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ORIGINAL RESEARCH

Comparison of *Acinetobacter calcoaceticus-baumannii* Complex Identification by MALDI-TOF Mass Spectrometry and Biochemical Method, Using Whole-genome Sequencing as the Reference Standard

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Background: Given the limited treatment options for *Acinetobacter* infections due to drug resistance, timely and accurate diagnosis is crucial for effective management. This study evaluated the performances of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) in identifying *Acinetobacter* bacteria.

Methods: Retrospective forty bacterial isolates from Thailand previously identified as *A. baumannii* using biochemical method were recruited. The retrospective diagnostic performances of biochemical method and MALDI-TOF MS were compared, considering whole-genome sequencing (WGS) as the reference standard.

Results: For identification performance, accuracy was 70% for the biochemical method, 82.5% for MALDI-TOF MS using direct colony samples, and 80% for MALDI-TOF MS using protein extract samples. In comparison to WGS, the direct colony method achieved the highest typing concordance. Regarding processing speed, MALDI-TOF MS effectively reduces the turnaround time compared to the biochemical method ($p < 0.0001$).

Conclusion: MALDI-TOF MS significantly outperforms biochemical method in the species-level identification of *Acinetobacter*. The superior efficacies in terms of accuracy, resolution, and speed emphasize the technical robustness of MALDI-TOF MS and position the method as an excellent identification technique for *Acinetobacter* isolates.

Keywords: *Acinetobacter*, MALDI-TOF MS, whole-genome sequencing, microbiology, bacterial identification

Introduction

Nosocomial infections caused by *Acinetobacter* isolates pose a significant global threat.[1] *Acinetobacter baumannii*, in particular, exhibits remarkable ability to adapt and acquire drug-resistance genes, complicating treatment options.[2] Therefore, there is a pressing need for timely and accurate diagnosis of *A. baumannii* infections to allow for appropriate patient's management.

The *Acinetobacter calcoaceticus-baumannii* (ACB) complex, including *A. baumannii, A. calcoaceticus, A. nosocomialis, A. pittii, A. seifertii* and *A. dijkshoorniae*, is a lead cause of hospital-acquired *Acinetobacter* infections, with reports of community-acquired infection also on the rise.[2,3] Infections caused by *Acinetobacter* species other than *A. baumannii* are

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increasingly emerging;[4,5] unfortunately, these cases often remain overshadowed by the prevalence of *A. baumannii* in routine identification processes.[3] This oversight not only obscures the true roles of lesser-known *Acinetobacter* species in pathogenesis but also poses risks of misidentification as *A. baumannii*. Such errors can lead to inappropriate clinical management practices.

In many clinical microbiology laboratories, including those in Thailand, routine biochemical methods cannot reliably distinguish species, so isolates are often reported simply as ACB complex.[6] As a result, the true epidemiology of individual ACB species remains largely unknown. Whole-genome sequencing (WGS) can provide definitive species identification but is too costly and labor-intensive to apply to all clinical isolates in routine practice.[7] One clinically important reason to differentiate *A. baumannii* from non-baumannii ACB species is that *A. baumannii* generally exhibits higher rates of multidrug and carbapenem resistance, with implications for empirical therapy, infection control and surveillance.[3] In this context, there is a critical need for practical, high-throughput identification tools for accurately identify bacterial species within *Acinetobacter*.

Over the years, several microbiological diagnostic approaches have been developed to enhance bacterial identification performance. Phenotypic-based identification through automated biochemical testing is conventionally utilized in routine medical laboratories due to their rapid turnaround and reliable results.[8] However, phenotypic similarities within the genus *Acinetobacter* can lead to challenges in distinguishing between species using biochemical tests alone.[9] In response to these limitations, genotypic systems have been established and applied, including both polymerase chain reaction (PCR)-based[10] and sequence-based[11] typing methods, which demonstrate excellent specificity. Nonetheless, these genotypic approaches can be labour-intensive and costly compared to conventional phenotyping systems. In recent decades, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has gained recognition in microbiology laboratories as a solution to these challenges. MALDI-TOF MS undertakes protein fingerprinting for bacterial typing, offering a straightforward workflow as well as cost-effective and rapid identification.[12] This promising method has proven to be highly effective in identifying difficult-to-detect bacterial strains, including *Mycobacterium tuberculosis*[13] and *Burkholderia pseudomallei*,[14] among others.[15] However, the effectiveness of MALDI-TOF MS is contingent upon the comprehensiveness of relevant protein databases,[16,17] raising concerns about the epidemiological distinctiveness of various clinical *Acinetobacter* species.[18,19] Therefore, understanding performance variability in a particular epidemiological area is crucial before integrating this innovative technique into routine clinical practice.

