peaks for *B. pseudomallei* C006 (BPC006) and six

Table 2 Results of VITEK MS plus system in identification of the validation strains using the newly created SuperSpectra

Validation strains	No. of strains	Identification by VITEK MS	Confidence values (%)	Percentage (%)
<i>B. pseudomallei</i>	14	<i>B. pseudomallei</i>	99.9	53.8
	8	<i>B. pseudomallei</i>	90.0–99.9	30.8
	4	<i>B. pseudomallei</i>	80.0–90.0	15.4
Subtotal	26	<i>B. pseudomallei</i> accuracy rate		100.0
<i>B. thailandensis</i>	11	<i>B. thailandensis</i>	99.9	55.0
	5	<i>B. thailandensis</i>	90.0–99.9	25.0
	4	<i>B. thailandensis</i>	80.0–90.0	20.0
Subtotal	20	<i>B. thailandensis</i> accuracy rate		100.0
<i>B. cepacia</i>	6	<i>B. cepacia</i>	99.9	30.0
	8	<i>B. cepacia</i>	90.0–99.9	40.0
	6	<i>B. cepacia</i>	80.0–90.0	30.0
Subtotal	20	<i>B. cepacia</i> accuracy rate		100.0
<i>B. multivorans</i>	8	<i>B. multivorans</i>	99.9	40.0
	8	<i>B. multivorans</i>	90.0–99.9	40.0
	4	<i>B. multivorans</i>	80.0–90.0	20.0
Subtotal	20	<i>B. multivorans</i> accuracy rate		100.0
<i>B. cenocepacia</i>	8	<i>B. cenocepacia</i>	99.9	40.0
	6	<i>B. cenocepacia</i>	90.0–99.9	30.0
	6	<i>B. cenocepacia</i>	80.0–90.0	30.0
Subtotal	20	<i>B. cenocepacia</i> accuracy rate		100.0
Total	106	Total accuracy rate		100.0

A total of 106 validation isolates were tested using the new SuperSpectra implemented in the Saramis database. The validation results showed that all these validation strains were identified correctly by VITEK MS plus system

specific peaks for *B. thailandensis* E264 (ATCC700388) by using Launchpad software (Fig. 1). Based on the Saramis dendrogram threshold of 65%, the mass spectrum peaks of 20 established strains were divided into two groups by the VITEK MS plus system, as shown in Fig. 2, indicating that there may be different peaks between the mass spectra of *B. pseudomallei* and *B. thailandensis* (Fig. 1). This finding would support the hypothesis that both species can be separated by MALDI-TOF MS. Then, the established strains (10 strains of *B. pseudomallei* and 10 strains of *B. thailandensis*) were tested eight times by MALDI-TOF MS, and the spectra of *B. pseudomallei* and *B. thailandensis* were obtained. All the spectra were added to the Saramis database as reference spectra. For each species, we selected the specific masses and weighted them by checking the weight table to create the SuperSpectra (Table 1). Finally, a total of 106 validation isolates were tested using the new SuperSpectra implemented in the Saramis database. The validation results showed that all these validation strains were identified correctly by the VITEK MS plus system. The SuperSpectra evaluation results are summarized in Table 2. All quality control (QC) results were within the designated acceptable range.

Discussion

Traditional molecular epidemiology analyses are unable to control the rapid spread of nosocomial infections when facing an outbreak infection of *B. pseudomallei* and *B. thailandensis* [12, 22]. Therefore, the need to develop a new method for detecting *B. pseudomallei* and *B. thailandensis* isolates is urgent. As an emerging proteomic tool for microbial identification, MALDI-TOF MS is superior to other methods in saving time and reducing cost [12, 20, 21, 23, 24]. Based on these studies, we hope that MALDI-TOF MS can correctly identify isolates of *B. pseudomallei* and *B. thailandensis*.

In this study, all the validation strains were correctly identified at the species level by using our newly created database. These findings show that MALDI-TOF MS is an efficient and robust tool for the rapid identification of *B. pseudomallei* and *B. thailandensis*. However, this successful application of MALDI-TOF can be regarded only as a pilot study due to the small sample size, which needs independent validation before it is used as a routine technique for clinical identification.

The limitation of this study is that we chose only 10 strains of *B. pseudomallei* and 10 strains of *B. thailandensis* as the established strains, and we need to further expand the number of established strains to obtain better identification results in later research. Strains in different countries and regions may also have obvious geographical differences, which indicates that we need to continuously update the existing information

on the Saramis database [12]. Furthermore, the validation strains are also limited in this study. To ensure the accuracy and reliability of the identification results, we need to further expand the number and types of established strains and validation strains and continuously update the VITEK MS database to obtain better identification results. Therefore, we can conclude that MALDI-TOF MS is a promising, rapid, and economical method to monitor the outbreaks and spread of *B. pseudomallei* and *B. thailandensis* isolates.

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