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REVIEW



## How MALDI-TOF mass spectrometry can aid the diagnosis of hard-to-identify pathogenic bacteria – the rare and the unknown

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### ABSTRACT

**Introduction:** Ten years after its introduction into clinical microbiology, MALDI-TOF mass spectrometry has become the standard routine identification tool for bacteria in most laboratories. The technology has accelerated analyses and improved the quality of results. The greatest significance has been observed for bacteria that were challenging to be identified by traditional methods.

**Areas covered:** We searched in existing literature (Pubmed) for reports how MALDI-TOF MS has contributed to identification of rare and unknown bacteria from different groups. We describe how this has improved the diagnostics in different groups of bacteria. Reference patterns for strains which yet cannot be assigned to a known species even enable the search for related bacteria in studies as well as in routine diagnostics. MALDI-TOF MS can help to discover and investigate new species and their clinical relevance. It is a powerful tool in the elucidation of the bacterial composition of complex microbiota in culturomics studies.

**Expert opinion:** MALDI-TOF MS has improved the diagnosis of bacterial infections. It also enables knowledge generation for prospective diagnostics. The term 'hard-to-identify' might only be rarely attributed to bacteria in the future. Novel applications are being developed, e.g. subspecies differentiation, typing, and antibiotic resistance testing which may further contribute to improved microbial diagnostics.

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MALDI-TOF MS; mass spectrometry; bacterial identification; fastidious; anaerobes; mycobacteria; rare bacteria; unknown species; culturomics; spectral database

## 1. Introduction

Before MALDI-TOF mass spectrometry (MS) had become a routine technology in laboratories of clinical microbiology, classical phenotypical tests, in particular, based on biochemical properties like the fermentation of sugars, were the common standard methods to identify and describe yet unknown, potentially pathogenic microorganisms. Generally, these tests start from pure colony material and need at least an overnight incubation. Thereby, the identification of even fast-growing bacteria is delayed by one day, and this delay becomes even longer for slow-growers.

Further, the differentiation of closely related bacteria just based on biochemical characteristics is difficult or impossible in a considerable number of cases. This is especially a problem for microorganisms with few characteristic biochemical reactions like non-fermenting gram-negative bacteria or anaerobes. This might lead to misidentifications with negative consequences for treatment.

Even more problematic are very rare or yet undescribed bacteria for which reliable references for biochemical profiles may be missing completely. MALDI-TOF MS has changed this situation dramatically [1]. Many previously hard-to-identify bacteria now can be easily characterized by their protein profile spectra. The time needed for definitive identification has reduced to minutes starting with a single small colony,

regardless of the kind of microbe, thereby the result is available within one day for most bacteria. And even many bacteria with very similar phenotypical characteristics can now be easily differentiated.

This review will give an overview of the progress which has been made in recent years. Dedicated custom databases for research studies and the improvement of the commercially available databases have recently enhanced the capability to detect bacteria from groups that have been difficult to identify. Large studies and clinical routine usage of MALDI-TOF MS systems have uncovered bacteria which were not previously recognized as clinically relevant, some of them even not defined as an individual species.

## 2. MALDI-TOF MS for identification of bacteria

Generally, cultured microorganisms are necessary for MALDI-TOF MS identification because a sufficient number of cells is necessary for spectra acquisition, i.e. about  $5 \times 10^5$  cells on a target spot [2]. The standard routine workflow in most laboratories starts with the transfer of a small amount of biomass from a colony grown on an agar plate to a sample position of a MALDI target plate. The bacterial biomass is overlaid with one  $\mu\text{l}$  of a solution containing a special matrix which is necessary for the MALDI process. After drying, the target plate is introduced into the mass spectrometer

**Article highlights**

- MALDI-TOF MS has significantly improved the identification of many previously hard-to-identify bacterial groups. This is particularly true for gram-negative nonfermenting bacteria, bacteria from the HACEK group, anaerobes, and mycobacteria.
- New species which are introduced by new phylogenetic insights or new discovery can easily be integrated into MALDI-TOF MS databases.
- Renaming of species is a further challenge in clinical microbiology.
- Permanent database update and curation is required.
- Culturomics studies benefit from MALDI-TOF MS and are feeding the databases.
- Novel algorithms can further improve differentiation of closely related species or subspecies.
- Detection of subtypes, e.g. for resistance or virulence detection, is extending the scope of MALDI-TOF-MS in the microbiology laboratory.
- MALDI-TOF MS-based phenotypic susceptibility testing might be a promising option for the future.