However, the extent of species misidentification within the ACB complex and how much MALDI-TOF improves on routine biochemical methods when compared with a genomic reference remains unclear. In this study, we compare the performance of biochemical method (phenotypic-based identification) and MALDI-TOF MS, employing whole-genome sequence data as the reference standard. Our aim is to determine whether MALDI-TOF MS resolution and reliability are sufficient for identifying *Acinetobacter* isolates in Northeast Thailand, a region where multidrug-resistant *A. baumannii* is prevalent and other *Acinetobacter* species are emerging.[3,20]

Materials and Methods

Ethics, Data Collection and Bacterial Isolates

The research protocol utilizing retrospective bacterial samples and anonymous patient's records was reviewed and approved by the Institutional Review Board of Khon Kaen University (Approval number HE661102), in accordance with the Declaration of Helsinki. Since there is no human sample collection, the IRB approved a waiver of informed consent for this study.

A total of 40 leftover *Acinetobacter* isolates including 21 isolates were collected on 1st January – 31st December 2019 from Nakhon Phanom Hospital and 19 isolates were collected on 1st January – 31st December 2022 from Srinagarind Hospital, both located in Northeast Thailand. All bacterial isolates used in this study were previously identified as *A. baumannii* using the biochemical method (Vitex2 Compact system, bioMérieux, France). The bacterial isolates, originally obtained from triple sugar iron agar, were preserved in glycerol stock and stored at −80°C. Upon thawing, the bacterial isolates were sub-cultured onto nutrient broth agar plates, and isolated colonies from each sample were used for further experimentation. Clinical characteristics of patients were recruited from corresponding hospitals.

For turnaround times comparison, anonymous electronic records of the time between specimen check-in and report issuance were exported from Srinagarind Hospital on 3rd April 2024. For biochemical method (Vitex2 Compact system), we retrieved records of 345 cases during 1st January 2019 to 30th November 2020. For MALDI-TOF MS (Bruker Daltonics, Germany), we retrieved records of 285 cases during 1st March 2023 to 31st December 2023.

Genomic DNA Extraction

Bacterial genomic DNA extraction was conducted using the QIAGEN DNeasy Ultraclean Microbial Kit. All samples exhibited an acceptable ratio of over 1.80 at 260/280 nm, evaluated by Nanodrop spectrophotometer (Thermo Fisher). The yield of genomic DNA was calculated from the optical absorbance at 260 nm.

Multilocus Sequence Typing (MLST) Analysis

The assembled sequences were analyzed using multi-locus sequence-typing (MLST) profiles through in silico methods with the Bacterial Isolates Genome Sequence Database (BIGSdb) tool version 2.23.0 (https://github.com/tseemann/mlst),[21] utilizing the Pasteur scheme.[22]

Whole-Genome Sequencing and Phylogenetic Analysis

As a reference method for species identification, short-read sequencing was conducted on all genomic DNA samples using Illumina next-generation sequencing. A previously validated pipeline for bacterial WGS analysis was utilized in our study.[14] In brief, the fastq files were assessed for sequence quality, subsequently trimmed, and de novo assembled. For phylogenetic analysis, validated sequences were mapped to the reference genome of *A. baumannii* ATCC19606 (accession number: AP022836.1). Single nucleotide polymorphism (SNP) variants and insertions/deletions (indels) were identified. Mapping quality was set to a minimum of 50, while the base alignment quality (Q score) was maintained at 20. Variants were filtered based on a minimum depth of coverage of 10-fold and a Q score of 20. Subsequently, intersections of variation sets and analysis of nucleotide frequencies across strains were determined using an in-house Python script. In total, 155,811 SNP variants from all samples were filtered and a maximum-likelihood phylogenetic tree was constructed based on 14,022 high-confidence SNPs using RAxML[23] with 1000 bootstrap replicates and a general time-reversible (GTR) model with gamma distribution. The phylogenetic tree was subsequently visualized using iTOL (https://itol.embl.de/).

