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A head-to-head comparison of three MALDI-TOF mass spectrometry systems with 16S rRNA gene sequencing

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ABSTRACT Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionized diagnostics in culture-based microbiology. Commonly used MALDI-TOF MS systems in clinical microbiology laboratories are MALDI Biotyper (Bruker Daltonics) and Vitek MS (bioMérieux), but recently the new EXS2600 (Zybio) has been launched. This study aimed to evaluate the performance of the three devices by comparing the results to 16S rRNA gene sequencing. A set of 356 previously collected difficult-to-identify bacteria was tested in parallel with the three systems. Only the direct smear method and simple formic acid extraction were applied. Valid results were achieved for 98.6%, 94.4%, and 93.3% of all isolates by MALDI Biotyper, EXS2600, and Vitek MS, respectively. Of all valid results, agreement with sequencing data was achieved in 98.9%, 98.5%, and 99.7% by MALDI Biotyper, EXS2600, and Vitek MS, respectively. Considering only the isolates with valid measurements at the single-species level, misidentification rates were 0%, 2.6%, and 1.1% for MALDI Biotyper, EXS2600, and Vitek MS, respectively. Apart from minor performance differences, our data demonstrate that the three systems provide comparable results and are suitable for use in medical diagnostic laboratories.

Early and accurate identification of pathogens from clinical specimens is pivotal to guide the therapy of infectious diseases and to ensure a favorable outcome (1). Despite the increasing significance of molecular techniques, which are characterized by short turnaround times, cultivation still represents the most common method for the detection of bacteria and fungi. Significant benefits of culture-based diagnostics are comparably low costs and the fact that it is not necessary to define the pathogen to be investigated in advance, which is in contrast to pathogen-specific methods such as PCR or serology. All colonies grown on agar plates can be subjected to phenotypic and biochemical analyses. However, even with automated systems, identification of isolates via those methods requires a significant amount of time, which typically delays the result for a minimum of 1 day (2). Identification using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has overcome the previous limitations since it offers timely and cost-effective results (2, 3). To date, MALDI-TOF MS has become an essential technique and the diagnostic gold standard for virtually all medical microbiology laboratories in developed healthcare systems.

As great as the importance of MALDI-TOF MS is in diagnostics, the choice of instruments and databases is particularly small (4). This puts laboratories in a weak position as customers and limits the possibilities for customization to meet specific needs. Currently, there are only two manufacturers offering FDA-approved and IVD-certified MALDI-TOF MS systems: Bruker Daltonics (Bremen, Germany) and bioMérieux (Marcy-l'Étoile, France). Recently, two novel competitors have entered the market, namely, ASTA (Suwon, South Korea) and Zybio (Chongqing, People's Republic of China).

However, until very recently, the availability of their respective MALDI-TOF MS systems has been limited to local markets. Hence, data about the performance of these devices are scarce and comprehensive comparisons with the more established MALDI-TOF MS systems are lacking (5, 6). Conducting such comparative studies is challenging since laboratories typically have access to only one device and database. Furthermore, the measurements, particularly those with discordant results, must be compared to a reference standard of identification.

In this study, we aimed to evaluate the performance of three different MALDI-TOF MS systems in a head-to-head comparison, with 16S rRNA gene sequences as reference.

MATERIALS AND METHODS

Bacterial strains

This study relies on a collection of isolates from human specimens sampled for diagnostic analysis at the Diagnostic and Research Institute for Hygiene, Microbiology and Environmental Medicine of the Medical University of Graz. This academic institution hosts the central microbiology laboratory of the University Hospital Graz, a 1,600-bed tertiary care center in Graz, Austria. In 2010, 1,145 consecutive isolates were analyzed in parallel using the MALDI Biotyper instrument with related software (Bruker Daltonics) and the AXIMA Assurance instrument (Shimadzu Company, Kyoto, Japan) with the Saramis software (AnagonsTec GmbH, Potsdam, Germany) applying the respective then-current databases. All isolates with discordant or invalid MALDI-TOF MS results were identified by 16S rRNA gene sequencing [database: GenBank (National Center for Biotechnology Information)] with sequence identities ≥98% being considered to represent a valid result. Notably, this allowed for situations, in which differing results of two MALDI-TOF MS systems were still both categorized to be correct since the two identified species demonstrate sequence identities ≥98%. In this study, 356 precharacterized strains were included. The distribution of orders and genera is summarized in Table 1.

