Research on the establishment and application of protein fingerprint spectrum database of Burkholderia pseudomallei in Hainan Province China

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Research article

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Abstract

**Background:** The aim of this study was to establish a SuperSpectrum of *Burkholderia pseudomallei* (*B. pseudomallei*) in Hainan and evaluate its application value in the rapid identification of clinical isolates of *B. pseudomallei*.

**Methods:** Using a collection of 167 isolates of *B. pseudomallei* from June 2010 to May 2019 in different regions of Hainan province, multilocus sequence typing (MLST) was performed, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was applied for spectrum acquisition. A SuperSpectrum was created based on the selection of 80 representative average spectra. In a second step, we validated the SuperSpectra with 137 strains of *B. pseudomallei*, 8 strains of *Burkholderia thailandensis* (*B. thailandensis*), 2 strains of *Burkholderia cepacia* (*B. cepacia*), 1 strain of *Burkholderia cenocepacia* (*B. cenocepacia*) and 1 strain of *Burkholderia multivorans* (*B. multivorans*), as well as 1 strain of *Burkholderia gladioli* (*B. gladioli*) identified by MLST typing and 16S rRNA gene sequencing.

**Results:** The results showed that there was 100% agreement between the validation strains analyzed by MALDI-TOF MS and those evaluated by MLST typing and 16S rRNA gene-sequencing analysis methods. Protein fingerprints spectra showed that specific peaks occurred in *B. pseudomallei* from the Hainan region. The result of clustering typing indicated that *B. pseudomallei* and its closely related species could be well classified by MALDI-TOF MS at the protein level.

**Conclusions:** MALDI-TOF MS is a promising, rapid, and economical method to monitor the outbreaks and spread of *B. pseudomallei* isolates. The establishment of an accurate and objective SuperSpectrum database can provide a new platform for the clinical rapid diagnosis of melioidosis.

Background

Melioidosis is a zoonotic infectious disease caused by *Burkholderia pseudomallei* (*B. pseudomallei*). Acute systemic melioidosis has a case fatality rate of up to 50% even if treated[1] and is the third most common cause of death from infectious diseases[2]. *B. pseudomallei* is widely distributed in water and soil throughout tropical and subtropical zones, and is largely prevalent in northeastern Thailand[3], southern Taiwan[4], and northern Australia[3]. The Hainan province is an international tourist island and the main epidemic source of melioidosis in China. Due to increased travel activity, international trade, climate change and the potential biothreat of *B. pseudomallei*, infection caused by *B. pseudomallei* has become a serious problem. Therefore, rapid and accurate identification of the bacteria is of great significance. Currently, the methods of detecting *B. pseudomallei* primarily include biochemical identification, molecular biology methods and mass spectrometry. Rapid biochemical analysis systems, such as API 20NE, Vitek 1 and Vitek 2 database, all contain *B. pseudomallei*, but it takes about 6h-18 h, and the correct identification rate is unstable (accuracy was 53%–98%)[5]. Molecular biology methods, including 16s rRNA, multilocus sequence typing (MLST)[6] and whole-gene sequencing, are highly...
accurate and sensitive, but because of the high technical requirements and the need for specialized instruments, they cannot be routinely applied in clinical laboratories. MALDI-TOF MS has the potential to identify pathogens rapidly and reliably, but _B.pseudomallei_ is not in the FDA-approved database. Thus, the establishment of localized SuperSpectrum of _B.pseudomallei_ is the premise for rapid and accurate identification of the bacteria. In this study, the characteristic fingerprint spectrum of _B.pseudomallei_ was acquired by MALDI-TOF MS, and the SuperSpectrum of _B.pseudomallei_ was created to realize rapid and correct identification of _B.pseudomallei_ to provide technical support for the epidemic outbreak and traceability of melioidosis.

**Materials And Methods**

A total of 167 _B.pseudomallei_ were collected from clinical samples of melioidosis patients in different regions of Hainan. In addition, 7 _B.thailandensis_ isolates were acquired from the soil in parts of Hainan, including Sanya (4 strains), Wanning (2 strains) and Ledong (1 strain). The reference strains of _B.thailandensis_(ATCC700388), _B.cepacia_ (ATCC25416) and _B.cenocepacia_ (ATCC25608) were all purchased from Beijing Zhongyuan company. Strains of _B. cepacia_, _B.multivorans_ and _B.gladioli_ were all obtained from clinical isolates of Hainan General Hospital, accounting for one strain each. A total of 30 representative _B.pseudomallei_ isolates from different regions of Hainan were selected as the established strains.

