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Research article

Comparison of MALDI-TOF mass spectrometry and *rpoB* gene sequencing for the identification of clinical isolates of *Aeromonas* spp.

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HIGHLIGHTS

- Mass spectrometry (MS)-based methods evaluated for *Aeromonas* spp. identification.
- Housekeeping gene *rpoB* sequencing most accurately identified *Aeromonas* species.
- MALDI Biotyper and VITEK MS differed in accuracy depending on the species.
- Database extension will help improve identification accuracy of MS-based methods.

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ABSTRACT

Aeromonas spp., widely present in rivers and soil, cause mild gastroenteritis, severe septicemia, and soft tissue infections in humans. Treatment of these infections require accurate identification of pathogenic *Aeromonas* spp. However, identification at the species level using conventional methods is highly challenging. In this study, we aimed to compare the accuracy of two different approaches developed for bacterial identification: (i) housekeeping gene sequencing (*rpoB*) in conjunction with phylogenetic analysis and (ii) matrix-assisted laser desorption ionization mass spectrometry-time of flight (MALDI-TOF MS) (MALDI Biotyper and VITEK MS), for differentiating *Aeromonas* spp. We analyzed 58 *Aeromonas* isolates recovered from patients at different medical institutions in Japan using both identification methods. The *rpo*B sequencing method was the most accurate, identifying all *Aeromonas* isolates at the species level. Meanwhile, the MALDI Biotyper system correctly identified 53 (91.4%) isolates at the genus level and an additional 30 (51.7%) at the species level. The VITEK MS system correctly identified 58 (100%) isolates at the genus level and an additional 34 (58.6%) at the species level. Thus, MALDI Biotyper and VITEK MS accurately identified isolates at the genus level, but differences were found in the accuracy of identification of species. However, the low cost and ease of analysis make MALDI-TOF MS-based methods strong candidates for use in clinical laboratories that require easy-to-use identification methods.

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1. Introduction

The genus *Aeromonas* is a glucose-fermentable, oxidase-positive, Gram-negative bacillus that is widely found in rivers and soil. In humans *Aeromonas* spp. cause intestinal infections, hepatobiliary infections, skin and soft tissue infections, and septicemia [1, 2, 3, 4, 5, 6, 7]. Currently, 36 species of *Aeromonas* have been identified [8]. Among them, *A. hydrophila*, *A. caviae*, *A. veronii* biovar *sobria*, and *A. dhakensis* are human pathogens most associated with clinical cases [1, 9, 10]. However, it is difficult to identify isolates of *Aeromonas* at the species level using the biochemical identification techniques currently used in many microbiology laboratories owing to the limited accuracy of these methods [11, 12, 13, 14]. Although genetic analysis using methods such as DNA–DNA hybridization is known to be highly accurate for *Aeromonas* identification [2], it is rarely used in clinical laboratories because of high cost and long time required for analysis. Similar problems exist in identification based on sequences of 16S ribosomal RNAs and housekeeping genes such as *gyrB*, *dnaJ*, and *rpoB* [2, 15, 16]. To counter this issue, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently been used as an alternative to conventional identification methods owing to its favorable speed of analysis and applicability [11, 17, 18]. In this study, we aimed to compare the accuracy and applicability of MALDI-TOF MS and housekeeping gene sequencing (*rpoB*) methods in identifying *Aeromonas* at the species level, which can help evaluate the feasibility of using MS-based methods for rapid clinical identification.