MALDI-TOF MS systems, scoring scheme, and statistical analysis

The isolates were used for a comparison of three MALDI-TOF MS systems of different manufacturers and their respective current databases (as of October 2022): (i) Bruker's MALDI Biotyper (Biotyper) using the "MBT IVD Library Revision G (Claim 6)" database, (ii) bioMérieux's VITEK MS (Vitek MS) using the "Knowledge Base V3.2" database, and (iii) Zybio's EXS2600 (EXS) using the "V.1.0.0.0" database.

All MALDI-TOF MS systems indicate the reliability of identification results by a color-coded categorization. For Biotyper and EXS, green results (identification score: ≥2.00) represent measurements valid at the species level and yellow results (1.70–1.99) represent measurements valid at the genus level. However, in certain cases, it is possible to get green results that do not feature a decisive species, for example, "*Fusobacterium* sp." (Bruker). With red scores (<1.70), no valid identification results were obtained. Although the traffic light coding is also applied by Vitek MS, the classification is based on a considerably different algorithm: green results (confidence levels ≥ 60%) include results with one organism at the species level as well as results with a group of organisms at the species level, for example, "99.9% *Lactobacillus casei/paracasei/rhamnosus.*" Yellow results consist of a group of results that add up to a confidence level of 100% caused by isolates that are not distinguishable by Vitek MS, for example, "33.3% *Moraxella lacunata*/33.3% *Neisseria avis*/33.3% *Moraxella catarrhalis.*" Identification results with more than four organisms or no concordant spectra in the database are categorized as red. Therefore, the Vitek MS performance cannot be easily compared to the performance of Biotyper and EXS. To facilitate a quantitative comparison, this study applies different identification levels: (i) single species level, which includes all green results featuring only one distinct species, (ii) level of green results, and (iii) level of valid results, that is, all green and

TABLE 1 Distribution of orders and genera of the isolates included in the study

Order	Genus	n	%
All isolates		356	100.0
Lactobacillales		71	19.9
	Streptococcus	39	11.0
	Enterococcus	20	5.6
	Lactobacillus	9	2.5
	Others	3	0.8
Bacteroidales		63	17.7
	Prevotella	42	11.8
	Bacteroides	21	5.9
Pseudomonadales		45	12.6
	Acinetobacter	18	5.1
	Pseudomonas	18	5.1
	Moraxella	9	2.5
Enterobacterales		40	11.2
Bacillales		36	10.1
	Staphylococcus	32	9.0
	Others	4	1.1
Pasteurellales		17	4.8
Micrococcales		10	2.8
Burkholderiales		12	3.4
Actinomycetales		10	2.8
Others		52	14.6

yellow results. Descriptive statistics were calculated with IBM SPSS Statistics for Windows, Version 28.0 (IBM, Armonk, USA).

Sample processing

Each measurement was performed from fresh colonies following overnight culture on Columbia sheep blood agar (BD; ref. no. 254071) or chocolate agar (bioMérieux; ref. no. 43109) at 5% CO_2 for fastidious organisms, or on Schaedler agar in anaerobic conditions for anaerobes (BD, Franklin Lakes, USA; ref. no. 254084). The processing was carried out in parallel, whereby one trained laboratory technician applied the strain onto the three targets (one spot each), according to the respective manufacturers' instructions using the corresponding reagents.

In a first attempt, all isolates were applied as smear directly from the agar plate to the respective MALDI-TOF MS targets. All measurements that did not yield a green result were repeated under the same conditions. When this approach also failed to generate a green result, a third measurement was performed, for which the cells were pretreated with formic acid, according to the manufacturer's protocols.

RESULTS

Validity of results

With the routinely used standard measurement procedure, that is, direct smear application of colonies to the target, green results were obtained for 85.4%, 77.2%, and 78.7% of isolates by the Biotyper, the EXS, and the Vitek MS MALDI-TOF MS systems, respectively. Upon failure to generate green results, re-testing and (if necessary) re-testing including formic acid treatment was performed, which increased the rates for measurement results at a green level to 92.1%, 87.4%, and 86.8% for Biotyper, EXS, and Vitek MS, respectively (Table 2).

