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Differentiation of *Acinetobacter* Genomic Species 13BJ/14TU from *Acinetobacter haemolyticus* by Use of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

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Acinetobacter genomic species 13BJ and genomic species 14TU were delineated independently in 1989 based on DNA-DNA hybridization among proteolytic strains of human origin (1, 2). Later studies suggested that these two taxa were congruent with each other at the species level and represented a distinct phylogroup within the hemolytic-proteolytic clade of the genus *Acinetobacter* (3). Reports from different countries showed that strains of *Acinetobacter* genomic species 13BJ/14TU were found in clinical specimens at a frequency similar to or even higher than that of another hemolytic and proteolytic species, *Acinetobacter haemolyticus* (4–6). It has also been noted that strains of *Acinetobacter* genomic species 13BJ/14TU are intrinsically resistant to polymyxins (4, 7), which further underpins the clinical relevance of this taxon.

Previously, *Acinetobacter* genomic species 13BJ/14TU had not been correctly identified using some phenotypic identification systems and the VitekMS matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) microbial identification system, although correct identification was achieved using partial *rpoB* gene sequencing (4, 8). In the present study, we showed that *Acinetobacter* genomic species 13BJ/14TU can be reliably differentiated from other *Acinetobacter* species using MALDI-TOF mass spectrometry (MS).

The strains of *Acinetobacter* genomic species 13BJ/14TU and *A. haemolyticus* for this study were selected mostly from the collection of the Laboratory of Bacterial Genetics (National Institute of Public Health, Prague, Czech Republic) and also included reference strains analyzed in the original DNA hybridization studies (Table 1). The whole-genome sequences for seven of these reference strains were published recently (3, 9). The species identity of the seven strains was confirmed by whole-genome sequence comparison using average nucleotide identity based on BLAST (ANIb), which was calculated using the JSpecies web program (http://imedea.uib-csic.es/jspecies/) with the default setting (3). The identification of the 13 remaining strains was based on a combination of validated methods, including comparative analysis of the partial *rpoB* gene, amplified rRNA gene restriction analysis (ARDRA), and/or phenotypic analysis (5, 7). All strains were epidemiologically unrelated as confirmed by their unique ApaI macrorestriction profiles of their genomic DNA and/or differences in their *rpoB* gene sequences (data not shown).

A subculture of each of the strains investigated was streaked onto sheep blood agar (Difco Laboratories, Detroit, MI; 5% sheep blood from Oxoid, Hampshire, United Kingdom) and incubated at 37°C for 24 h. Cells from representative single bacterial colonies were directly smeared onto a target spot of a steel target plate by using a disposable loop, overlaid with 1 μl of matrix consisting of a saturated solution of sinapinic acid (catalog no. 49508; Sigma-Aldrich, Buchs, Switzerland) in 60% acetonitrile (catalog no. 154601; Sigma-Aldrich)-3% trifluoroacetic acid (catalog no. T6508; Sigma-Aldrich) or α-cyano-4-hydroxycinnamic acid (catalog no. 145505; Sigma-Aldrich, Buchs, Switzerland) in 33% ethanol-33% acetonitrile (catalog no. 154601; Sigma-Aldrich, Buchs, Switzerland)–3% trifluoroacetic acid (catalog no. T6508; Sigma-Aldrich), and air dried within minutes at room temperature. Matrices were tested in parallel to assess the impact of matrices on the ability to identify the strains.

Protein mass fingerprints were obtained using a MALDI-TOF Axima Confidence mass spectrometer (Shimadzu-Biotech Corp., Kyoto, Japan), with detection in the linear, positive mode at a laser frequency of 50 Hz and within a mass range of 3,000 to 30,000 Da. Acceleration voltage was 20 kV, and the extraction delay time was 200 ns. For each sample spot, a total of 100 spectral profiles, each consisting of 10 single spectra, were averaged and processed using Launchpad v.2.8 software (Shimadzu-Biotech Corp., Kyoto, Japan). This software was also used for peak processing of all raw spectra with the following settings: the advanced scenario was chosen from the Parent Peak Cleanup menu, peak width was set at 80 channels, the smoothing filter width was set at 50 channels, the baseline filter width was set at 500 channels, and for peak detection

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TABLE 1 Strains used in this study