2. Materials and methods

2.1. Bacterial isolates and identification

This study involved 58 *Aeromonas* isolates from patients at six different medical institutions in Japan, identified to the genus level using the VITEK 2 system (BioMérieux, Marcy-l'Étoile, France). Further species level identification was achieved using *rpoB* gene sequencing [16], which has been considered the gold standard technique [2]. Isolates were grown in duplicate on Mueller–Hinton agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 35 °C for 24 h under aerobic conditions. Amplification of *rpoB* was performed using primers *Pasrpob-L* (5′-GCAGTGAAAGARTTCTTTGTTC) and *Rpob-R* (5′-GTTGCATGTTNGNACCCAT) as described by Korczak et al. [19]. DNA sequencing was conducted using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA) and an ABI3730xl analyzer (Applied Biosystems). BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to process the *rpoB* sequencing data and to identify genes. A phylogenetic tree was generated using the neighbor-joining method based on the nucleotide sequences of clinical isolates with reference strain sequences retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) using Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7) [20].

2.2. Preparation of samples for MALDI-Biotyper analysis

Samples were analyzed using MALDI-Biotyper (Bruker Daltonics, Bremen, Germany) and Flex Control software ver. 3.4 (Brucker Daltonics) for the acquisition of mass spectra. Isolates were grown in duplicate on Mueller–Hinton agar plates (Becton Dickinson) at 35 °C for 24 h under aerobic conditions. Colonies from the medium were spotted onto a steel target plate (Becton Dickinson), and 0.5 μL of formic acid (Becton Dickinson) was added on it and dried; then, 1 μL of 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution was overlaid (Becton Dickinson).

2.3. Preparation of samples for VITEK MS analysis

For VITEK MS (BioMérieux) analysis, the MALDI-TOF MS method was used in combination with the VITEK MS database and its exclusive analysis software VITEK MS ver. KB3.2. Isolates were grown in duplicate on Mueller–Hinton agar plates (Becton Dickinson) at 35 °C for 24 h under aerobic conditions. Colonies from the medium were spotted onto a VITEK MS target slide (BioMérieux), and 0.5 μL of formic acid (BioMérieux) was added and dried; then, 1 μL of 10 mg/mL CHCA matrix solution (BioMérieux) was overlaid.

2.4. Data acquisition using MALDI-Biotyper

For each isolate, MALDI-Biotyper generates a score from 0 to 3 according to the similarity between the sample spectrum and reference spectra in the database, and displays the top 10 matching results. The scores were categorized as follows: ≥2.000, highly reliable identification; 1.999–1.800, less reliable identification; ≤1,799, non-reliable identification. In this study, considering the two best matches obtained from MALDI-TOF Biotyper, we evaluated the MALDI-TOF identification results in the following categories: A) the correct species is an unique match with a score ≥2.000; B) the correct species is the first match with a score ≥2.000, but the second different match also has a score ≥2.000; C) both first and second matches have a score ≥2.000, but the correct species is the second match, D) the first and second matches have a score >2.000, but the correct species is neither the first nor second match. Isolates belonging to category A with a match were considered correctly identified, isolates belonging to categories B and C were considered inconclusive, and isolates belonging to category D were considered misidentified.

2.5. Data acquisition using VITEK MS

The VITEK MS equipment generates a score ranging from identification probability of 0%–99.9%, showing similarity between sample and reference spectra, and displays matching results with the highest scores.

The reliability of identification was evaluated according to the standard manufacturing interpretative criteria: 60%–99.9%, identification at the species level with high confidence; <60%, non-reliable identification.

3. Results

Among the 58 isolates tested using *rpoB* sequencing, 19 isolates of *A. caviae*, 15 isolates of *A. hydrophila*, 12 isolates of *A. dhakensis*, 11 isolates of *A. veronii*, and 1 isolate of *A. jandaei* were identified to the species level (Table 1). All isolates showed ≥99.0% sequence homology with the corresponding reference strains [16]. Unrooted neighbor-joining phylogenetic trees based on *rpoB* showed relationships among *Aeromonas* spp. (Figure 1).

