

# A Rapid MALDI-TOF MS Identification Database at Genospecies Level for Clinical and Environmental *Aeromonas* Strains

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## Abstract

The genus *Aeromonas* has undergone a number of taxonomic and nomenclature revisions over the past 20 years, and new (sub)species and biogroups are continuously described. Standard identification methods such as biochemical characterization have deficiencies and do not allow clarification of the taxonomic position. This report describes the development of a matrix-assisted laser desorption/ionisation–time of flight mass spectrometry (MALDI-TOF MS) identification database for a rapid identification of clinical and environmental *Aeromonas* isolates.

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## Introduction

Bacteria belonging to the genus *Aeromonas* are widely distributed in freshwater and brackish environments, and have long been recognized as etiologic agents for fish diseases [1]. They are included into the class Gammaproteobacteria, comprising Gram-negative, non-spore-forming rod-shaped bacteria, are facultative anaerobic oxidase- and catalase-positive, glucose-fermenting, resistant to the vibriostatic agent O/129, and generally motile [2].

*Aeromonas* play also a significant role as opportunistic pathogens for humans causing gastroenteritis, septicemia, pneumonia, meningitis, and wound infections in immunocompetent as well as in compromised patients. *A. hydrophila*, *A. caviae* and *A. veronii* (biovar *sobria* and biovar *veronii*), are clinically the most significant species [3].

So far, the genus *Aeromonas* comprises 21 validly proposed species: *A. allosaccharophila*, *A. aquariorum*, *A. bestiarum*, *A. bivalvium*, *A. caviae* (synonym: *A. punctata*) *A. culicicola*, *A. encheleia* (corresponds to HG 11), *A. eurenophila*, *A. hydrophila*, *A. jandaei*, *A. media*, *A. molluscorum*, *A. popoffii*, *A. salmonicida*, *A. schubertii*, *A. sharmana*, *A. simiae*, *A. sobria*, *A. tecta*, *A. trota* (synonym: *A. enteropelogenes*), *A. veronii* (synonym: *A. ichthiosmia*). It has to be noted that within these proposed species the position of *A. allosaccharophila*, *A. culicicola* and *A. sharmana* has to be clarified since the first two might belong to *A. veronii* and the last one seems not belong to the genus *Aeromonas* at all [4,5].

Several phylogenetic studies on *Aeromonas* allowed the elevation of the genus name to the rank of family [2,6,7,8]. Nevertheless the taxonomy of this genus is rather complex and has been submitted to ongoing changes due to newly described species [9,10,11,12,13,14] and rearrangements of existing taxa

[15,16,17,18,19,20]. One major problem in *Aeromonas* identification relies on the fact that some species are phenotypically very similar (e.g. *A. caviae* and *A. media*, *A. veronii* and *A. sobria*). Several molecular methods have been therefore applied as an alternative to the laborious DNA-DNA hybridization technique for resolving the *Aeromonas* taxonomy and even though the sequence analysis of ribosomal RNA genes allowed for the discrimination of the genospecies [6,21,22], other more discriminating housekeeping genes such as *gyrB* and *rpoD* are now increasingly used [8,23,24,25,26]. Nevertheless, sequencing and phylogenetic methods are costly, time consuming and therefore not appropriate for a rapid species identification in the diagnostic laboratory. A valid alternative to conventional methods of bacterial identification and classification, based on the characterization of biomarker molecules, but definitely more rapid and reliable is the mass spectrometry technique [27]; MALDI-TOF MS (matrix assisted laser desorption ionization mass spectrometry – time of flight) combined with a reliable database is a powerful method for the identification and comparison of microbial isolates based on protein fingerprints analysis of whole cells [28]. MALDI-TOF MS applications in microbiology are important for proteomic and natural product analyses [29]. This technique can be used to detect non-volatile and thermally unstable molecules from a few to several hundred kDa, the most applicable range used for the analysis is 2–20 kDa. The identification of microorganisms by MALDI-TOF MS is based on the detection of mass signals from biomarkers that are specific at genus, species or sub-group level.