Bacterial Species Identification Using MALDI-TOF MS

Sample Preparation for MALDI-TOF MS with Direct Colony (DC) Method

A single colony was smeared and air dried onto the plate for MALDI-TOF analysis. Then, 1 µL of matrix solution, α-Cyano-4-hydroxycinnamic acid (CHCA), was added. All samples were tested in duplicate.

Sample Preparation for MALDI-TOF MS with Protein Extraction (PE) Method

A single colony was dissolved in 300 µL of sterile water and treated with 900 µL of cold ethanol. The mixture was centrifuged at 13,000 rpm at 4°C for 2 minutes twice, then 50 µL of 70% formic acid and 50 µL absolute acetonitrile were added to the pellets, followed by thorough mixing and centrifugation. One microliter of the protein extract was applied onto the MALDI-TOF MS target plate and covered with 1 µL of Bruker matrix (α-cyano-4-hydroxycinnamic acid). The target plate was left to dry. All samples were tested in duplicate.

MALDI-TOF MS Instrument Setting

The Bruker Daltonics Autoflex MALDI-TOF mass spectrometer from Germany was used for analysis. The system was controlled using flexControl software (GmbH Bremen, Germany). The analysis was conducted with specific instrumental settings: ion source 1 was set to an acceleration voltage of 25.00 kV, ion source 2 to 23.45 kV, and the lens voltage was configured at 6.0 kV.[14]

MALDI spectra of all isolates within a mass range of 2000 to 20,000 Da were used for pattern matching analysis to identify bacterial species using MALDI-Biotyper software version 4.1.100. A score between 0 and 3.000 was generated depending on whether database entries matched, as well as whether the peaks of the test isolate were present in entries

that did not match. A score of ≥2.000 indicates species-level identification, a score of 1.700–1.999 indicates genus-level identification, and a score of ≤1.700 indicates no identification.[24]

Data Analysis

Statistical analysis, including the Mann–Whitney and Chi-square tests, as well as assessments of normal distribution using the D'Agostino & Pearson test, were conducted with GraphPad Prism version 10.2.2 (GraphPad Software, San Diego, California, USA). To evaluate the typing concordance rate of tested methods with WGS, adjusted Wallace coefficients were employed. The coefficients were quantified on a scale from 0.000 to 1.000, ranging from the lowest to the highest concordance level.[25] Calculations and the statistical differences between adjusted Wallace coefficients were performed using the online tool "Comparing Partitions" (http://www.comparingpartitions.info/index.php?link=Tool).

Results

The Genomic Analysis Reveals Two Distinct Clades of *Acinetobacter* Bacteria in Northeast Thailand with the Different Distribution of Sequence Types

Demographic data of patients whose *Acinetobacter* isolates were used are presented in Supplementary Table S1. Most patients presented with pneumonia, accounting for 36 out of 40 cases (90%). Carbapenem-resistant *A. baumannii* (CRAB) was identified in 17 cases (42.5%) and multi-drug resistance was detected in 4 cases (10%). Among these, 28 isolates were confirmed to be *A. baumannii* (70%) by WGS, while the remaining 12 isolates comprised 4 *A. nosocomialis* (10%), 4 *A. pittii* (10%), and 1 isolate each from non-ACB complex species, including *A. soli* (2.5%), *A. seifertii* (2.5%), *A. junii* (2.5%), and *A. bereziniae* (2.5%) (Table 1). Through bioinformatics analysis of WGS data, 12 out of 28 (42.9%) *A. baumannii* isolates

