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Species Identification of Clinical *Prevotella* Isolates by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

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The performance of matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) for species identification of *Prevotella* was evaluated and compared with 16S rRNA gene sequencing. Using a Bruker database, 62.7% of the 102 clinical isolates were identified to the species level and 73.5% to the genus level. Extension of the commercial database improved these figures to, respectively, 83.3% and 89.2%. MALDI-TOF MS identification of *Prevotella* is reliable but needs a more extensive database.

Prevotella species are obligate anaerobic, Gram-negative, pleomorphic rods and were previously considered to belong to the genus *Bacteroides.* In 1990, 16 species were transferred from the genus *Bacteroides* to the new genus *Prevotella* (13). Since then, the description of several species belonging to this genus was amended. Novel species were also added to the genus, extended now up to 44 validated species, of which 20 have been described since 2004 (14). For most species the primary site of isolation is the oral cavity, and some are associated with periodontitis and dental diseases. They were also isolated from abscesses and soft-tissue infections at other sites in the human body. Due to the still-increasing number of species in this genus, identification by classical methods is challenging, time-consuming, and susceptible to erroneous results.

In recent years, matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) has become well established in the routine clinical microbiology laboratory setting as a means to identify bacterial pathogens (2, 6, 12). Highly reproducible and distinct mass spectra are obtained within a mass range of 2,000 to 20,000 Da by using whole-cell MALDI-TOF MS (8). Identification and differentiation of microorganisms is achieved by pattern analysis of the mass spectra using mathematical tools. The technique is very rapid, and only minimal amounts of bacteria are needed.

Reports show that MALDI-TOF MS can be applied for identification of *Bacteroides* (11) and *Clostridium* (7) isolates and suggest that it is also a useful method for the identification of other anaerobic bacteria (10, 15–18). We identified 102 clinical *Prevotella* isolates by 16S rRNA gene analysis and evaluated the performance of MALDI-TOF MS for the species identification of *Prevotella.*

Overall, 125 isolates were included in the study. Twenty-three reference strains (Table 1) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and the Culture Collection of the University of Göteborg (CCUG; Göteborg, Sweden). In addition, 102 clinical isolates isolated in our laboratory between March 2007 and December 2010 or during a Belgian antibiotic-susceptibility multicenter survey (19) were included. The clinical origins of isolates are represented in Table S1 in the supplemental material.

All strains were grown on fastidious anaerobic agar (LAB M, Bury, United Kingdom) with 5% horse blood (E&O, Bonnybridge, Scotland) at 35°C for 24 to 48 h in an anaerobic chamber. Routine identification was performed using biochemical and enzymatic tests and analysis of whole-cell fatty acids by using the Microbial Identification System (Microbial Identification Inc., Newark, DE) (3, 9).

Sequencing of the 16S rRNA gene was used as the reference method for species identification as recommended by CLSI (4). PCR amplification of nearly the full 16S rRNA gene was performed as previously described with the conserved UFPL and URPL primers (5). The PCR products, consisting about 1,400 bases, were purified and sequenced by VIB Genetic Service Facility (University of Antwerp, Wilrijk, Belgium) using an ABI 3730 DNA sequencer (Applied Biosystems, Halle, Belgium). The sequencing primers used were UFPL, URPL, 16SB1, and 16SB2 (5). Sequence assembly was performed by using a Bionumerics sequence type module (Bionumerics version 6.5; Applied Maths, Sint-Martens-Latem, Belgium). The unknown strain sequences were compared to sequences available in the GenBank database by BLASTn 2.2.25+ interrogation (http://blast.ncbi.nlm.nih.gov/). Sequence identities of ≥99% and ≥97% were used for species and genus identification, respectively, according to CLSI guidelines (4). Obtained sequences were deposited in GenBank (see below; see also Table S2 in the supplemental material).