Strain	Species	Country, yr of isolation	Specimen (human)	WGS[a] accession no.	Reference(s)
CIP 64.3^T	*A. haemolyticus*	Unknown	Sputum	APQQ00000000.1	3, 13
NIPH 1878 (=Tjernberg 197)	*A. haemolyticus*	Sweden, 1980s	Wound		2
NIPH 58	*A. haemolyticus*	Czech Republic, 1992	Blood		5
NIPH 261	*A. haemolyticus*	Czech Republic, 1993	Tissue	APQR00000000.1	3, 5
NIPH 939	*A. haemolyticus*	Czech Republic, 1998	Cervix		5
ANC 3857	*A. haemolyticus*	Czech Republic, 2010	Wound		
ANC 3923	*A. haemolyticus*	Czech Republic, 2010	Wound		
ANC 3927	*A. haemolyticus*	Czech Republic, 2010	Wound		
ANC 4581 (=CIP 70.27)	*A. haemolyticus*	Unknown	Nose	ADMT00000000.1	9, 13
ANC 4583 (=CIP 70.26)	*A. haemolyticus*	Unknown	Ocular pus	AMJB00000000.1	9, 13
NIPH 1860 (=CIP 64.2)	*Acinetobacter* genomic species 13BJ/14TU	Germany	Conjunctiva	APRT00000000.1	1, 2, 3
NIPH 1859 (=Tjernberg 71)	*Acinetobacter* genomic species 13BJ/14TU	Sweden, 1980s	Conjunctiva	APRZ00000000.1	2, 3
NIPH 1861 (=Bouvet 496)	*Acinetobacter* genomic species 13BJ/14TU	France	Skin		1
NIPH 2036 (=Bouvet 1191)	*Acinetobacter* genomic species 13BJ/14TU	Belgium	Catheter	ATGK00000000.1	1, 3
ANC 3881	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 2010	Lungs (autopsy)		
NIPH 378	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1995	Eye		5
NIPH 637	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1995	Blood		5
NIPH 669	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1997	Blood		5
NIPH 1035	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1998	Blood		5
ANC 4056	*Acinetobacter* genomic species 13BJ/14TU	Germany, 2004	Wound		
J80	*Acinetobacter* genomic species 13BJ/14TU	Japan	Unknown		

[a] WGS, whole-genome sequence.

method, the threshold apex was chosen. For the threshold apex peak detection, the threshold type was set as dynamic and the threshold offset was set to 0.025 mV with a threshold response factor of 1.25. The processed spectra were exported as peak lists with *m/z* values and signal intensities for each peak in the ASCII format. Calibration was conducted for each target plate using spectra of the reference strain *Escherichia coli* DH5α.

All 21 strains were analyzed in duplicate using two sample spots from the same colony. Generated protein mass fingerprints were analyzed with SARAMIS software (AnagnosTec, Potsdam-Golm, Germany) and the Putative Assigned Protein Masses for Identification Database (PAPMID) (Mabritec, Switzerland).

In the first step, a biomarker mass pattern, called a superspectrum, was calculated for the *Acinetobacter* genomic species 13BJ/14TU, using the SARAMIS SuperSpectrum tool. As ribosomes are known to be among the most abundant proteins (10), 23 ribosomal subunit protein masses, namely, L36 (4,265.1 Da), L34 (5,175.1 Da), L33 (6,104.3 Da), L30 (6,641.7 Da), L32 (6,930.8 Da), L35 (7,185.8 Da), L29 (7,448.6 Da), L31 (8,343.6 Da), S21 (8,349.9 Da), L27 (8,769.1 Da), L28 (8,986.7 Da), S17 (9,407.0 Da), S20 (9,570.2 Da), S15 (10,007.5 Da), S19 (10,063.9 Da), S14 (11,253.2 Da), L18 (12,390.1 Da), L17 (13,540.7 Da), S12 (13,649.0 Da), L13 (15,859.3 Da), S5 (17,008.7 Da), S7 (17,554.5 Da), and L6 (18,968.0 Da), were retrieved from the genome of *Acinetobacter* genomic species 13BJ/14TU strain CIP 64.2, making use of the PAPMID software. The spectra of the *Acinetobacter* genomic species 13BJ/14TU strains investigated in this study were matched against these reference masses for automatic identification, allowing for an error of 500 ppm.

The superspectrum for automated identification of *Acinetobacter* genomic species 13BJ/14TU was generated according to the user guidelines and validated against the entire in-house extended SARAMIS database (version 3.3.2), consisting of 2,087 bacterial species, including 18 *Acinetobacter* spp. with validly published names. These 18 species encompass all *Acinetobacter* spp. which are known to be associated with human clinical specimens, except for *Acinetobacter nosocomialis*, *Acinetobacter seifertii*, *Acinetobacter soli*, and *Acinetobacter variabilis*.

All 21 strains were identified using SARAMIS software, including the new *Acinetobacter* genomic species 13BJ/14TU superspectrum. Using only the SARAMIS database without the new *Acinetobacter* genomic species 13BJ/14TU superspectrum, all 10 *A. haemolyticus* strains were identified correctly as *A. haemolyticus* while the 11 *Acinetobacter* genomic species 13BJ/14TU strains had been identified only as *Acinetobacter* spp. However, with the implementation of the *Acinetobacter* genomic species 13BJ/14TU superspectrum, all strains were correctly identified as *A. haemolyticus* or *Acinetobacter* genomic species 13BJ/14TU, respectively. The choice of matrix used for spectral acquisition made no impact on the identification of the strains.

MALDI-TOF MS, as we have demonstrated, is a useful tool in the differentiation and identification of related species of bacteria such as *A. haemolyticus* and *Acinetobacter* genomic species 13BJ/14TU. Other studies detailing the use of a commercial MALDI mass spectrometer in comparison with other instruments such as the Vitek 2 and MicroScan reported that *Acinetobacter* genomic species 13BJ/14TU could not be accurately identified with these instruments (8). However, the method used here uses the same principle as commercial instruments used in routine identification procedures but with a more in-depth approach in the analysis of the spectra.

The use of calculated theoretical masses from sample genomes as identifying masses contributed to the improved discriminatory power of our method as observed in this study. Use of a sinapinic acid matrix may further increase the discriminatory power of the method due to its ability to better ionize proteins of higher molecular weights that can be used for identification compared to α-cyano-4-hydroxycinnamic acid (11, 12).

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