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MALDI-TOF MS VERSUS 16S rRNA SEQUENCING: MINOR DISCREPANCY BETWEEN TOOLS IN IDENTIFICATION OF *BACTEROIDES* ISOLATES

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Members of the genus *Bacteroides* are important components of the normal microbiota of gastrointestinal tract; however, as opportunistic pathogens are also associated with severe or even life-threatening infections with significant mortality. Various species within *Bacteroides fragilis* group are phenotypically very similar; thus, their identifications with traditional-automated biochemical methods are frequently inaccurate. The identification of the newly discovered or reclassified bacteria can be doubtful because of the lack of biochemical profile in the database of these tests. The aim of this study was to determine the accuracy of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method by testing of 400 Hungarian *Bacteroides* clinical isolates. Inaccurate identification results with MALDI-TOF MS were confirmed by 16S rRNA gene sequencing and findings were compared with traditional-automated biochemical test rapid ID 32A method as well.

Keywords: *Bacteroides fragilis* group, sequencing, rapid ID 32A

Introduction

Although members of the *Bacteroides* genus are components of the normal microbiota of human gut, these strains are frequently isolated from sepsis, skin and soft tissue, intraabdominal and postoperative wound infections, pelvic, brain, and lung abscesses with the mortality rate of more than 19% [1–4]. In the infections caused by *Bacteroides* isolates, the most frequently used antibiotics are

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beta-lactam/beta-lactamase inhibitors, cephamycins, carbapenems, clindamycin, fourth-generation fluoroquinolones, and nitroimidazoles [1]. In the last 20 years, increasing antibiotic resistance rate of *Bacteroides* isolates was reported against cefoxitin, clindamycin, and moxifloxacin; however, amoxicillin/clavulanic acid, piperacillin/tazobactam, carbapenems, metronidazole, and tigecycline remained very active drugs against these bacteria [5]. Due to various antibiotic resistance patterns of species within the genus, adequate identification of *Bacteroides* isolates is very important to achieve optimal antimicrobial therapy [6]. Presumptive phenotypic identification based on colony morphology, antibiotic disks (5 μg vancomycin, 1,000 μg kanamycin, and 10 μg colistin), spot tests, and microscopic morphology (Gram-stained smear) may provide information about the bacteria at genus or species level [4, 7]. For species-level identification, commercial biochemical tests are available detecting either preformed enzymes under aerobic environment or inducible enzymes under anaerobic conditions. The main disadvantages of phenotypic identification methods are that they cannot distinguish surely the closely related species, and the anaerobic bacteria grow relatively slowly, and some species are biochemically inactive [3, 6]. In the last decades, remarkable changes in the taxonomy and nomenclature of anaerobic bacteria have been documented, owing to the development in molecular methods, new genera and species have been described. Identification of these new species by phenotypic methods can be difficult due to the lack of their biochemical profiles in various commercially available databases [8]. 16S rRNA gene sequencing is considered to be the gold standard method for identification; however, the application in routine diagnostic laboratory is difficult because of its time-consuming, expensive, and technically challenging features [3, 9]. Among the molecular methods, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) provides accurate and fast identification results of *Bacteroides* species [3]. The aim of this study was to test the accuracy of MALDI-TOF MS identification system using 400 Hungarian *Bacteroides* clinical isolates and to retest the strains with contradictory results given using MALDI-TOF in different laboratory in contradictory cases, rapid ID 32A (bioMérieux, France), and 16S rRNA gene sequencing methods were applied.

Materials and Methods

Bacterial strains

A total of 400 *Bacteroides* clinical isolates received from five Hungarian clinical microbiological centers (two centers from Budapest, Debrecen, Szeged, and Pécs) were collected between 2014 and 2016. The strains were stored at

−80 °C in cryobank vials with brain–heart infusion medium and with 20% glycerol until use. Local laboratories cultured and identified the examined strains according to standard laboratory operating procedures for anaerobic bacteria. The first identification was performed by MALDI-TOF MS (Bruker Daltonik, Germany) in the participating clinical microbiological centers. Species were distributed as follows during the first routine identification: 233 *Bacteroides fragilis*, 5 *Bacteroides caccae*, 34 *Bacteroides ovatus*, 69 *Bacteroides thetaiotaomicron*, 2 *Bacteroides salyersiae*, 14 *Bacteroides uniformis*, 27 *Bacteroides vulgatus*, 1 *Bacteroides nordii*, 14 *Parabacteroides distasonis*, and 1 *Parabacteroides goldsteinii* (Table I). Before the final MALDI-TOF analysis in Szeged, all the examined 400 strains were cultured on Schaedler agar (bioMérieux) for 48 h at 37 °C in anaerobic chamber (PerkinElmer, UK) under anaerobic conditions (85% N_2, 10% CO_2, and 5% H_2).