Table 1 *Acinetobacter* Species Identified by Whole-Genome Sequencing

Species Identified	n (%)	MLST, n (%)
ACB complex		
A. baumannii	28 (70)	ST2, 12 (42.9)
		ST433, 3 (10.7)
		ST164, 2 (7.1)
		ST395, 2 (7.1)
		ST16, 1 (3.6)
		ST141, 1 (3.6)
		ST218, 1 (3.6)
		ST221, 1 (3.6)
		ST279, 1 (3.6)
		ST1479, 1 (3.6)
		Unknown, 3 (10.7)
A. nosocomialis	4 (10)	ST768, 2 (50)
		ST322, 1 (25)
		ST410, 1 (25)
A. pittii	4 (10)	ST207, 1 (25)
		ST220, 1 (25)
		Unknown, 2 (50)
Acinetobacter species		
A. soli	1 (2.5)	Unknown, 1 (100)
A. seifertii	1 (2.5)	Unknown, 1 (100)
A. junii	1 (2.5)	Unknown, 1 (100)
A. bereziniae	1 (2.5)	ST1317, 1 (100)
Total	40 (100)	

Abbreviations: ACB complex, *Acinetobacter calcoaceticus-baumannii* complex; MLST, multilocus sequence typing.

were classified as ST2, while other sequence types were distributed more broadly among the remaining isolates. A phylogenetic tree as illustrated in Figure 1 revealed that *A. baumannii* is divided into two distinct clades. The upper clade displays considerable variation among *Acinetobacter* species, being *A. baumannii* (eg, ST279, ST395, and ST433) closely related to *A. nosocomialis* and other species, including, *A. seifertii, A. pittii, A. junii*, and *A. bereziniae*. In contrast, the lower clade predominantly features ST2 *A. baumannii*, with other *A. baumannii* isolates (eg, ST16, ST141, ST164, ST221, ST279, and ST1479), and a single *A. soli* isolate.

MALDI-TOF MS Uncover Non-*baumannii Acinetobacter* Species in Phenotypic Reports of *A. baumannii*

From WGS, there were 12 isolates of non-*baumannii Acinetobacter* that were misidentified by the biochemical method (Figure 2a). For MALDI-TOF MS, resolution was nearly equivalent to that of WGS, with the exception of *A. bereziniae* (which was not detected by MALDI-TOF MS) (Figure 2a). This considerably improved the identification accuracy, especially for other *Acinetobacter* species (Figure 2b). The overall accuracy rates were 70% for biochemical method, 82.5% for MALDI-TOF MS using DC samples, and 80% for MALDI-TOF MS using PE samples. Therefore, MALDI-TOF MS demonstrates superior accuracy and offers a distinct advantage in identifying clinically significant *Acinetobacter* species compared to biochemical method.