All mass spectra were generated in positive linear mode by scanning the sample spot with the laser beam, and after signal acquisition, the raw mass spectra are processed automatically by smoothing, baseline correction and peak recognition [30]. The

essential information used for microbial identification is contained in a peak list containing m/z values and intensities. This list is analysed by comparison to the database SARAMIS™ (Spectral Archive And Microbial Identification System), in which the identification at the species level is based on a percentage of confidence referred to reference spectra (SuperSpectra™) that contain family, genus and species specific m/z biomarkers, as described in the SARAMIS™ user manual. For the generation of one SuperSpectra™ some representatives isolates of one species from different locations (hospitals, reference centers and strain culture collections) are needed. Beside the FingerprintSpectra every isolate will be determined by accredited and published microorganism identification procedures. The SuperSpectra™ are generated based on measurements of well known microorganisms and contain sets of genus, species and strain biomarkers which are characteristic for the respective group of microorganisms. Superspectra™ are computed from typical strains covering more than 90% of the intraspecific diversity in most species.

Accuracy of the identification strongly relies upon the robustness of the database and the choice of reference isolates. This is especially important when considering genera comprising species of clinical and environmental origin presenting a high genetic diversity.

There are excellent precedents for the application of MALDI-TOF MS for taxonomic studies [31,32,33,34], as well as for routine diagnostic [35].

Previous studies proved the applicability of this technique for the identification of the *Aeromonas* species [36,37,38]. The major aim of this study was to establish a rapid and reliable species identification tool for the genus *Aeromonas* using the SARAMIS™ identification system based on a relatively high number of phylogenetically well characterized isolates of clinical and environmental origin.

## Methods

### Bacterial Strains

92 morphologically and genetically well characterized strains (see supporting information Table S1) belonging to all known genospecies of the genus *Aeromonas* were used to create the m/z reference library system using the SARAMIS™ software. All strains were phylogenetically typed and assigned to the respective genetic species using the housekeeping gene *gyrB*. The obtained sequences were deposited in GenBank and accession numbers are listed in Table S1. The mass fingerprinting identification database produced was then evaluated on 741 clinical and environmental isolates. All strains were grown on Blood Agar at 30°C for 24 hours previous to the protein fingerprinting mass spectrometry analysis.

### DNA Extraction

Genomic DNA was extracted from colonies grown on blood agar according to Demarta et al. [39], and resuspended in TrisEDTA buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

### PCR Amplification and Sequencing

The sets of primers used for amplification and sequencing of the *gyrB* gene have been reported elsewhere [40,41].

### Phylogenetic Analyses

Nucleotide sequences of *gyrB* gene (fragment of 1100 bp) was aligned and phylogenetically analysed using MEGA version 3.1 [42].

Phylogenetic tree was constructed using the Neighbour-Joining method with genetic distances computed by employing Kimura's 2-parameter method [41].

### MALDI-TOF MS

Strains were transferred from the colony directly on a 48-position stainless steel FlexiMass™ target plate (Shimadzu Biotech, Kyoto, Japan) using a plastic loop. The transferred colony material was then overlaid with 0.5 µl of Matrix (DHB 75%) solution containing 75 mg/ml 2, 5-dihydroxybenzoic acid in acetonitrile/ethanol/water (1:1:1) supplemented with 3% trifluoroacetic acid. All mass spectra were acquired using an AXIMA Confidence™ (Shimadzu Biotech, Kyoto, Japan) mass spectrometer, equipped with a nitrogen laser (pulse width: 3 ns) operated in positive linear mode. The measured mass range of spectra was 2000–20,000 Da. A minimum of 20 laser shots per sample was used to generate each ion spectrum. For each bacterial sample, 50 protein mass fingerprints were averaged and processed.

All spectra were processed by the MALDI-TOF MS Launchpad 2.8 software (Shimadzu Biotech, Kyoto, Japan).

### Data Analysis

A database identification system was established analyzing 92 morphologically and genetically well characterized *Aeromonas* strains belonging to all known species of the genus. The resulting peak lists of these samples were exported to the SARAMIS™ software package (bioMérieux, France) and submitted to single-linkage cluster analysis to produce taxonomic trees. These trees were compared to a *gyrB* phylogenetic tree (Neighbour-Joining). Specific biomarkers containing sets of genus, species and strain characteristic masses were used for the creation of species-specific SuperSpectra™ recognizing the most frequently encountered species. 11 different SuperSpectra™ were created that allow identifications of: *A. hydrophila*, *A. caviae*, *A. media*, *A. tecta*, *A. popoffi*, *A. eucrenophila*, *A. encheleia*, *A. bestiarum*, *A. salmonicida*, *A. sobria* and *A. veronii*.