When all (green and yellow) results were accepted, the respective rates increased by up to 16% by the first measurement (Table 2). Following the protocol of re-tests with

TABLE 2 Rates of valid measurement results of the three tested MALDI-TOF MS systems[a]

|	Biotyper	EXS	Vitek MS
Single-species results			
Direct smear	84.3 (80.1–87.9)	75.6 (70.8–79.9)	74.4 (69.4–78.9)
Re-testing	88.2 (84.4–91.4)	82.0 (77.6–85.9)	79.5 (74.9–83-6)
Formic acid treatment	91.0 (87.5–93.8)	85.7 (81.6–89.1)	81.7 (82.8–90.1)
Green results			
Direct smear	85.4 (81.3–88.9)	77.2 (72.5–81.5)	78.7 (69.6–78.9)
Re-testing	89.3 (85.6–92.3)	83.7 (79.5–87.4)	84.3 (80.1–87.9)
Formic acid treatment	92.1 (88.8–94.7)	87.4 (83.5–90.6)	86.8 (82.8–90.1)
All valid results			
Direct smear	94.9 (92.1–97.0)	91.9 (88.5–94.5)	85.4 (81.3–88.9)
Re-testing	97.8 (95.6–99.0)	93.8 (90.8–96.1)	91.6 (88.2–94.2)
Formic acid treatment	98.6 (96.8–99.5)	94.4 (91.5–96.5)	93.3 (90.1–95.6)

[a]Measurements of all 356 isolates were performed with Bruker MALDI Biotyper (Biotyper), Zybio EXS2600 (EXS), and bioMérieux VITEK MS (Vitek MS). Validity is displayed at the single-species level (Biotyper and EXS: green results featuring only one distinct species; Vitek: green and yellow results featuring only one distinct species), the level of green results, and the level of all valid results (green or yellow). When the first analysis failed to yield results at the desired level, the isolate was re-tested. When the re-test also failed, the isolates were re-tested using the formic acid extraction protocol. All rates are presented as percentages with 95% confidence intervals in parentheses.

and without formic acid, a maximum success rate of 98.6% was achieved by the Biotyper system.

Notably, a direct comparison of the categories "green" and "yellow" is restricted by the different definitions by the respective manufacturers, that is, Bruker and Zybio vs bioMérieux. For instance, *Streptococcus dysgalactiae* was identified by all MALDI-TOF MS systems at the species level. While Biotyper and EXS categorized this finding as a green result, the Vitek MS result was only yellow, since decisive discrimination between *S. dysgalactiae* subspecies dysgalactiae and *S. dysgalactiae* subspecies equisimilis failed—a differentiation level that is not even targeted by Biotyper or EXS. Consequently, an evaluation that considers only the "green result" vs "yellow result" categories would incorrectly understate the performance of the Vitek MS in this case. In other cases, however, the structure of the Vitek MS database results in differentiation results being presented as green that would be deemed invalid by the other MALDI-TOF MS systems: the Vitek MS database includes entries, in which for one spectrum results of "organism groups" are deposited. These groups can consist of up to four species, which can even be members of different genera and still are defined as valid measurements. To facilitate a quantitative evaluation, we included another comparison, in which only those results were accepted, which featured one single species (Table 2). After up to three measurements, valid results at the single-species level were obtained for 91.0%, 85.7%, and 81.7% of isolates by the Biotyper, the EXS, and the Vitek MS system, respectively (Table S1).

Some isolates did not yield valid results since no accurate spectra could be generated. For a certain group, however, not the quality of spectra but the database is the reason for identification failure: two of the five (40.0%) invalid Biotyper, seven of the 20 (35.0%) invalid EXS, and 12 of the 24 (50.0%) invalid Vitek MS results were attributable to missing database entries. The overlap between all measurement failures of the different MALDI-TOF MS systems is depicted in Fig. 1A (failure to generate green or yellow results) and Fig. 1B (failure to generate green results). In only seven isolates (2.0%), it was observed that all devices failed to generate a result at the species level (Biotyper and EXS) or organism group level (Vitek MS): one isolate each of *Achromobacter xylosoxidans*, *Bifidobacterium longum*, *Prevotella melaninogenica*, *Propionimicrobium lymphophilum*, *Pseudomonas aeruginosa*, *Pseudomonas pseudoalcaligenes*, and *Prevotella multiformis*. For the latter one, no valid measurement results at all were obtained (red results with all MALDI-TOF MS systems).