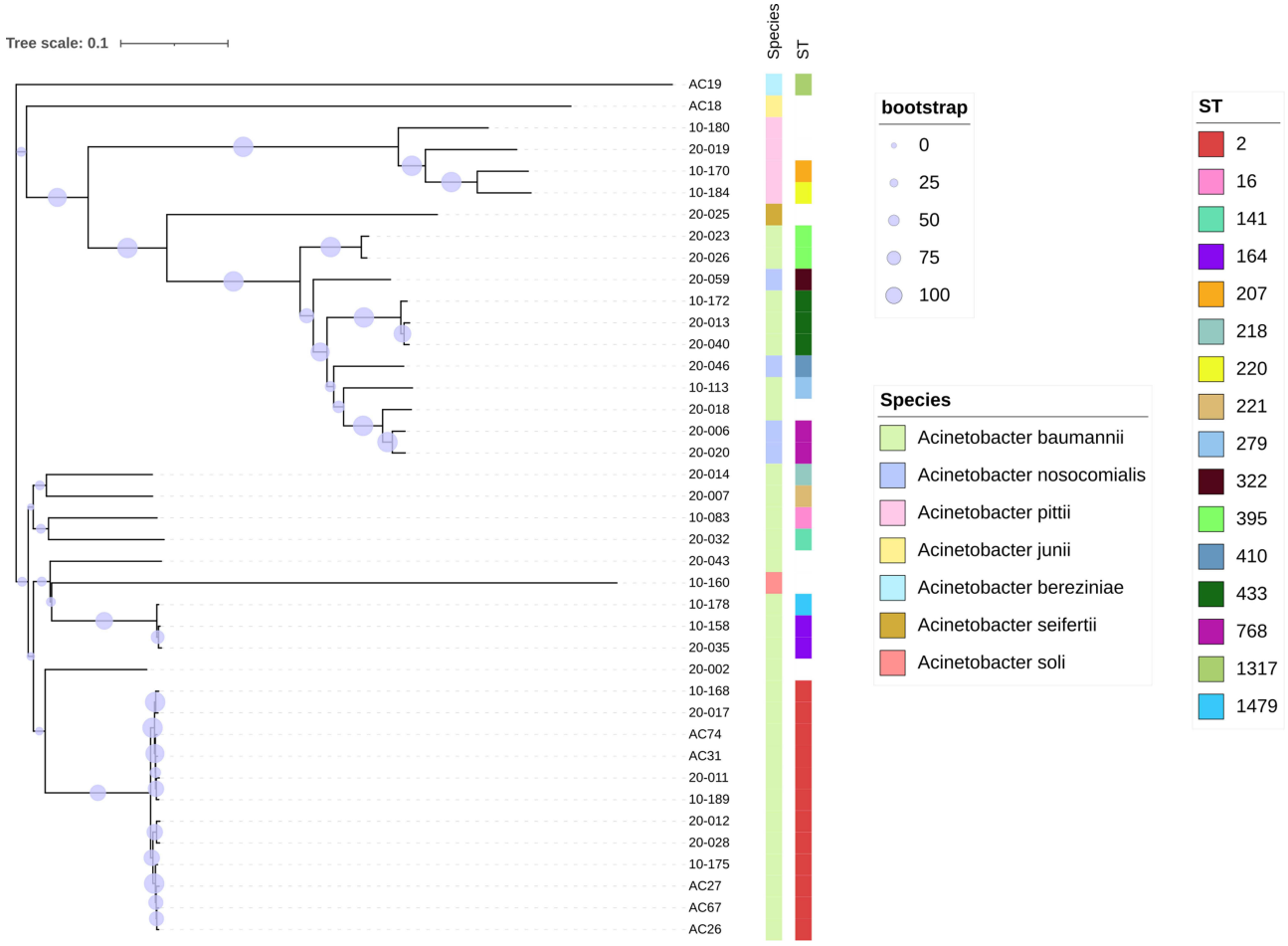

Figure 1 Phylogenetic tree of *Acinetobacter* species based on SNP analysis from whole-genome sequencing. A total of 14,022 filtered SNP variants were used to construct a maximum-likelihood phylogenetic tree using RAxML. The bootstrap percentages, calculated from 1000 replicates, are indicated by circle size at the branch nodes. Each individual sample is identified by a specific number, and *Acinetobacter* species along with their corresponding sequence types are color-coded for clarity. Instances of non-identification from MALDI-TOF MS are indicated.

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MALDI-TOF MS Using Direct Colony Samples Demonstrates Superior Identification Performance

Among the methods tested, MALDI-TOF MS DC exhibited the highest accuracy for both the ACB complex (83.33%) and other *Acinetobacter* species (75.00%), yielding an overall accuracy of 82.5% (Figure 2b). We further examined the concordance rate in comparison to the reference method, WGS (Table 2). The highest concordance rate with WGS was observed with MALDI-TOF MS DC (adjusted Wallace coefficient = 0.639, 95% CI: 0.286–0.992), followed by MALDI-TOF MS PE (adjusted Wallace coefficient = 0.556, 95% CI: 0.167–0.943).