MALDI-TOF MS measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) provided with a 20-Hz nitrogen laser. Spectra were obtained in the linear positive mode with an accelerating voltage of 20 kV and analyzed within a mass range of 2,000 to 20,000 Da.

Main spectra (MSPs) of 23 reference strains of species known to occur in humans and, in a later phase, of 7 sequenced clinical isolates were added (Table 1). They were created by processing spectra obtained with MALDI Biotyper (Bruker Daltonik) from each isolate after ethanol/formic extraction. For ethanol/formic extraction, a few colonies were suspended in 300 μl of water. Pure

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TABLE 1 Main spectra (MSPs) present in the database[a]

Species	Bruker Reference Library 3.2.1.0 MSP	MSP of reference strain created in this study	MSP of clinical isolate sequenced in this study
P. amnii	407 RLT	CCUG 53648 UZB	
P. baroniae	HU30410_3 PNU	DSM 16972 UZB	
P. bergensis	DSM 17361T DSM		
P. bivia	0807M220682 IBS	CCUG 9557 UZB (= DSM20514T)	
	DSM 20514T DSM		
	HU26382 PNU		
	HU33416_4 PNU		
	HU33518_4 PNU		
	HU44067_1 PNU		
	HU51442_4 PNU		
P. buccae	DSM 20615 DSM		
	HU21568_4 PNU		
	HU28914_4 PNU		
	HU29415 PNU		
	HU47210_2 PNU		
	IBS_MS_33 IBS		
P. buccalis	DSM 20616T DSM		UZB SEQ057 UZB
			UZB SEQ077 UZB
P. copri		DSM 18205 UZB	
P corporis	DSM 18810T DSM	DSM 18810 UZB	
P. dentalis		DSM 3688 UZB	
P. denticola		DSM 20614 UZB	
P. disiens	DSM 20516T DSM	CCUG 9558 UZB (= DSM20516)	
	HU28371_5 PNU		
	HU28371_6 PNU		
P. heparinolytica			UZB SEQ003 UZB
P. histicola		DSM 19854 UZB	
P. intermedia	DSM 20706T DSM	CCUG 24041 UZB (= DSM20706)	
P. loescheii		DSM 19665 UZB	UZB SEQ062 UZB
P. maculosa	DSM 19339T DSM	DSM 19339 UZB	
P. marshii		DSM 16973 UZB	
P. melaninogenica	DSM 7089T DSM	CCUG 4944B UZB (= DSM20706)	
	IBS_MS_43 IBS		
P. micans		DSM 21469 UZB	
P. multisaccharivorax	DSM 17128T DSM		
P. nanceiensis	DSM 19126T DSM	DSM 19126 UZB	
	IBS_MS_20 IBS		
P. nigrescens	DSM 13386T DSM		
P. oralis	DSM 20702T DSM		UZB SEQ054 UZB
P. oris	DSM 18711T DSM	CCUG 15405 UZB (= DSM18711)	
P. oulorum		CCUG 54769 UZB	
P. pallens	DSM 18710T DSM		
	VA20903_1_10 ERL		
P. pleuritidis		CCUG 54350 UZB	
P. salivae	DSM 15606T DSM		UZB SEQ071 UZB
P. shahii	DSM 15611T DSM		
P. stercorea	DSM 18206T DSM	DSM 18206 UZB	
P. tannerae		CCUG 34292 UZB	
P. timonensis		DSM 22865 UZB	
P. veroralis		CCUG 15422 UZB	UZB SEQ061 UZB
Prevotella species	176 RLT		

[a] Data represent MSPs of strains from the Bruker Reference Library database 3.2.1.0 ($n = 37$), MSPs of added reference strains ($n = 23$), and MSPs of added sequenced clinical isolates ($n = 7$). The name of the MSP consists of the abbreviation of culture collection, the strain number, and the creator of the MSP. CCUG, Culture Collection of the University of Göteborg; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; UZB, Universitair Ziekenhuis Brussel.