## Results and Discussion

The protein mass fingerprint analysis emerging from the MALDI-TOF MS data of 92 genetically well characterized *Aeromonas* strains provided a good separation at genospecies (Fig. 1) level comparable with the phylogenetic tree obtained by *gyrB* gene sequencing.

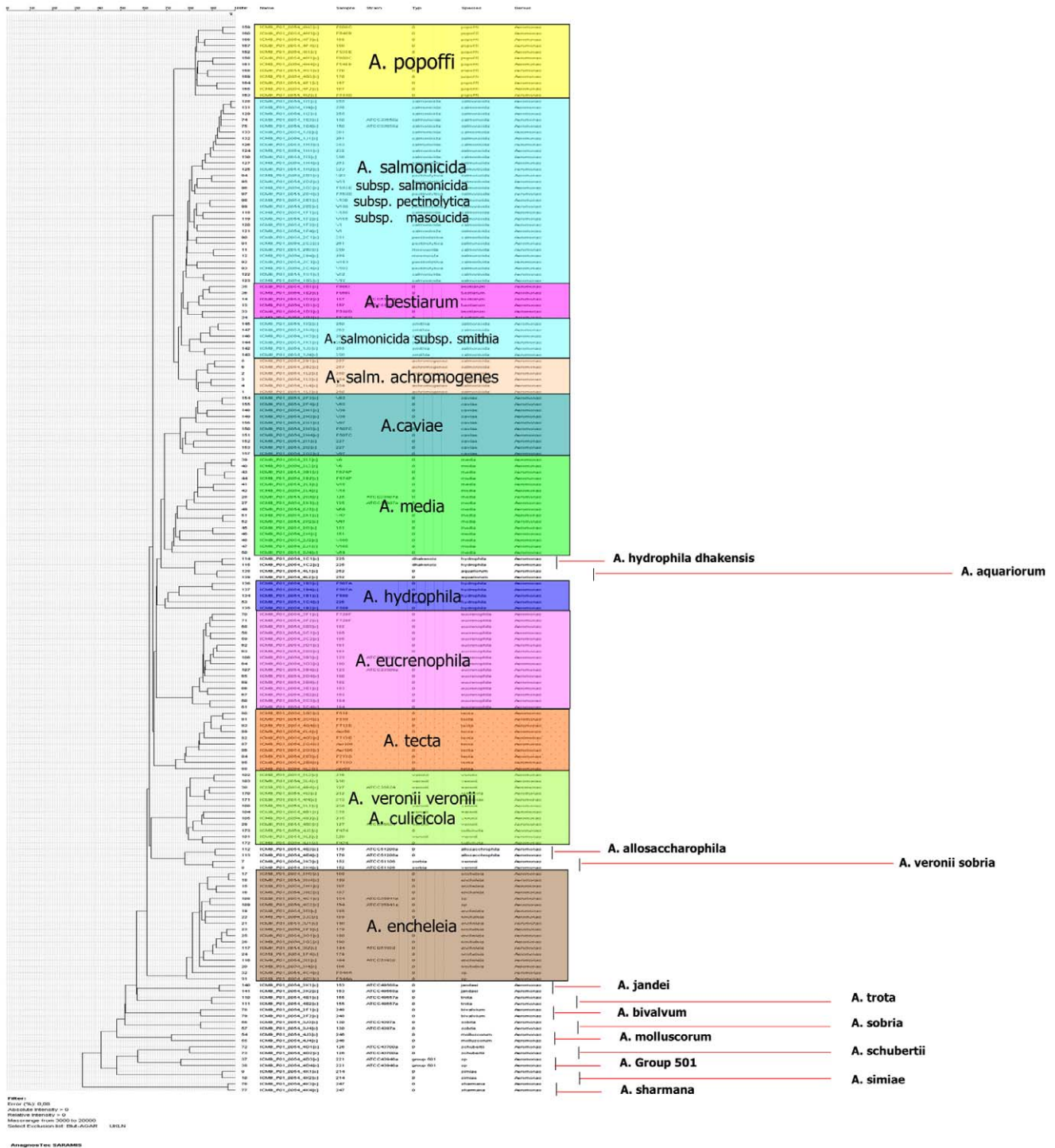
In fact both trees clustered the species *A. veronii* (*A. veronii* biovar *sobria*, *A. veronii* biovar *sobria*), *A. culicicola*, and *A. allosaccarophila* together, confirming the hypothesis that this group in fact represents only one genospecies [18].