For EXS and Vitek MS, trends were observable that specific groups of organisms were particularly challenging: 10 of 20 (50.0%) invalid EXS results were obtained analyzing
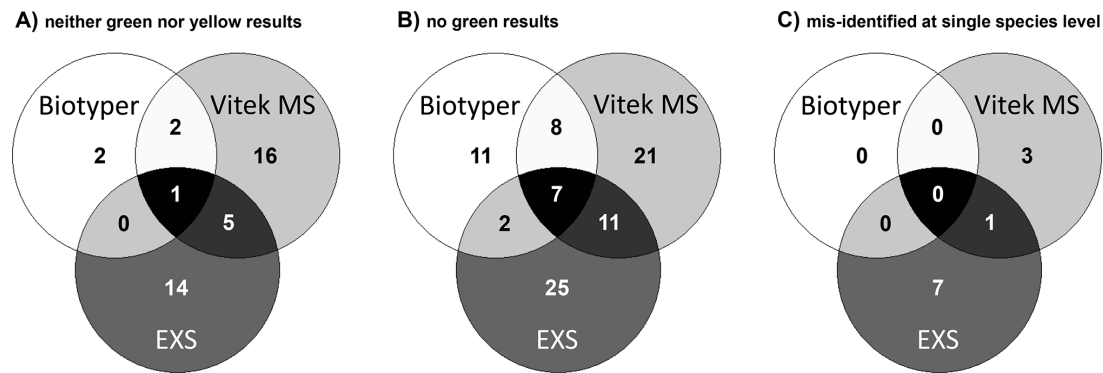
FIG 1 Venn diagrams visualizing the overlap of isolates with neither green nor yellow results (A), no green results (B), or incorrectly identified isolates (C). The latter group included all cases, in which the respective MALDI-TOF system (Bruker MALDI Biotyper: Biotyper; bioMérieux VITEK MS: Vitek; and Zybio EXS2600: EXS) yielded a valid result at single-species level, that is, green with only one distinct species, but false species identification.

Prevotella spp. Aerobe gram-negative rods such as *Pseudomonas* spp. and *Acinetobacter* spp. were very prevalent [13/24 (54.1 %)] in the group of invalid Vitek MS measurements.

Accuracy of results

All MALDI-TOF MS results were compared to the results of 16S rRNA gene sequencing. When only results featuring one distinct species were considered, agreement with sequencing results was 91.0% for the Biotyper, 83.4% for the EXS, and 80.6% for the Vitek MS after up to three measurements. However, those numbers do not allow us to calculate the rate of misidentifications since the aforementioned rates refer to the overall number of isolates—including those without valid identification results. Considering only the isolates with valid measurements at the single-species level, misidentification rates were 0% (0/324) for Bruker, 2.6% for EXS (8/305), and 1.4% for Vitek MS (4/291).

The performance for correct identification at the other validity levels is summarized in Table 3. Except for single-species-level analysis, MALDI-TOF MS results featuring more than one species were accepted to be correct, when at least one of the species was in concordance with 16S rRNA gene sequencing results.

TABLE 3 Concordance of 16S rRNA gene sequencing and MALDI-TOF MS measurements[a]

	Valid results	True results		
	n	*n*	Of valid tests	Of all tests
Biotyper				
Single-species results	324	324	100.0 (98.9–100)	91.0 (87.5–93.8)
Green results	328	324	98.8 (96.9–99.7)	91.0 (87.5–93.8)
All valid results	351	347	98.9 (97.1–99.7)	97.5 (95.3–98.8)
EXS				
Single-species results	305	297	97.4 (94.9–98.9)	83.4 (79.1–87.1)
Green results	311	300	96.5 (93.8–98.2)	84.3 (80.1–87.9)
All valid results	336	331	98.5 (96.6–99.5)	93.0 (89.8–95.4)
Vitek MS				
Single-species results	291	287	98.6 (96.5–99.6)	80.6 (76.1–84.6)
Green results	309	303	98.1 (95.8–99.3)	85.1 (81.0–88.6)
All valid results	332	331	99.7 (98.3–100)	93.0 (89.8–95.4)

[a] The rate of MALDI-TOF MS tests, which yielded results in agreement with sequencing results (true), refers to the total measurements that yielded results at the respective validity level, that is, single-species level, level of green results, and level of valid results (green or yellow). Except for single-species-level analysis, MALDI-TOF MS results featuring more than one species were accepted to be correct, when at least one of the species was in concordance with 16S rRNA gene sequencing results. For the category "all valid results," results featuring the correct genus according to the reference results were classified as true. Total number of isolates included in the analysis: 356; all rates are presented as percentages with 95% confidence intervals in parentheses.