ethanol (900 μl) was added to the suspension and mixed. The suspensions were centrifuged twice at 13,000 × *g* for 2 min to remove the supernatant. The pellets were dried at room temperature and resuspended in 50 μl of 70% formic acid (Sigma-Aldrich, Bornem, Belgium) and 50 μl of acetonitrile (Sigma-Aldrich). After centrifugation at 13,000 × *g* for 2 min, 1 μl of supernatant was spotted 10 times on a polished steel target plate. Immediately after drying, all spots were overlaid with 1 μl of matrix, a saturated solution of alfa-cyano-4-hydroxycinnamic acid (Bruker Daltonik) dissolved in 50% acetonitrile (Sigma-Aldrich) and 2.5% trifluoroacetic acid (Sigma-Aldrich). Each spot was measured 3 times by averaging data for 240 laser shots. The 30 spectra obtained were analyzed in the flexAnalysis program (Bruker Daltonik). A minimum of 20 accurate spectra was downloaded in MALDI Biotyper software to create a MSP.

All isolates were analyzed by the direct transfer method (6) routinely used in our laboratory. A small amount of a single colony was smeared directly on a ground steel target plate in a thin film. Spots were overlaid with 1 μl of matrix solution. Spectra were analyzed with MALDI Biotyper 3.0 software and Reference Library 3.2.1.0 (Bruker Daltonik), which contained 37 *Prevotella* MSPs comprising 20 species (Table 1). The spectra were reanalyzed with the extended Reference Library. The MALDI Biotyper output is a log (score) in the range 0 to 3.0. Thresholds for species and genus identification are, respectively, a log (score) of ≥2 and ≥1.7.

The identification results obtained by 16S rRNA gene sequencing are shown in Table 2 and Table S2 in the supplemental material together with MALDI-TOF MS results. Identification to the species level with 16S rRNA gene sequencing was obtained for 98 of 102 isolates (96.1%). Isolate SEQ053 (JN867222) displayed 99% identity with an isolate previously described in a blood culture study and provisionally designated "*P. massiliensis*" (1). For 3 of 102 isolates, sequence identity with a validly named strain was ≥97% but was <99% with good separation from strains of other genera and species (>0.8% sequence divergence); SEQ116 (JN867246) and SEQ065 (JN867234) were closely related to *P. melaninogenica*, and SEQ72 (JN867238) was related to *P. oralis*, displaying 98.7%, 98.5%, and 97.7% identity with the type strain, respectively. Of 98 isolates genetically identified to the species level, routine identification was confirmed for 42 (42.9%). For 39 isolates (39.8%) it was incomplete, and for 17 isolates (17.3%) it was discordant (see Table S1 in the supplemental material). Those results illustrate the difficulty of accurate species identification of this genus by phenotypic testing in a routine clinical laboratory setting.

MALDI-TOF MS results before and after extension of the commercial database are represented in Table 2. Using the commercial database, 64 of 102 (62.7%) isolates were identified to the species level. MALDI-TOF MS results were concordant with 16S rRNA gene sequencing for these isolates. Eleven of 102 isolates (10.8%) were identified only to the genus level with MALDI-TOF MS. Twenty-seven isolates (26.5%) were not identified.

Extension of the commercial database with spectra of 23 reference strains (13 additional species) improved identification to the genus and species level from 73.5% (75/102) to 89.2% (91/102) and from 62.7% (64/102) to 83.3% (85/102), respectively. As we had no reference strain for *P. heparinolytica* and because this species was not represented in the commercial database, we added a sequenced clinical isolate (SEQ003; GenBank accession no. JN867221) to the database. This made it possible to identify the two other *P. heparinolytica* isolates in the collection.