**VITEK2 Compact biochemical identification**

After recovering all 180 strains stored at -80°C, they were inoculated with quality-control strain _Enterobacter cloacae_ ATCC700323 onto the Columbia Sheep Blood Agar Plate (CBA)(ZhengZhou AnTu Biological Company,ZhengZhou City,China), MacConkey Agar Plate (MAC)(Zheng Zhou AnTu Biological Company,ZhengZhou City,China), and Influenza Blood Chocolate Plate (Vancomycin Chocolate Agar Plate, CHA,Zheng Zhou AnTu Biological Company,ZhengZhou City,China) and incubated in 5% CO\textsubscript{2} at 35°C for 18-24 h. Vitek 2 compact(BioMerieux,French) VT2.R 7.01 of the GN(BioMerieux,French) card was used to identify the colonies grown on three different media, respectively, and the specific operation was conducted according to the standard operating procedure in the instrument manual.

**Molecular biological identification**

Using 16s rRNA gene sequencing

The identification of 13 strains (8 strains of _B. thailandensis_, 2 strains of _B. cepacia_, 1 strain of _B. cenocepacia_, 1 strain of _B. multivorans_ and 1 strain of _B. gladioli_) used in this study was confirmed at the species level using the primers described by Brett et al.[7-9]. The PCR products were sent to Tianyihui Yuan Company (guangzhou,China) for sequencing, and the sequences were searched against the GenBank database using the BLAST algorithm(https://blast.ncbi.nlm.nih.gov/Blast.cgi).

MLST sequencing and typing of _B.pseudomallei_ pathogenic strains
The specific methods of MLST analysis referred in detail to the *B. pseudomallei* MLST research scheme established by Godoy *et al.* [10]. The sequencing results of alleles on seven housekeeping genes of each *B. pseudomallei* pathogenic strain were compared with gene sequences in the MLST database to find their respective allele numbers. The composition of all allele numbers was the *B. pseudomallei* sequence type (ST).

**MLST sequencing and typing of *B. thailandensis* [10]**

A total of seven isolates of *B. thailandensis* other than the reference strain (ATCC700388) were recovered and their chromosomal DNA was extracted, followed by PCR amplification and sequencing on seven alleles (ace, dtB, gmhD, lepA, lipA, narK, ndh). TaKaRa LA Taq with GC buffer system and 50 μl reaction volumes were used for PCR. Amplification conditions: 95°C for 2 min; 30 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 40 s; 72°C for 7 min. PCR products were sequenced bidirectionally. The sequencing results were collated and submitted to the MLST database ([http://pubmlst.org/bpseudomallei/](http://pubmlst.org/bpseudomallei/)) to obtain the allele sequence number and determine the ST of strains.

**MALDI-TOF MS acquisition**

All of the isolates were inoculated onto the Columbia Sheep Blood Agar Plate (CBA), and 30 established strains were incubated onto the Columbia Sheep Blood Agar Plate (CBA), MacConkey Agar Plate (MAC) and Influenza Blood Chocolate Plate (Vancomycin Chocolate Agar Plate, CHA). All of the plates were inoculated in 5% CO₂ at 37°C for 24 h. The 30 established strains were from different regions of Hainan island. The monoclonal colonies were picked up by 1 μl volume of sterile inoculation loop and uniformly spread on the target plate. After drying, 1 μl of CHCA matrix solution was added, and then dried at room temperature to form crystals. The VITEK MS (BioMerieux, French) was calibrated, and then mass spectra data were collected and analyzed as well as identified through SARAMS Premium software. After the spectrum acquisition, the data were transferred from a VITEK MS acquisition station to the Saramis analysis server.

**Selection of reference spectra**

A total of 100 mass spectra were collected for each established strain, and an average spectrum was generated for the 100 original spectra. SARAMS Premium software was used to transfer the average mass spectrum of *B. pseudomallei* bacteria from each sample and remove the debased spectra, and then aggregate these mass spectra to generate the reference spectra.

**Creation of SuperSpectra**

According to the specificity, intensity, mass-to-charge ratio (m/z) and other comprehensive parameters of the peak, a SuperSpectra containing all of the characteristic peaks was created. The original Spectra of the SuperSpectra should meet the following conditions: (1) The similarity between different strains of the same species should be 65% or more; (2) the original peak number of the database establishment spectra
was controlled between 100 and 200; (3) select 39 specific masses (the common rate of each specific masses was $\geq 80\%$); (4) the weight of the obtained specific masses was 31, and the sum of the weights was $31 \times 39 = 1209 < 1400$; (5) the error range of mass number (%) was 0.0013-0.0746 < 0.08; (6) the absolute peak and relative peak intensity (%) range: 0.30-43.5 > 0. The SuperSpectra were activated for subsequent automated identification at the species level after it have been created.