Identification by MALDI-TOF MS Significantly Reduces Time to Report

MALDI-TOF MS DC reporting speed was significantly faster than that of the biochemical method (p-value < 0.0001) illustrated in Figure 3a. The majority of turnaround times fell within the 24 to 48 hours (Figure 3b). However, no reports were issued within 24 hours using the biochemical method in our setting. Notably, MALDI-TOF MS DC produced reports within 48 hours (<24 hours and 24–48 hours) more frequently than the biochemical method (p-value = 0.0141). Therefore, MALDI-TOF MS DC effectively reduces the turnaround time for *Acinetobacter* identification compared to biochemical method.

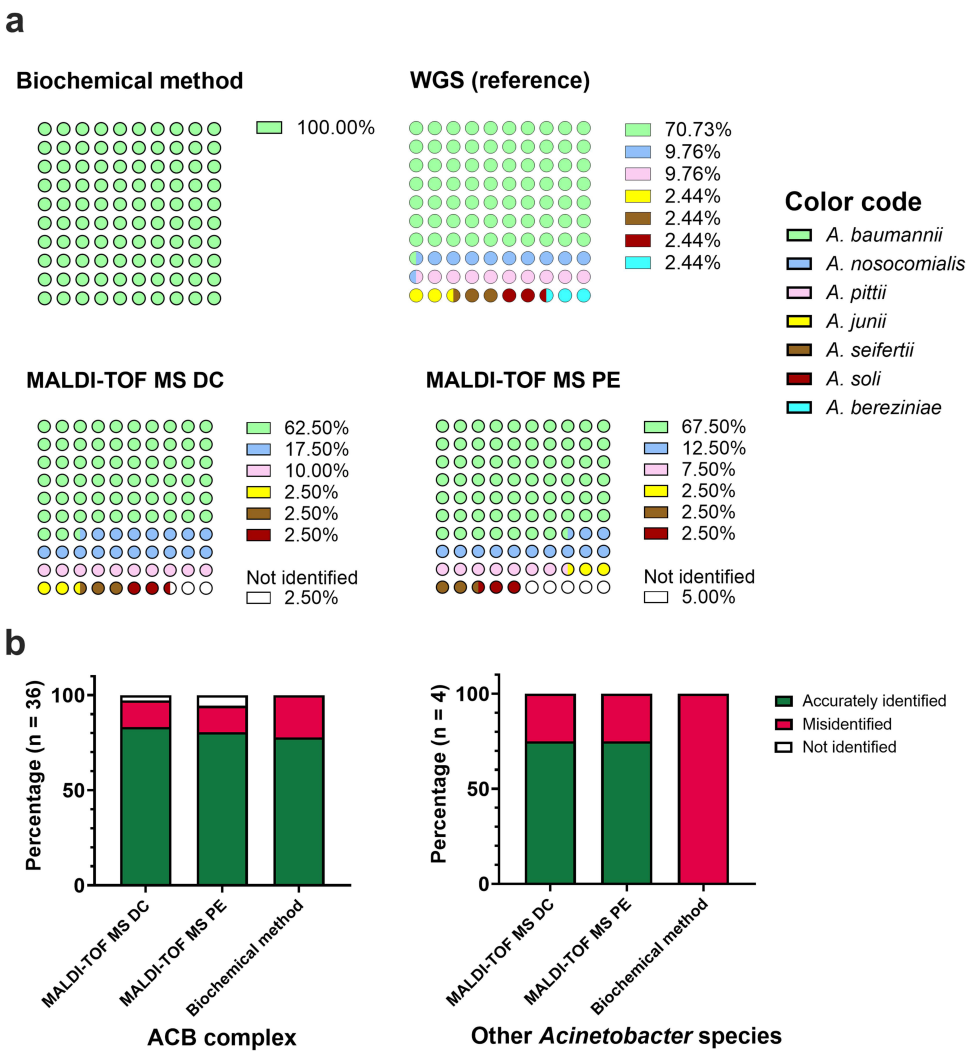

Figure 2 Identification resolution and accuracy of MALDI-TOF MS using direct colony (DC) and protein extract (PE) methods, compared to biochemical technique. (**a**) Percentages of *Acinetobacter* species identified by each method, with each color representing a different species. Whole-genome sequencing (WGS) was employed as the reference method. (**b**) Accuracy of each method in identifying the *Acinetobacter calcoaceticus-baumannii* complex (ACB Complex) and other *Acinetobacter* species.