Interesting the m/z profiles analysis allowed to separate the two biovars *veronii* and *sobria*, furthermore the profile of the strain ATCC 51106 *A. veronii* biovar *sobria* was more closely related to that of *A. allosaccarophila* ATCC 51208 than to that of *A. veronii* biovar *veronii*, confirming the results obtained with the *gyrB* sequences.

Moreover MALDI-TOF MS analysis categorized in a single cluster *A. encheleia* and the unnamed *Aeromonas* sp. HG11 [23] and allowed the segregation in the different genospecies of the *A. salmonicida*/*A. bestiarum*/*A. popoffii* group.

*A. salmonicida* and *A. bestiarum* are difficult to separate on the basis of 16S rRNA (differ in only 2 nucleotide positions) [2] but they could be separated using *gyrB* as well as other housekeeping genes such as *rpoB* or *rpoD*.

At the subspecies level, *A. salmonicida* formed a very uniform group, with respective intraspecies substitution rates of 1.3 and



**Figure 1. Dendrogram resulting from single-linkage cluster analysis of MALDI-TOF mass spectra.** Error 0.08%; Mass range from  $m/z$  2,000 to 20,000. doi:10.1371/journal.pone.0048441.g001

0.8% for *gyrB* and *rpoB*, rendering very difficult to classify strains at the subspecies level [41]. MALDI-TOF MS seemed to allow a better differentiation of the strains in study. The type strains of each subspecies were well differentiated and formed a defined group in the MALDI-TOF MS dendrogram (Fig. 1).

A branch in the MALDI-TOF MS dendrogram groups in one single cluster strains assigned to the species *A. aquariorum* and

*A. hydrophila* subsp. *dhakensis* (Fig.1). Data based on phylogenetic analysis by sequencing *gyrB*, *rpoD* and 16S rRNA [43], strongly suggested that strains of *A. hydrophila* subsp. *dhakensis* belongs in fact to the species *A. aquariorum*, confirming the results obtained with MALDI-TOF MS (Fig. 1).

Due to the reliable identification at species level, it was possible to create 11 different SuperSpectra™ for *A. hydrophila*, *A. caviae*,

**Table 1.** Characteristic masses retained for the creation of SuperSpectra™.

	<i>A. hydrophila</i>	<i>A. caviae</i>	<i>A. popoffi</i>	<i>A. tecta</i>	<i>A. eucrenophila</i>	<i>A. media</i>	<i>A. media</i>	<i>A. media</i>	<i>A. media</i>	<i>A. bestiarum</i>	<i>A. encheleia</i>	<i>A. salmonicida</i>	<i>A. veronii</i>	<i>A. veronii</i>	<i>A. veronii</i>	<i>A. sobria</i>
3332	3150	3683	3047	2007	2006	3150	3828	3151	3828	3772	3153	2241	3590			
3871	3435	3827	3435	3156	2039	3844	4346	3899	4346	3930	3606	3047	4172			
4169	4302	4257	3665	3671	2071	3863	4591	4259	4591	4189	4174	4170	4260			
4256	4394	4322	4258	4265	2087	4257	4699	4487	4699	4309	4262	4257	4348			
4318	4974	4393	4317	4325	2093	4440	5050	4600	5050	4393	4366	4309	4650			
4445	5051	4879	4460	4468	2514	4591	5584	4701	5584	5071	4504	4361	5052			
4698	5187	5203	4591	4599	2614	4655	5675	5007	5675	5186	4646	4490	6104			
5003	5394	5665	4700	4707	6107	5070	5700	5144	5700	5393	4704	4518	6307			
5049	5687	6064	5462	5694	6313	5155	5877	5351	5877	5590	4989	4670	6934			
5706	5885	6329	6083	5903	8615	5603	6085	6071	6085	6197	5161	5155	7184			
6022	6213	6914	6305	6109	8923	5637	6305	6307	6305	6859	6313	7234	7336			
6304	7210	7194	6481	6315	9193	6305	6480	6482	6480	7236	6867	7410	7920			
7208	7410	7220	6861	6490	9220	6480	6919	6951	6919	7408	9195	7749	8831			
7347	7463	7492	7333	7206	9408	7566	7195	7197	7195	7934	9384	8624	8941			
7477	8606	7904	7369	7343	10318	7730	7332	7335	7332	8160	9980	9042	9204			
7746	8979	8060	7473	7684	10931	8343	7658	8263	7658	8621	11164	10280	9231			
8637	8998	9184	7915	8617	11376	9400	8343	8607	8343	9040	11191	10904	10904			
8913	9401	9399	8343	9197	12205	10136	9400	9201	9400	11385	12216	11235	11235			
9183	9949	9682	9185	9221	11273	11273	11166	9403	11166	12406	12406	11422	11422			
9398	11373	11329	11193	9412	10313	10313	11348	10313	11348	12412	12412	12412	12412			
10008			12197	10323	10648	10648		10648		12461	12461	12461	12461			
			12197	10937												
			11383													