**Verification of SuperSpectra for B.pseudomallei isolates**

To assess the capability and stability of the newly created SuperSpectra, external validation was performed for the remaining 150 validation isolates. Of the 150 strains, 137 were *B.pseudomallei* strains and their closely related species (8 strains of *B. thailandensis*, 2 strains of *B. cepacia*, 1 strain of *B. cenocepacia*, 1 strain of *B. multivorans* and 1 strain of *B. gladioli*). The 150 spectra were imported into SARAMS Premium software for pattern matching with the newly created SuperSpectrum database and the obtained identification results were analyzed for evaluating the clinical identification capabilities of the VITEK MS super mass spectrum of *B.pseudomallei*.

**MALDI-TOF MS dendrogram**

To construct the dendrogram, Flexanalysis software (version 3.4) was used to adjust the baseline and smoothness of the spectra. Cluster analysis was performed based on comparison the similarity of Main Spectra Projection by calculated the pattern matching.

**Results**

**Vitek 2 Compact biochemical identification**

The number of 137 *B.pseudomallei* strains cultured on three different media that were accurately identified as *B.pseudomallei* was as follows: 134 strains on CBA, 128 strains on MAC and 125 strains on CHA. Additionally, a total of eight strains of *B. thailandensis*, two strains of *B. cepacia* and one strain of *B. gladioli* were all accurately identified. In addition, one reference strain of *B.cenocepacia* and one strain of *B.multivorans* were misidentified as *B. cepacia*.

**Molecular biological identification results**

Using 16S rRNA gene sequencing, a total of eight strains of *B.thailandensis*, two strains of *B.cepacia*, one strain of *B.cenocepacia*, one strain of *B.multivorans* and one strain of *B. gladioli* were all identified correctly.

Through identification and typing of MLST, a total of 167 isolates including the strains established database were all *B.pseudomallei* strains, and the most common STs were ST46, ST55, ST50 and ST58. Furthermore, all of the eight isolates of *B.thailandensis* were identified accurately as *B.thailandensis*, and the most common ST was ST345.
MALDI-TOF MS identification results

The identification results of 150 *Burkholderia* isolates were consistent with gene sequencing. In addition, a total of 137 *B.pseudomallei* strains, 8 strains of *B.thailandensis*, 2 strains of *B. cepacia*, 1 strain of *B.cenocepacia* and 1 strain of *B.multivorans* as well as 1 strain of *B.gladioli* were all identified accurately. The correct identification rate of *B.pseudomallei* database was 100% and the confidence interval was 75.5%-99.9%. At the same time, the identification repeatability of the same isolates was 100%, and cross-error identification did not occur. All of the original spectra obtained before the SuperSpectrum established of 137 *B.pseudomallei* isolates were no match by comparing with SARAMS Premium database.

Identification of discriminatory peaks

In this study, it was demonstrated that the specific peaks of Hainan were partially different from those of Thailand[3,11], Taiwan [4], Australia[3] and other endemic regions. *B.pseudomallei* isolates in the above areas were all found in 2049 Da, 4410 Da, 5149 Da, 6551 Da and 7169 Da, respectively.see text Fig 1. Furthermore, the specific peaks of Hainan were extremely similar to those of *B. thailandensis* (around 2206 Da, 2597 Da, 3112 Da, 3585 Da, 4043 Da, 4115 Da, 4410 Da, 5194 Da, 6226 Da, 7170 Da, 9621 Da and 10486 Da ),see text Fig 1.Moreover, nine specific peaks (2876 Da, 3275 Da, 3658 Da, 3762 Da, 3997 Da, 4810 Da, 6551 Da, 7526 Da and 8094 Da ) have not previously been reported in other regions, which may be a unique biomarkers to *B.pseudomallei* strains in Hainan.

Phylogeny based on MALDI-TOF MS protein profiles

Based on the Saramis dendrogram threshold of 65%, the mass spectrum peaks of 150 *Burkholderia* strains were divided into three groups by the VITEK MS system. These three groups were *B.pseudomallei*, *B. thailandensis* and other closely related species of *Burkholderia*,see text Fig 2.