Table 2 Identification Accuracy in Species Level of MALDI-TOF MS Using Direct Colony (DC), Protein Extract (PE), and Biochemical Technique Compared to Whole Genome Sequencing (WGS) as Reference Method

Methods	Number of *Acinetobacter* Species Identified								Types of Results	Adjusted Wallace Coefficient (95% CI)
	A. bau mannii	*A. noso comialis*	*A. pittii*	*A. soli*	*A. seifertii*	*A. junii*	*A. bere ziniae*	Not Identified		
WGS (reference)	28	4	4	1	1	1	1	0	7	
MALDI-TOF MS DC										
Concordance	23	3	4	1	1	1	0	1	7	0.639 (0.286–0.992)
Discordance	5[1]	1	0	0	0	0	1			
MALDI-TOF MS PE										
Concordance	24	2	3	1	1	1	0	2	7	0.556 (0.1669–0.943)
Discordance	4[1]	2[1]	1	0	0	0	1			
Biochemical method										
Concordance	28	0	0	0	0	0	0	0	1	NA
Discordance	0	4	4	1	1	1	1			

Notes: Adjusted Wallace coefficients quantify the concordance rate of tested methods based on WGS, [1]; included by 1 result of "Not identified".
Abbreviations: CI, confident interval; NA, not applicable.

Discussion

Accurate and timely identification of *Acinetobacter* species is crucial to guide appropriate antibiotic therapy and improve patient outcomes. Our previous research revealed a concerning level of misidentification, particularly when non-*baumannii Acinetobacter* species were mistakenly reported as *A. baumannii*.[3] This study demonstrates the superior performance of MALDI-TOF MS for *Acinetobacter* identification, surpassing traditional methods within terms of resolution, accuracy, and turnaround time. Our findings underscore the critical need to adopt MALDI-TOF MS as the primary method for *Acinetobacter* identification in clinical microbiology laboratories.

While *Acinetobacter* infections often present with similar initial symptoms,[26] mortality rates and drug-resistance profiles vary significantly between *A. baumannii* and other species in the genus.[26,27] *A. baumannii* poses a particular challenge due to widespread antibiotic resistance, limiting therapeutic options. Misidentification can lead to overprescription of broad-spectrum antibiotics, resulting in adverse side effects and unnecessary financial burdens. Therefore, precise identification of clinically relevant species is crucial for optimizing treatment strategies.