With MALDI-Biotyper, all *Aeromonas* isolates were correctly identified to the genus level, and important differences were detected among different species in the isolates correctly identified to the species level (Table 2). Identification of *Aeromonas* at the species level is usually based only on the first best match on the identification ranking list analyzed using MALDI-Biotyper [21, 22]. In the case of *A. hydrophila*, the best result (93.3%) was obtained when the identification was performed using the first match score. Most of the single isolates of *A. hydrophila* were correctly identified, whereas the frequencies of correct identification were 78.9%, 72.3%, 0%, and 0% for isolates of *A. caviae*, *A. veronii*, *A. dhakensis*, and *A. jandaei*, respectively (score >2.0). Two isolates of *A. caviae* were identified as *A. hydrophila*, and there were two isolates with no peak value. One isolate of *A. hydrophila* was identified as *A. jandaei*. Although an isolate of *A. dhakensis* was identified as *A. hydrophila* (7/12) and *A. jandaei* (3/12), there were two isolates that had no peak value. Three isolates of *A. veronii* were identified as *A. ichthiosmia* (Table 3).

With VITEK MS, all isolates were accurately identified at the genus level. Additionally, all isolates of *A. hydrophila* and *A. caviae* were accurately identified at the species level. However, except for *A. hydrophila* and *A. caviae*, isolates of all other species were detected as more than one

Table 1. Comparison of rpoB gene sequencing, MALDI-biotyper, VITEK MS.

rpoB identification	n	Identification by	
		MALDI-Biotyper	VITEK MS
A. caviae	19	*A. caviae (15)*	*A. punctata (caviae) (19)*
		A. hydrophila (2)	
		Unidentified (2)	
A. hydrophila	15	*A. hydrophila (14)*	*A. hydrophila (15)*
		A. jandaei (1)	
A. dhakensis	12	*A. hydrophila (7)*	*A. sobria or A. veronii (5)*
		A. jandaei (3)	*A. hydrophila (2)*
		Unidentified (2)	*A. punctata(caviae) or A. sobria or A. veronii or A. jandaei (2)*
			A. hydrophila or A. sobria or A. veronii (1)
			A. punctata(caviae) or A. sobria or A. veronii (1)
			A. hydrophila or A. punctata(caviae) or A. sobria or A. veronii (1)
A. veronii	11	*A. veronii (8)*	*A. sobria or A. veronii (11)*
		A. ichthiosmia (3)	
A. jandaei	1	*Unidentified (1)*	*A. sobria or A. veronii (1)*

species (Table 4). All isolates of *A. veronii* were identified as *A. veronii/A. sobria*, whereas identification based on the results for *A. dhakensis* and *A. jandaei* was inconsistent.

4. Discussion

Among the 36 species of *Aeromonas* identified to date [8], only a few species, *A. hydrophila*, *A. caviae*, *A. veronii* biovar *sobria*, and *A. dhakensis*, have been established as human pathogens [2]. Therefore, identification of *Aeromonas* isolates to the species level has clinically important implications [23]. Several methods have been implemented to accurately identify *Aeromonas*, including biochemical methods and sequencing of the 16S rRNA gene and multiple molecular markers, among which DNA–DNA hybridization is considered the gold standard method [24]. In this study, the housekeeping gene sequence (*rpoB*) was used as the standard. All obtained phylogenetic trees derived from the housekeeping gene sequences showed considerable divergence among all *Aeromonas* species studies. This result is in agreement with the findings of previously reported phylogenetic studies based on housekeeping gene sequences such as *rpoB* [16, 24]. As demonstrated in the present phylogenetic analysis, the use of housekeeping genes is a valuable approach for the classification of *Aeromonas* species and for the accurate identification of novel, closely related isolates.

Previous studies have shown that in the identification results using MALDI-Biotyper, considering only the first match may not the best approach for accurate identification, and the scores of the second identification option should also be evaluated [17, 18, 19, 20, 21, 22, 23, 24, 25]. Therefore, we evaluated the consistency of identification using MALDI-TOF by considering the two best scores provided by MALDI-Biotyper for the first and second identification matches (Table 3). Using this approach, 93.3% and 73.7% of *A. hydrophila* and *A. caviae* isolates, respectively, were identified accurately, whereas the identification of most isolates of *A. veronii* was inconclusive (Table 3). In the latter case, the difference between the first and second identification scores was small, with both scores above 2.0 in most cases (Table 2, categories B to C), which hindered accurate identification. All isolates of *A. dhakensis* (12/12) were misidentified as different species of *Aeromonas* (Tables 3 and 4). *Aeromonas dhakensis* is not considered to be included in the MALDI-Biotyper's database [26].