Discussion

MALDI-TOF MS is a time-of-flight mass spectrometry technique for microbial identification and typing, which was developed in recent years. It uses a specific mass-to-charge ratio among different bacteria as a biomarker molecule to identify bacteria, which has the advantages of rapid, accurate and high throughput. Moreover, compared with the VITEK 2 Compact automatic microbial analysis system and 16S rRNA sequencing method, MALDI-TOF MS can shorten the identification time of bacteria from 6 h to 48 h to several minutes. However, its identification and typing ability of unknown bacteria must be established in the spectral database with enough known strains to search in order to realize the best matching and true and reliable strain identification results. Due to the apparent geographical distribution characteristics of *B.pseudomallei* and the bacteria is not listed in the current version of FDA approved database. Therefore, the laboratory can establish a high-quality protein fingerprint database of local *B.pseudomallei* strains on the basis of the original database according to the epidemic characteristics of the region to realize the rapid diagnosis of melioidosis.
At present, there are few data on the accurate identification of \textit{B.pseudomallei} by MALDI-TOF MS, which is still in the Basic research stage and has not been widely used in clinical practice. Thus, we need to acquire more data to provide theoretical basis for the comprehensive application of this technology in clinical practice in the future. Since \textit{B.pseudomallei} bacteria is not contained in the commercialized database, a localized \textit{B.pseudomallei} database should be established firstly to identify \textit{B.pseudomallei} strains by mass spectrometry. The first published study of its application to \textit{Burkholderia} spp. was in 2012. Karger\cite{12} et al. established the database using 10 \textit{B.pseudomallei} strains and 17 \textit{B. mallei} strains. Then 9 \textit{B.pseudomallei} strains (including 2 strains of database established) and 16 \textit{B. mallei} strains (including 12 strains of database established) were used for verification, with an accuracy of 100\% \cite{12}. The limitation of the above study was that there were fewer strains within the database for construction and verification, and the validation of strains contains the established strains, which resulted in less representative. Since then several related studies have been published. Wang \textit{et al.}\cite{4} used 5 \textit{B.pseudomallei} strains from National Taiwan University Hospital and Peking Union Medical College Hospital to establish a database in 2016. Additionally, they used 57 \textit{B.pseudomallei} strains and 60 strains of \textit{B. cepacia} and \textit{B. putida} to verify the newly created database, in which \textit{B.pseudomallei} can be correctly identified and \textit{B. cepacia} and \textit{B. putida} were not identified mistakenly for \textit{B.pseudomallei}\cite{4}. The largest evaluation of \textit{B. pseudomallei}, to date, has been undertaken using 26 strains of \textit{B.pseudomallei} from Thailand, Laos, Cambodia, Australia and 21 other \textit{Burkholderia} strains to construct the database\cite{3}. They then tested the accuracy of the new database for the identification of 581 \textit{B.pseudomallei} strains, 19 \textit{B. mallei} strains, 6 \textit{B. thailandensis} strains and 23 other strains\cite{3}. All of the 581 \textit{B. pseudomallei} were correctly identified, with 100\% sensitivity and specificity\cite{3}. In 2018, Li \textit{et al.}\cite{13} used 10 strains of \textit{B.pseudomallei} and 10 strains of \textit{B. thailandensis} strains to establish database, and selected 20 strains of \textit{B.pseudomallei}, 20 strains of \textit{B. thailandensis}, 20 strains of \textit{B. cepacia} and 20 strain of \textit{B. multivorans} identified the newly established database, and the accuracy was 100\%.

The characteristic fingerprint spectrum was acquired and construction of SuperSpectrum database by collecting data of 30 \textit{B.pseudomallei} isolates from different times and regions in this study. What's more, the database was verified by 150 \textit{Burkholderia} spp., and the results indicated that the confidence level of \textit{B.pseudomallei} was 75.5\%-99.9\%, and that accurate identification of \textit{B.pseudomallei} can be realized through MALD-TOF MS. The results demonstrated that the identification accuracy rate of \textit{B.pseudomallei} was 100\%, the confidence interval was 75.5\%-99.9\%, and the correct repeated identification rate of the same strain was 100\%. Our findings confirm that the quality of the self-built laboratory database was ideal, rapid and reliable for identification of \textit{B.pseudomallei} and its related species. This advantage is particularly significant for prevention and control of infectious diseases caused by the “bioterrorist bacteria,” such as \textit{B.pseudomallei}, which can prompt the laboratory staff to take personal protection as soon as possible. Moreover, this patient sample and its culture could be operated in strict accordance with the relevant biosafety level requirements to reduce the risk of laboratory infection. Compared with the previously reported, \textit{B.pseudomallei} strains from different regions of Hainan Island were selected to construct the database in this study, and the number of strains for establishing and verifying the database was more than ever before (except Suttisunhakul \textit{et al.}\cite{3} 2017 report), has very good
representativeness and stability. In addition, our self-built database spectra can not only be saved and implanted into bioMerieux's mass spectrometer database system or other institutions but also the data sharing can improve the identification rate of such bacteria. What's more, a total of 150 *B. pseudomallei* strains were clustered and typed according to the spectra characteristics of different strains while establishing the mass spectrum database of *B. pseudomallei* bacteria in this study. On the one hand, the result of mass spectrometry clustering typing was more consistent with the category of molecular biology typing, on the other hand, it was similar to the result reported in other literatures, indicating that mass spectrometry technology can be well applied to the clustering typing of bacteria and provides technical support for the prevention and traceability of the outbreak and epidemic of *B. pseudomallei* bacteria.