Characterization of misidentified isolates

There was a notable overlap of the misidentification results observable: most isolates with results at the single-species level that were misidentified by any MALDI-TOF MS system also yielded incorrect or invalid results for at least one other MALDI-TOF MS device (Table 4; Fig. 1C). For three Vitek MS misidentifications, it must be considered that the current database does not include the analyzed species. No isolate was misidentified by all three systems. All misidentifications (EXS: eight; Vitek MS: four) represented minor errors with results at least correct at the genus level. In one case, the misidentification is particularly noteworthy since the respective false results might have triggered a misdiagnosis that also could have had therapeutic consequences. A *Streptococcus mitis* strain, a commensal causing rare but sometimes severe infections, was misidentified by EXS and Vitek MS as the closely related *Streptococcus pneumoniae*, which is much more often related to the disease. Considerably, it is good and common laboratory practice to verify the MALDI-TOF MS finding of *S. pneumoniae* with traditional methods, for example, bile solubility or optochin sensitivity, since the limitations of MALDI-TOF MS to differentiate these germs are well known.

DISCUSSION

Already in 2014, McElvania TeKippe and Burnham evaluated the performance of Biotyper and Vitek MS for analyzing "unusual and/or difficult-to-identify microorganisms" and reported identification rates comparable to our findings despite relying on much less comprehensive databases (7). However, their collection of 172 isolates representing 50 genera featured a very specific focus on organisms rarely encountered in routine diagnostics. In this setting, MALDI-TOF MS became the primary tool for rapid, accurate, and cheap identification of cultivated bacteria and yeasts. Accordingly, there is plenty of data analyzing Biotyper and Vitek MS performance (8–15). Contrarily, studies evaluating the EXS system are very scarce: to date, only three studies have been published. However, focusing on the detection of *Shewanella* (16), on antifungal susceptibility testing in *Talaromyces marneffei* (17), and classification of *Listeria* species (18), those data do not feature an insight into the capability of EXS to support routine diagnostics in a medical microbiology laboratory.

This study attempted to present the results not only as a narrative enumeration of individual results but to make these results evaluable with the help of quantitative indicators. These indicators aim to give an overview of the MALDI-TOF MS performance in terms of providing (i) valid and (ii) correct identification results. With valid results ranging from 93.3% to 98.6% and correct results of 98.5% to 99.7% in this cohort, all

TABLE 4 Summary of isolates that yielded valid but incorrect identification results at single-species level by any MALDI-TOF MS system[a]

Reference result	Biotyper result	Score	EXS result	Score	BMX result	Conf. level
Bacteroides dorei	*Bacteroides vulgatus*[b,c]	2.22	*B. vulgatus*	2.43	*B. dorei*	99.9
Brevibacterium massiliense	No green result		Invalid result		*Brevibacterium casei*	99.9
Brevundimonas nasdae	No green result		*Brevundimonas aurantiaca*	2.17	Invalid result	
Fusobacterium gonidiaformans	No green result		*Fusobacterium necrophorum*	2.16	Invalid result	
Moraxella canis	*M. canis*	2.43	No green result		*M. catarrhalis*	99.9
Neisseria flavescens	*Neisseria subflava*	2.16	*Neisseria lactamica*	2.25	No single-species result	
Neisseria flavescens	*Neisseria subflava*	2.14	*N. lactamica*	2.16	No single-species result	
Prevotella timonensis	*P. timonensis*	2.13	*Prevotella buccae*	2.32	*Prevotella timonensis*	99.9
P. timonensis	*P. timonensis*	2,06	*P. buccae*	2.05	*P. timonensis*	99.9
Staphylococcus croceolyticus	*Staphylococcus petrasii*	2.08	Invalid result		*Staphylococcus haemolyticus*[b]	99.9
S. mitis	*S. mitis*	2.13	*Streptococcus pneumoniae*	2.04	*S. pneumoniae*	99.1

[a]Misidentified isolates are marked by underlining. For all isolates with results at the single-species level, the respective score (Biotyper and EXS) or confidence level (Vitek MS) is indicated.

[b]Cases of misidentification, in which the analyzed species is not included in the respective MALDI-TOF system's database.