Aeromonas identification using VITEK MS showed that all isolates of *A. hydrophila* and *A. caviae* were correctly identified at the species level.

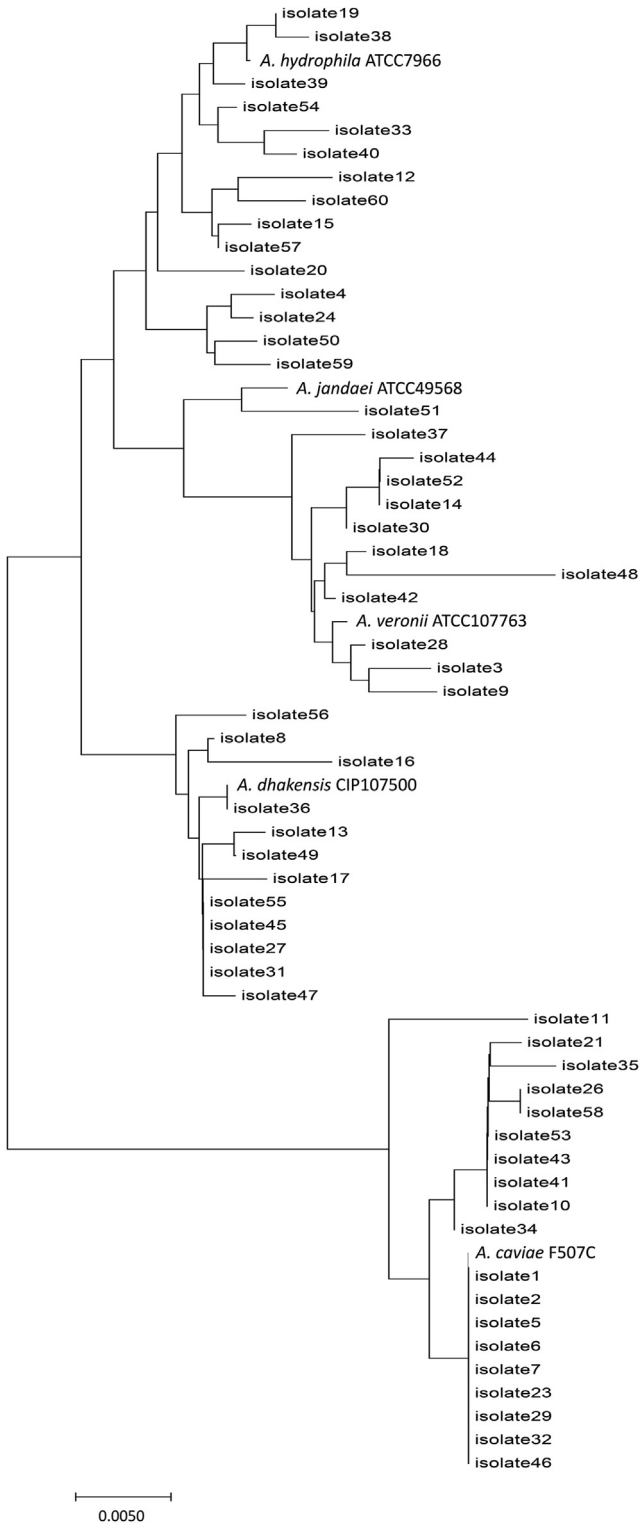

Figure 1. Unrooted neighbor-joining phylogenetic tree based on rpoB, showing relationships among *Aeromonas* spp. type strains are included.