MALDI-TOF MS

The strains were identified in each center and also in Szeged by MALDI-TOF MS (Bruker Daltonik) with Biotyper version 3.0 software containing 5,989 mass spectra of reference strains of aerobic, anaerobic bacteria, and fungi. The reidentification of each strain in Szeged was performed by one person with three simultaneous measurements and three persons repeated it with one measurement per person with the same conditions (strains and chemicals). Results with the best log score values were accepted. The measurement mode was microflex, the parameters were:

Table I. Distribution of different *Bacteroides* species identified by MALDI-TOF MS (n = 400) in the original laboratory and reidentification in the laboratory of Szeged

Species	ID Budapest I	RD Budapest I	ID Budapest II	RD Budapest II	ID Debrecen	RD Debrecen	ID Szeged	RD	ID Pécs	RD
B. fragilis	49	48	52	49	63	64	60	60	9	9
B. caccae	1	1	2	1	1	1	1	1	–	–
B. ovatus	8	8	6	5	10	8	9	8	1	1
B. thetaiotaomicron	14	13	23	24	19	24	13	13	–	–
B. salyersiae	–	–	1	2	1	–	–	–	–	–
B. uniformis	8	8	–	–	3	1	3	3	–	–
B. vulgatus	11	10	6	6	2	1	8	8	–	–
P. distasonis	8	9	–	–	1	1	5	5	–	–
Bacteroides cellulosilyticus	–	–	–	1	–	–	–	–	–	–
Bacteroides stercoris	–	–	–	1	–	–	–	–	–	–
P. goldsteinii	1	–	–	–	–	–	–	–	–	–
B. nordii	–	1	–	1	–	–	1	2	–	–

Note: ID: first identification; RD: reidentification.

linear positive ion mode with a laser frequency of 20 Hz, LT: ISI 20 kV, IS2 18.5 kV, lens 8.5 kV, PIE 250 ns, no gating, range: 20–20,000 Da [6]. A small amount of one colony was spotted on the target plate, 1 µl of 70% aqueous formic acid, and after drying, 1 µl of MALDI matrix (α-cyano-4-hydroxycinannamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) were added to the spot. Interpretation of log score values was as follows: 0.000–1.699: unreliable identification; 1.700–1.999: genus-level identification; ≥2.000: species-level identification. We applied *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29742 strains as controls.

Rapid ID 32A

Twenty-one strains with contradictory results of identification and reidentification obtained by MALDI-TOF MS were checked by traditional biochemical test kit of rapid ID 32A method according to the manufacturer's instructions. Suspension with 4 McFarland turbidity was prepared from 48 h subculture in 2 ml of sterile suspension medium and dropped 55 µl into each cupule. The cupule for urease enzyme was overlaid with mineral oil. After covering the strips, they were incubated under aerobic conditions for 4 h at 37 °C. To nitrate and indole cupules, the appropriate reagents were added and these tests were read after 5 min. Catalase production was also directly investigated with 15% hydrogen peroxide. The biochemical profile was analyzed by computer with a specific database (analytic profile index, version 3.2) provided by the manufacturer. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29742 were used as control strains.

Reverse transcription polymerase chain reaction (RT-PCR)

Twenty-one strains with contradictory results were identified by 16S rRNA gene sequencing as well. DNA templates for PCR analyses were prepared as follows: one colony of each isolate was suspended in 100 µl of distilled water and heated at 99.5 °C for 12 min in a dry bath. The RT-PCRs for amplification of 16S rRNA gene were performed using 30 µl total volumes, containing 15 µl of 2× SYBR Green qPCR Master Mix (BioTool, USA), 10.2 µl water, 0.6 µl of E8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and E533R (5′-TIACCGIIICTICTGGCAC-3′) primers (concentrations: 35–35 pmol/µl), 0.6 µl ROX (BioTool Swiss AG, Switzerland), and 3 µl of DNA templates. StepOne RT-PCR machine (Applied Biosystems, USA) was used for the PCR cycling and detection: 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s, 56 °C for 20 s, 72 °C for 30 s, and 1 cycle of 72 °C for 75 s and a melting curve detection from 72 to 95 °C.

16S rRNA gene sequencing

The DNA amplicons from RT-PCRs (proportional scale up to 30 µl) were purified using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan). Purified templates were sequenced with ABI BigDye® Terminator version 3.1 kit in Series Genome Analyzer 3500 (Life Technologies, USA). The obtained sequencing data were analyzed by NCBI BLAST (http://www.ncbi.nim.nih.gov/blast/cgi) and leBiBi software (http://pbil.univ-lyon1.fr/bibi), the reliable identification level was set to 98.00%.