For all identifications, the 10 best matches of the database (with and without addition of new MSPs) were carefully assessed. In all cases where identification to the species level was obtained (the score of the top match ≥ 2.0), the following species or genus

TABLE 2 Comparison of MALDI-TOF MS identification with 16S rRNA gene sequencing identification results for 102 clinical *Prevotella* isolates

Identification by 16S rRNA gene sequencing	Total no. of isolates	No. of isolates with indicated level of MALDI-TOF MS result using the Bruker Reference Library 3.2.1.0 database			No. of isolates with indicated level of MALDI-TOF MS result using the Bruker Reference Library 3.2.1.0 database and the main spectra (MSPs) of reference strains created in this study		
		Species	Genus	No identification	Species	Genus	No identification
P. baroniae	3	3	0	0	3	0	0
P. bergensis	2	2	0	0	2	0	0
P. bivia	23	19	4	0	23	0	0
P. buccae	10	10	0	0	10	0	0
P. buccalis	3	0	1	2	0	1	2
P. corporis	3	3	0	0	3	0	0
P. denticola	9	0	0	9	9	0	0
P. disiens	7	7	0	0	7	0	0
P. heparinolytica	3	0	0	3	0	0	3
P. histicola	3	0	0	3	3	0	0
P. intermedia	3	3	0	0	3	0	0
P. loescheii	1	0	0	1	1	0	0
P. melaninogenica	8	6	1	1	6	1	1
P. nanceiensis	9	6	3	0	8	1	0
P. nigrescens	2	2	0	0	2	0	0
P. oralis	1	0	1	0	0	1	0
P. oris	3	3	0	0	3	0	0
P. salivae	2	0	0	2	0	0	2
P. timonensis	2	0	0	2	2	0	0
P. veroralis	1	0	0	1	0	1	0
Species provisionally identified as *P. massiliensis*	1	0	0	1	0	0	1
Prevotella species most closely related to *P. melaninogenica*	2	0	0	2	0	0	2
Prevotella species most closely related to *P. oralis*	1	0	1	0	0	1	0
Total (%)	102 (100)	64 (62.7)	11 (10.8)	27 (26.5)	85 (83.3)	6 (5.9)	11 (10.8)

match always had a score < 1.7. MALDI-TOF MS differentiated well between the *Prevotella* species. In the cases where only identification to the genus level (score between 1.7 and 1.999) could be made, all the matches with a score > 1.7 belonged to the genus *Prevotella*.

Identification results were improved not only by adding lacking species or additional strains to cover intraspecies variability but sometimes also by adding a spectrum of a reference strain already present in the commercial database. For instance, a MSP of reference strain *P. bivia* CCUG9557 was already present in Bruker database 3.2.1.0 (*P. bivia* DSM 20514T DSM), but for 19 of 23 *P. bivia* isolates, MALDI-TOF MS identification scores improved by addition of an in-house-made MSP of this strain and species identification was then obtained for all included isolates (see Table S2 in the supplemental material). The use of media or incubation conditions differing from those used for the creation of reference database by the manufacturer could be responsible for lower identification scores.

In this study, the majority of strains (96.1%) could be identified to the species level with 16S rRNA sequencing. In addition, one isolate displayed 99% identity with an unvalidated *P. massiliensis* species (1) and 3 isolates (2 closely related to *P. melaninogenica* and 1 closely related to *P. oralis*) did not display 99% identity with a validly named strain. This could have been due to limitations in the composition of the databases, or these isolates could represent species not yet established. As 16S rRNA gene analysis is still too expensive and time-consuming for routine use, MALDI-TOF MS makes it possible to identify clinical isolates with high accuracy and high speed for low cost.

We showed that MALDI-TOF MS is more accurate than conventional identification methods and represents a reliable alternative for 16S rRNA gene sequencing for the routine identification of *Prevotella* isolates. However, the Bruker MALDI-TOF MS system needs optimization by adding reference spectra for species not yet represented and by expanding the number of available spectra per species. Accurate identification of clinical isolates would improve knowledge about the pathogenicity, epidemiology, and clinical relevance of *Prevotella* species.

Nucleotide sequence accession numbers. The sequences obtained in this work were deposited in GenBank (accession no. JN867221 to JN867322).

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