SARAMS Premium software was used to analyze the spectral data of *B. pseudomallei* bacteria and its related species statistically in order to understand whether the protein fingerprint spectra of *B. pseudomallei* strains in Hainan was different from other endemic areas. Our observation that the specific peaks of 2049 Da, 4410 Da, 5194 Da, 6551 Da and 7169 Da were the same as the specific peaks of *B. pseudomallei* isolates from endemic regions such as Thailand[3,11], Taiwan[3] and Australia[3], and had an extremely high similarity (a total of 12 mass spectra peaks between 2206 Da, 2597 Da, 3112 Da, 3585 Da, 4043 Da, 4115 Da, 4410 Da, 5194 Da, 6226 Da, 7170 Da, 9621 Da and 10486 Da were identical) with *B. thailandensis* isolates from different areas of this province. However, the peaks of 2876 Da, 3275 Da, 3658 Da, 3762 Da, 3997 Da, 4810 Da, 6551 Da, 7526 Da and 8094 Da were significantly different from those of other regions, which may be a unique biomarker of *B. pseudomallei* in Hainan.

**Conclusion**

Our findings show that MALDI-TOF MS is an efficient and robust tool for the rapid identification of *B. pseudomallei* and their closely related species after the self-created *B. pseudomallei* database was established. Therefore, MALDI-TOF MS has a high application value in the clinical diagnosis of melioidosis, so that patients can be treated timely and effectively, and take appropriate hospital infection control measures to prevent the spread of the epidemic. Additionally, it could strengthen biosafety management to avoid laboratory-acquired infections.

**Abbreviations**

*B. pseudomallei*: Burkholderia pseudomallei; MLST: multilocus sequence typing; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; *B. thailandensis*: Burkholderia thailandensis; *B. cepacia*: Burkholderia cepacia; *B. cenocepacia*: Burkholderia cenocepacia; *B. multivorans*: Burkholderia multivorans; *B. gladioli*: Burkholderia gladioli; CBA: Columbia Sheep Blood Agar Plate; MAC: MacConkey Agar Plate; CHA: Inuenza Blood Chocolate Plate; ST: sequence type; m/z: mass-to-charge ratio
Declarations

Acknowledgements

Not applicable.

Authors’ contributions

Xu-Ming Wang, Ling-Li Liu, Hua Wu and Mei-Hui Huang isolated bacteria and performed the laboratory measurements. Xu-Ming Wang and Mei-Hui Huang made substantial contributions to conception and design, and drafted the manuscript. All authors read and approved the final manuscript.

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 Availability of data and materials

All the data and material involved in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The institutional review boards at the Hainan general hospital approved the study protocol.

Consent for publication

Written informed consent was obtained from all the participants for publication of this study. A copy of the written consent is available for review by the Editor of this journal.

Conflict of interest statement

No potential conflicts of interests with respect to publication of this article is stated here.
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**Figures**

![Characteristic MALDI-TOF MS spectra of B. pseudomallei.](image)

**Figure 1**

Characteristic MALDI-TOF MS spectra of B. pseudomallei. The vertical axis shows relative intensities of ions and the horizontal axis shows mass to charge ratio (m/z) or masses of ions (Da).
Clustering of mass spectra of Burkholderia spp. Based on the Saramis dendrogram threshold of 65%, the mass spectrum peaks of *B. pseudomallei* and its related species (8 strains of *B. thailandensis*, 2 strains of *B. cepacia*, 1 strain of *B. cenocepacia*, 1 strain *B. gladioli* and 1 strain *B. multivorans*) were divided into three groups by the VITEK MS system. For details, see text.