Results

During the routine identification in local laboratories and reidentification in Szeged, out of 400 strains, 379 (94.75%) were correctly identified to species level with the log score value of ≥2.000 (log score value range: 2.020–2.525, average log score value: 2.249) and the best log score values were chosen for analysis. Among the results of parallel MALDI-TOF MS reidentification measurements performed by three persons in Szeged, the best log score from the identification results was chosen, and the distribution of the identification results is summarized in Table I. MALDI-TOF MS reidentification results of 21 strains with log score value range of 1.855–2.458 were confirmed by 16S rRNA gene sequencing method and rapid ID 32A. These isolates with discrepancy according to the reidentification in Szeged are 4 *B. fragilis* and 17 non-*B. fragilis* strains (seven *B. thetaiotaomicron*, one *P. distasonis*, one *B. cellulosilyticus*, one *B. salyersiae*, one *B. stercoris*, one *Bacteroides intestinalis*, one *B. vulgatus*, two *B. ovatus*, and two *B. nordii*). The log score value of five strains among the examined 21 isolates with contradictory results was under 2.000 (1.993–1.802), and these strains belong to non-*B. fragilis* group. The same identification results with MALDI-TOF MS and sequencing were obtained in case of 15 (71.42%) isolates. Excellent identification results (>95.00%) were obtained with rapid ID 32A only in case of eight strains (8/21, 31.01%) (Table II). The database of rapid ID 32A does not contain the biochemical profiles of *B. cellulosilyticus, B. nordii, B. salyersiae*, and *Bacteroides xylanisolvens*, for this reason, four strains were not acceptable, whereas *P. distasonis* strains SY2 were identified as *Capnocytophaga* sp. In comparison of identification results with MALDI-TOF MS and rapid ID 32A, we reported only five concordant results (5/21, 23.81%). The quality of sequencing results was ≥98.00%, with the exception of strain SY9, which was identified with the level of 95.00%. In case of *Bacteroides* strains SY9, SY64, and SY81, the MALDI-TOF MS and 16S rRNA gene sequencing

Table II. Comparison of results of three different identification methods of 21 *Bacteroides* strains

Number	Original identification with MALDI-TOF MS	Reidentification in Szeged with MALDI-TOF MS	Log score value	Results of 16S rDNA sequencing (BLAST)	Quality of sequencing (%)	Results of rapid ID 32A identification	Quality of identification by rapid ID 32A (%)
D2	*B. ovatus*	*B. thetaiotaomicron*	1.871	*B. thetaiotaomicron*	99.00	*B. thetaiotaomicron*	99.80
D4	*B. fragilis*	*B. thetaiotaomicron*	1.993	*B. thetaiotaomicron*	99.00	*B. thetaiotaomicron*	99.70
D39	*B. salyersiae*	*B. fragilis*	2.237	*B. fragilis*	95.00	*B. uniformis*	39.60
D46	*B. vulgatus*	*B. thetaiotaomicron*	2.188	*B. thetaiotaomicron*	99.00	*B. thetaiotaomicron*	Very good quality
D63	*B. ovatus*	*B. fragilis*	2.458	*B. fragilis*	99.00	*B. fragilis*	97.10
D69	*B. uniformis*	*B. thetaiotaomicron*	2.129	*B. thetaiotaomicron*	99.00	*B. caccae*	52.40
D71	*B. uniformis*	*B. thetaiotaomicron*	1.855	*B. thetaiotaomicron*	99.00	*B. uniformis*	Not acceptable profile
SY2	*B. fragilis*	*P. distasonis*	2.359	*P. distasonis*	99.00	*Capnocytophaga* sp.	Not acceptable profile
SY9	*B. vulgatus*	*B. intestinalis*	1.916	*B. cellulosilyticus*	95.00	*P. distasonis*	82.00
SY23	*B. fragilis*	*B. vulgatus*	2.072	*B. fragilis*	99.00	*B. uniformis*	Not acceptable profile
SY25	*B. thetaiotaomicron*	*B. fragilis*	2.382	*B. fragilis*	99.00	*B. thetaiotaomicron*	Not acceptable profile
SY53	*B. vulgatus*	*B. ovatus*	2.072	*B. thetaiotaomicron*	99.00	*B. uniformis*	61.70
SY64	*B. ovatus*	*B. nordii*	1.802	*B. salyersiae*	99.00	*B. ovatus*	84.50
SY77	*B. ovatus*	*B. fragilis*	2.081	*B. fragilis*	99.00	*B. fragilis*	97.80
SY81	*B. fragilis*	*B. ovatus*	2.232	*B. xylanisolvens*	99.00	*B. caccae*	52.80
SE33	*B. fragilis*	*B. thetaiotaomicron*	2.39	*B. fragilis*	99.00	*B. fragilis*	96.50
SE56	*B. thetaiotaomicron*	*B. stercoris*	2.357	*B. stercoris*	99.00	*B. fragilis*	97.20
SE57	*B. caccae*	*B. salyersiae*	2.008	*B. salyersiae*	99.00	*B. caccae*	54.30
SE59	*B. fragilis*	*B. thetaiotaomicron*	2.135	*B. thetaiotaomicron*	99.00	*B. fragilis*	96.60
SE67	*B. fragilis*	*B. cellulosilyticus*	2.327	*B. cellulosilyticus*	99.00	*B. fragilis*	97.70
SZ80	*B. ovatus*	*B. nordii*	2.043	*B. nordii*	98.00	*B. ovatus*	43.30