However, *A. dhakensis*, *A. veronii*, and *A. jandaei* isolates were identified as more than one bacterial species, and it was difficult to narrow down the identification to one species (Table 4). However, in *A. veronii*, the probability that *rpo*B sequencing results were included in the results of multiple bacteria was 100% (11/11). In this case, the ornithine test can be performed to distinguish *A. veronii/A. sobria*. Previous studies have identified that species with a positive result for ornithine test is *A. veronii* and that with a negative result is *A. sobria* [27], indicating that it is

Table 2. Identification results using the Biotyper database for 58 Aeromonas isolates.

Species	n	First best match	Score	Second best match	Score	Consistency
A. caviae	11	*A. caviae*	2.010–2.307	*A. caviae*	1.889–2.261	A
	3	*A. caviae*	2.053–2.075	*A. hydrophila*	1.786–1.899	A
	1	*A. caviae*	2.216	*A. hydrophila*	2.205	B
	2	*A. hydrophila*	2.014–2.214	*A. caviae*	2.003–2.175	C
	2	No peaks found				E
A. hydrophila	13	*A. hydrophila*	2.01–2.368	*A. hydrophila*	2.034–2.322	A
	1	*A. hydrophila*	2.098	*A. veronii*	1.899	A
	1	*A. jandaei*	1.907	*A. hydrophila*	1.848	E
A. dhakensis	3	*A. hydrophila*	2.161–2.203	*A. hydrophila*	2.07–2.117	D
	1	*A. hydrophila*	1.886	*A. hydrophila*	1.88	E
	1	*A. hydrophila*	2.102	*A. caviae*	1.987	D
	2	*A. hydrophila*	2.049–2.166	*A. jandaei*	1.992–2.095	D
	2	*A. jandaei*	1.138–2.182	*A. hydrophila*	2.129–2.167	D
	1	*A. jandaei*	1.915	*A. veronii*	1.835	E
	2	No peaks found				E
A. veronii	6	*A. veronii*	2.089–2.262	*A. veronii*	1.975–2.216	A
	1	*A. veronii*	2.16	*A. ichthiosmia*	2.085	B
	1	*A. veronii*	2.206	*A. hydrophila*	2.101	B
	3	*A. ichthiosmia*	2.095–2.143	*A. veronii*	2.061–2.073	C
A. jandaei	1	No peaks found				E

Note: [a]First and second identification best matches with their respective scores provided by the Byotyper identification list.
[b]For the evaluation of the identification results, the following variables were considered: (i) the score according to the MALDI Biotyper Compass (≥2.000: highly reliable identification, 1.999–1.800: less reliable identification, ≤1799: non-reliable identification), and (ii) the consistency list of the first two best matches, where (A) the correct species is the unique species with a score ≥2.000, (B) the correct species is the first match, but the second match also has a score ≥2.000, (C) the first and second matches have a score ≥2.000, but the correct species is the second match, (D) the first and second matches have a score >2.000, but the correct species is neither the first nor second match. (E) Score <2.000 for the first and second matches, or unidentifiable species. Isolates belonging to category A with a match are considered correctly identified, isolates belonging to categories B and C are considered inconclusive, isolates belonging to category D are considered misidentified, and isolates in category E are considered unidentifiable.

Table 3. Identification using the Biotyper database of N Aeromonas isolates (%).

Species	n	Accurate	Inconclusive	Missidentified
A. caviae	19	14 (73.7)	3 (15.8)	2 (10.5)
A. hydrophila	15	14 (93.3)	0	1 (6.7)
A. dhakensis	12	0	0	12 (100)
A. veronii	11	6 (54.5)	5 (45.5)	0
A. jandaei	1	0	0	1

Table 4. Identification results using the VITEK MS database for 58 Aeromonas isolates.