[c]Classified as correct since the Biotyper provides the hint that upon the result *Bacteroides vulgatus* a differentiation between *B. vulgatus* and *B. dorei* is not possible.

three MALDI-TOF MS systems proved to be valuable tools for the microbiology labora-tory. Inevitably, details suffer in this attempt to aggregate and summarize data. As an example, 17 isolates (4.8%) with a Biotyper score greater than 1.90 but just not the target score of 2.00 (=green result) had to be measured repeatedly by the Biotyper system. In a practical and pragmatic approach, which might be the daily routine in many diagnostic laboratories that aim to provide results for the attending physicians as soon as possible, such close results would probably not have triggered retesting— even when this practice must be validated and the results should be interpreted with caution. Consequently, it can be assumed that the critical evaluation of this study even tends to underestimate the performance of all three MALDIs.

Previously, anaerobe bacteria represented a particular challenge for MALDI-TOF MS analysis with notably lower identification rates compared to other groups of microor-ganisms (11, 12). Due to the continuous expansion and improvement of the spectra databases, identification rates for anaerobes today exceed 80% at the species level and 90% at the genus level, with a slight advantage reported favoring Vitek MS (13). Contrarily, the identification of *Enterobacterales* and non-fermenting Gram-nega-tive bacilli was reported to be a specific strength of Biotyper (14, 15). The methodic limitations to differentiate between closely related Gram-negative species are reflec-ted by the peculiarity of the Vitek MS not to commit to a single species ID for certain organism groups, for example, "*Citrobacter braakii/freundii*" or "*Enterobacter cloacae/asburiae*" (19). Also in our study, invalid Vitek MS results were observed more frequently with those organisms. A comparable finding occurred for EXS and anaerobe bacteria. However, the strain collection of this investigation had no particular focus on those groups and therefore lacks an adequate number of respective organisms to substantiate this finding, which should be addressed in more specific comparisons. Contrarily, the Biotyper system has some sort of intrinsic limitations regarding some organism groups: Bruker is the only manufacturer to provide a modular database with add-ons that are not available for all users but must be purchased separately. Depending on whether the respective databases are installed, identification of specific organism groups, for example, filamentous fungi or Mycobacteria, is not possible. Notably, the sole mycobacterial isolate included in this study (*Mycobacterium abscessus*) was not identified by the Bruker Biotyper using the IVD reference library but only after the optional Mycobacteria IVD Module was applied. Furthermore, the required database must be selected as a reference before analyzing the isolate, which has certain implications for laboratories and their workflows.

To our knowledge, this is the first study presenting a parallel comparison of three different MALDI-TOF MS devices and databases. Furthermore, all MALDI-TOF MS results were compared to the results of 16S rRNA gene sequencing representing the gold standard of identification. However, this study also has some limitations, that is, (i) the low sample number, (ii) the specific species spectrum, and (iii) the applied test systems. In the case of the latter, it should be considered that neither the bioMérieux nor the Bruker device used in this work represent the latest models. On the other hand, the respective differences between the bioMérieux VITEK MS PRIME (market launch: 2022) and the Bruker MALDI Biotyper sirius (market launch: 2019) are less to be found in the measurement technology but rather in the handling and the capability for high-throughput measurement. Both the most recent and the precursor models use the same spectrum database of the respective manufacturer. Hence, this study still allows a comparison of the performance in terms of measurement validity and database quality. With regard to the overall low sample size, it is evident that a study based on 356 isolates cannot provide a definitive comparison of the MALDI-TOF MS systems. However, the identity of all analyzed isolates was specified by 16S rRNA DNA gene sequencing representing a high-quality reference method. This gives this study an edge over other investigations just relying on a method comparison between two devices, which would not allow the detection of concordantly incorrect results. Notably, such results were observed in our analysis. Finally, the species included in this analysis are not

representative of the spectrum of microorganisms that we are encountering in the daily routine of medical microbiology. However, with respect to the excellent performance reported for the identification of the most common pathogens, for example, *Staphylococcus aureus* or *Klebsiella pneumoniae*, it would not have strengthened the significance of this study to expand the sample collection with strains that do not represent any challenge for MALDI-TOF MS (8–10). Instead, this study focused on species that neither Biotyper nor Vitek MS has been successful in identifying in the past, which makes that organism collection an interesting study object.

Altogether, our data suggest that all three devices are suitable for the use in a diagnostic laboratory for medical microbiology, although specific weaknesses for each MALDI-TOF MS system were observed. Further studies are necessary to provide a more profound comparison.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplementary Table 1 (JCM01913-22-s0001.pdf). MALDI-TOF measurement results for each included isolate.

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