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**Table 2.** Identification values at species level obtained with the created SuperSpectra™.

		>99%	90–99%	<90%	NI	n
<i>Aeromonas</i>	<i>hydrophila</i>	158	5	2	2	167
<i>Aeromonas</i>	<i>caviae</i>	176	6	7	8	197
<i>Aeromonas</i>	<i>media</i>	76	9	3	3	91
<i>Aeromonas</i>	<i>tecta</i>	12				12
<i>Aeromonas</i>	<i>popoffii</i>	13			6	19
<i>Aeromonas</i>	<i>eucrenophila</i>	21	3		1	25
<i>Aeromonas</i>	<i>encheleia</i>	8	1			9
<i>Aeromonas</i>	<i>bestiarum</i>	25	5			30
<i>Aeromonas</i>	<i>salmonicida</i>	41	1	1	1	44
<i>Aeromonas</i>	<i>veronii</i>	90	5		8	103
<i>Aeromonas</i>	<i>sobria</i>	21				21
<i>Aeromonas</i>	<i>spp</i>				23	23
<b>n</b>		<b>641</b>	<b>35</b>	<b>13</b>	<b>52</b>	<b>741</b>

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*A. veronii*, *A. media*, *A. tecta*, *A. popoffii*, *A. eucrenophila*, *A. encheleia*, *A. bestiarum*, *A. sobria* and *A. salmonicida* to be used for the identification of the strains at the species level (Table 1).

We tested the new SuperSpectra™ with 741 strains of *Aeromonas*. 93% of these strains were successfully identified (Table 2), 93% of them with an identification value greater than 99%.

52 of 741 strains (7%) could not be identified mostly due to the absence of SuperSpectra™ (23 strains, *A. allosaccharophila*, *A. aquariorum*, *A. bivalvium*, *A. culicicola*, *A. jandaei*, *A. molluscorum*, *A. schubertii*, *A. sharmiana*, *A. simiae*, *A. trota*), or for the absence of SuperSpectra™ with sufficient coverage in our database (29 strains, Table 2).

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These results demonstrate that the mass spectral data of the strains contained sufficient protein information to distinguish between genera, species, and strains (Table 2).

Another mass spectrometry study of intact-cell with *Aeromonas* strains [37] also confirmed that the signals generated from the analysis of the protein masses could be used as specific biomarkers for the differentiation below the species level. For the the majority of the species analysed the identification was successful.

With *A. tecta* and *A. sobria* we obtained a correct identification for all the strains, whereas for *A. eucrenophila*, *A. salmonicida*, and *A. hydrophila* only 1 strains for the first two and 2 strains for the last species could not be identified.

Identification of *A. popoffii* with the created SuperSpectra™ was possible only in 46% of the cases. These failure could be due to insufficient coverage of the specific SuperSpectra™ or lack of performance of the last.

The approach presented in this paper uses the technique MALDI-TOF MS to develop a rapid, sensitive and specific method to detect isolates of the genus *Aeromonas*.

Our work highlighted the importance of testing well characterized strains of different origins for producing high quality MALDI-TOF MS databases as rapid identification tools. In conclusion, we can affirm that MALDI-TOF MS is a rapid and relatively inexpensive method for the identification of *Aeromonas* species and constitutes a valid alternative to conventional methods of identification and classification.

## Supporting Information

### Table S1 Strains used in this study.

(DOC)

## Author Contributions

Conceived and designed the experiments: CB AD OP MT. Performed the experiments: CB. Analyzed the data: CB APC DZ MT. Contributed reagents/materials/analysis tools: OP. Wrote the paper: CB OP.



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