Species	n	Identified bacterial species	Score
A. caviae	19	*A. punctata (caviae)*	99.9%
A. hydrophila	15	*A. hydrophila*	99.9%
A. dhakensis	5	*A. sobria*	50.0%
		A. veronii	50.0%
	2	*A. hydrophila*	99.9%
	2	*A. punctata (caviae)*	25.0%
		A. sobria	25.0%
		A. veronii	25.0%
		A. jandaei	25.0%
	1	*A. hydrophila*	33.0%
		A. sobria	33.0%
		A. veronii	33.0%
	1	*A. punctata (caviae)*	33.0%
		A. sobria	33.0%
		A. veronii	33.0%
	1	*A. hydrophila*	25.0%
		A. punctata (caviae)	25.0%
		A. sobria	25.0%
		A. veronii	25.0%
A. veronii	11	*A. sobria*	50.0%
		A. veronii	50.0%
A. jandaei	1	*A. sobria*	50.0%
		A. veronii	50.0%

possible to use this test to differentiate the two species. *Aeromonas dhakensis* was not found in the identification results, probably because *A. dhakensis* is among the species that do not exist in the VITEK MS ver. 3.2 database. When the database of *A. dhakensis* isolates is updated in the future, the ability to distinguish *A. dhakensis* using cluster analysis from other species may increase [21].

In our study, the MALDI-TOF MS method showed some deviations from the identification results of *rpoB* sequencing. Microbial identification using MALDI-TOF MS relies on the evaluation of microbial proteins, and some strains in the environment may show unique biomarkers that can be used to distinguish strains but are not supported by databases [28]. The result "unidentified" obtained with some of the isolates in this study cannot be currently explained, but it may be attributed to the lack of a library of mass spectra and fingerprints for identification. Using MALDI-TOF MS, Donohue et al. [29] and Shin et al. [30] reported a successful identification rate of 93%, with 7% identification failure. In contrast, the corresponding values in our study with MALDI-Biotyper (first best match only) were 63.8% and 36.2%, and with VITEK MS (containing *rpoB*-matched stock in the results), they were 77.6% and 22.4%, respectively (Table 1). This could be attributed to the high detection of *A. dhakensis* among the isolates, which is not described in both MALDI-TOF MS databases. Considering the discussion on the novelty of *A. dhakensis* [21, 29], identification using MALDI-TOF MS alone may be unreliable. *Aeromonas dhakensis* was not detected in the study of Shin et al. [30], and in our study, many isolates were *A. dhakensis*, which may have caused the difference. However, the high discrimination ability

of *A. hydrophila* and *A. caviae*, which are frequently detected, is consistent with the report of Shin et al. [30]. Furthermore, the validation data of VITEK MS against *Aeromonas* in this study may be novel. Therefore, we conclude that MALDI-TOF MS cannot easily distinguish *Aeromonas* at the species level, and differences in the genus levels may show some discrepancies, as shown in Table 1. However, MALDI-TOF MS offers the advantages of speed of analysis and low cost over other methods. In the present study, MALDI-TOF MS showed good accuracy in differentiating *A. hydrophila* and *A. caviae*, but was not able to accurately identify some of the pathogenic species such as *A. dhakensis* and *A. veronii*. Information on *A. dhakensis* is not included in the database, which makes it difficult to analyze this species at present [31]. Thus, it is evident from the results that additional information on the biochemical properties of different *Aeromonas* species and upgradation of the MS database are required to clearly understand the current status of MALDI-TOF MS in the differentiation of *Aeromonas* species and to further establish its applicability in routine clinical practice.

Declarations

Author contribution statement

Daisuke Kitagawa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yuki Suzuki: Performed the experiments; Analyzed and interpreted the data.

Noriyuki Abe, Koji Ui, Keitaro Suzuki, Takaya Yamashita, Atsuko Sakaguchi, Soma Suzuki, Kazue Masuo: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Akiyo Nakano, Ryuichi Nakano: Analyzed and interpreted the data.

Masatoshi Sato, Koichi Maeda, Fumihiko Nakamura, Hisakazu Yano: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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