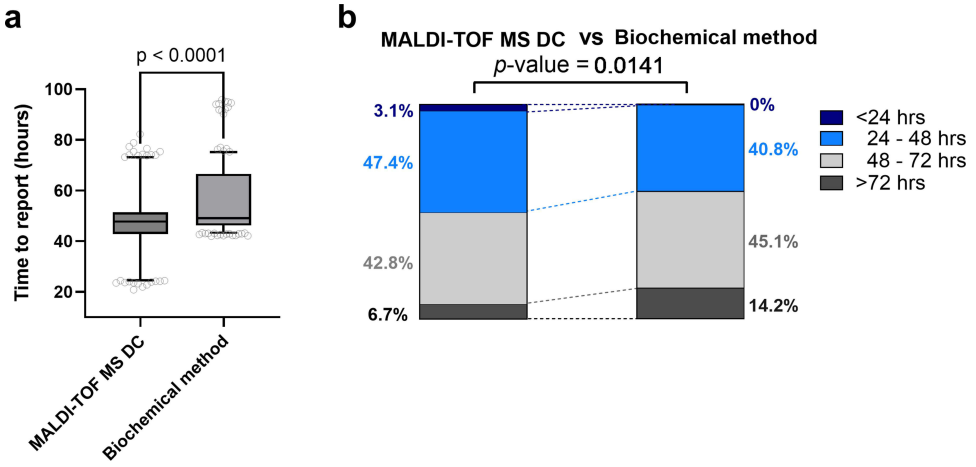

Figure 3 Comparison of time to report between MALDI-TOF MS and biochemical method. (**a**) The time (in hours) for *Acinetobacter* identification reports generated by MALDI-TOF MS using the direct colony (DC) and the biochemical method (phenotypic-based identification) is illustrated in a dot plot, displaying the median and interquartile range. A Mann–Whitney test was conducted to assess the significance of the difference between the two methods. (**b**) The comparison of reporting times, categorized as <24 hours, 24–48 hours, 48–72 hours, and >72 hours, between MALDI-TOF MS using the direct colony method (DC) and the biochemical method is presented in stacked bar format. A Chi-square test was used to evaluate the significance of reports generated within 48 hours (both <24 hours and 24–48 hours) across all cases between the two methods. The p-values are provided as indicated.

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We proved that the efficiency of MALDI-TOF MS extends beyond its accuracy. The streamlined pre-analytical process, using direct colony sampling and rapid protein profiling, significantly reduces turnaround time. This streamlined workflow allows clinicians to make informed antibiotic selection and adjustment decisions within 2–3 days after symptom onset,[28,29] minimizing the risk of delayed proper therapy and promoting adherence to antibiotic guidelines. The rapid identification provided by MALDI-TOF MS also contributes to antimicrobial stewardship by minimizing the use of broad-spectrum antibiotics, which can help combat the emergence of antibiotic resistance. Furthermore, MALDI-TOF MS offers significant financial advantages by reducing the need for prolonged hospital stays and unnecessary medical services, ultimately lowering healthcare costs for both patients and laboratories.[30]

Our study involved a comprehensive MALDI-TOF MS database, including both Bruker-provided and in-house databases validated by genotyping.[11,14] While MALDI-TOF MS accurately identified most isolates, a few strains remained unidentified, including ST220-*A. pittii*, ST279-*A. baumannii*, and ST2-*A. baumannii*. Additionally, *A. bereziniae*, which was misidentified by all tested methods, presented a relatively low bootstrap value and long branch. This underscores the need for continued expansion and refinement of proteomics databases, particularly for geographically diverse strains, and further development of advanced software algorithms.

Multiplex PCR served as a representative practical genomic-based method in our study. The development and validation of new primer sets for accurately detecting novel *Acinetobacter* species is crucial but often time-consuming.[31] In contrast, MALDI-TOF MS provides a comprehensive protein profile at a single laser shot and benefits from a continuously expanding proteomics database. This, coupled with ongoing advances in the technology, makes MALDI-TOF MS a highly promising tool for bacterial identification in this era.[16,32]

We acknowledge the limitations of our study related to sample size and the restricted diversity of *Acinetobacter* species included. Due to the limited sample size, we were unable to assess the impact of misidentification on clinical outcomes, such as an intensive care unit (ICU) admission and mortality. Despite these limitations, our study demonstrates the superior performance of MALDI-TOF MS, highlighting its potential to improve accuracy, efficiency, and turnaround time in *Acinetobacter* identification.

Conclusions

This study highlights the limitations of conventional phenotypic method for *Acinetobacter* identification, particularly in distinguishing between closely related species. We observed that automated biochemical method exhibited significantly lower resolution compared to genomic and protein-based identification methods. Our genomic analyses revealed a high prevalence of *A. baumannii*, with ST2 accounting for the largest proportion (42.9%) among *A. baumannii* isolates in our setting, consistent with national reports in Thailand.[18] Phylogenetic tree showed that among *A. baumannii* cases, there was a considerable proportion of non-ST2 *A. baumannii* in our setting (57.1%) that presented a close relationship to other *Acinetobacter* species, especially *A. nosocomialis*, as indicated by a separated clade from ST2 *A. baumannii* (common sequence type). This finding suggests that phenotypic characteristics may be insufficient to discriminate between these subtle phylogenetically distinct *Acinetobacter* species, potentially leading to misidentification of non-*baumannii* species as *A. baumannii*.

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Author Contributions

All authors made a significant contribution to the work reported.

Conceptualization and supervision: W.K., L.W., C.K., G.L., K.F., A.N.

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Writing – original draft: P.S., A.P. and A.N.

All authors took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

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