results were different (SY9: *B. intestinalis/B. cellulosilyticus*; SY64: *B. nordii/ B. salyeriae*; and SY81: *B. ovatus/B. xylanisolvens*), because these strains are phylogenetically closely related and the protein patterns of these species are so similar that makes identification by mass spectrometry difficult [10, 11]. We accepted the sequencing results of *B. fragilis* SY23, *B. thetaiotaomicron* SY53, and *B. fragilis* SE33 strains. In the case of five isolates, the log score value of MALDI-TOF MS was bit lower than 2.00 (D2: 1.871; D4: 1.993; D71: 1.855; SY9:1.916; and SY64: 1.802).

Discussion

As several studies demonstrated the increasing rate of antibiotic-resistant strains among anaerobic bacteria, adequate species-level identification is getting very important. The traditional-automated methods have some limitations, e.g., the discrimination ability of biochemically similar strains is not sufficient. On the other hand, the results of identification may depend on the proper anaerobic environment and the deposited species in the library. Rapid ID 32A, as well as some other tests, cannot make difference between Gram-negative and Gram-positive bacteria (one fits all). The database always needs to be improved and expanded with the newly recognized species. According to the literature data, the correct identification with preformed enzyme kits is only 78%–79% of the *B. fragilis* group isolates [6]. Another disadvantage of the biochemical test can be the length of incubation time [(rapid ID 32A: 4 h; API 20 A (bioMérieux): 24 h; remel rapid ID ANA II (Thermo Fisher Scientific, USA): 4 h] and different incubation conditions, depending on the kind and the principle of kits. 16S rRNA gene sequencing is the most accurate method, but its complicated, time-consuming, and expensive features inhibit the application in routine clinical microbiology. The MALDI-TOF MS system revolutionized and simplified the identification of various clinical isolates. This method is easy to perform within a short period of time and reproducible, and this has a high discriminatory power. This study demonstrated that 94.75% of *Bacteroides* isolates were correctly identified with Biotyper software 3.0. According to the *dnaJ, gyrB, hsp60, recA, rpoB*, and 16S rRNA gene sequencing data, the phylogenetically related *Bacteroides* species classify to clades, e.g., species pairs: *B. intestinalis/B. cellulosilyticus, B. nordii/B. salyersiae, and B. ovatus/B. xylanisolvens* [10]. The differences among the results by MALDI-TOF MS and 16S rRNA gene sequencing can be explained with the classification in the same phylogenetical clade of *Bacteroides* strains SY9, SY64, and SY81. In the case of *Bacteroides* isolates SY23 (*B. vulgatus/B. fragilis*), SY53 (*B. ovatus/B. thetaiotaomicron*), and SE33

(*B. thetaiotaomicron/B. fragilis*), we accepted the 16S rRNA sequencing results. Culebras et al. [6] reported that the accurate, species-level identification of *Bacteroides* strains with MALDI-TOF MS system is 87% in comparison with 16S rRNA sequencing method. On the other hand, the rate of correct identification with rapid ID 32A method was 52.3%. Nagy et al. [4] reported that the unequivocal identification rate of *Bacteroides* isolates was 98.60% of with MALDI-TOF MS. According to the data of study by Handal et al. [11], the species-level identification of *Bacteroides* and Gram-positive anaerobic cocci blood culture isolates is 86.6% by MALDI-TOF MS. This study proved the superiority of MALDI-TOF MS system to traditional-automated biochemical tests. For validation of the method, two types of measurements were applied: one person with three simultaneous measurements and three persons repeated the investigation with one measurement per person with the same conditions (strains and chemicals). Good reproducibility of MALDI-TOF MS identification method of *Bacteroides* species was proved with this study.

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Conflict of Interest

None.

Ethical Approval

None.

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