for measurement. For some microorganisms, well-adapted sample preparation protocols have been established to improve the efficacy of protein extraction from the biomass. In the vacuum of the MALDI-TOF instrument, the matrix including the embedded proteins is evaporated by a pulsed laser beam. The biomolecules are ionized and accelerated by high voltage, then separated according to their mass/charge ratio ( $m/z$ ) in a flight tube. The resulting mass spectrum depicts the  $m/z$  on the x-axis and the intensity (number of molecules at a certain mass) on the y-axis. The spectra are compared to pre-established databases according to the algorithms of the respective manufacturers. A comparison of MALDI-TOF MS with other frequently used methods for identification of bacteria is given in [Table 1](#).

Today, systems provided predominantly by two different manufacturers are in use in the vast majority of laboratories performing MALDI-TOF MS-based microorganism identification, i.e. the MALDI Biotyper (Bruker Daltonik GmbH, Germany) and the Vitek MS (bioMérieux, France). Both are available as research-use-only (RUO) and in-vitro diagnostic (IVD) versions. These two different systems both acquire protein/peptide mass spectra in the range of 2000–20,000  $m/z$ ; however, the bioinformatic approaches differ fundamentally. The MALDI Biotyper uses a pattern matching approach [3] with a database containing individual references, each called MSP. A newly acquired spectrum is converted to a peak list which is then compared to all MSPs of the MALDI-TOF reference database. The similarity of the spectrum to each reference is calculated and expressed as a 'log(score)' with values between 0 and 3. The reference with the highest log(score) value, i.e. the highest similarity, is the basis of the identification. A log(score)  $\geq 2$  indicates a 'high confidence identification', log(score)  $\geq 1.7$  and  $< 2$  gives a 'low confidence identification' for gram-positive and gram-negative bacteria. Log(scores)  $< 1.7$  are interpreted as 'no reliable identification'. For mycobacteria, the thresholds are adopted to log(score)  $\geq 1.8$  (high confidence) and  $\geq 1.6$  (low confidence), respectively [4]. For research purposes, these acceptance criteria have often been modified according to the personal experience of the authors. The bioinformatic approach is the same for both the RUO and IVD version of the MALDI Biotyper. In contrast,

the Vitek MS is using two different bioinformatic systems in the IVD and RUO mode. The Vitek MS IVD uses an algorithm based on supervised machine learning, the 'Advanced Spectra Classifier', for identification [5]. The spectra between 3,000 and 17,000 Daltons are divided into 1300 segments ('bins') which are weighted according to their importance for identification/differentiation of a species. An unknown spectrum is also 'binned' and compared to the Vitek MS 'Knowledge Base'. Results are given as percentages, with a perfect match having a value of 99.9%,  $>60\%$  to 99.8% are considered as good. Scores  $<60\%$  are considered as no valid identification. The SARAMIS system (RUO of Vitek MS) is working with a database of so-called 'Super Spectra', reference peak lists with weighted marker peaks. SuperSpectra are computed from typical strains covering intraspecific diversity in species. Identification of unknown strains is performed by comparison to all SuperSpectra in the database followed by a ranking according to similarity. Confidence levels are given as high ( $>98\%$ ) to medium (85% to 98%) to low (75% to 85%) [6].

Detailed information can be found in the product information presentations on the websites of each manufacturer of the MALDI Biotyper [7] and Vitek MS [8]. The ASTA MicroIDSys [9] is not currently in use in Europe or the United States, and limited information is available on this system.

### 3. MALDI-TOF MS: performance for challenging organism groups

#### 3.1. Aerobic bacteria

Aerobic bacteria from several taxonomic entities have been difficult to identify or differentiate by traditional methods. Soon after the introduction of MALDI-TOF MS into clinical routine, a significant improvement in these areas could be observed. The continuous enhancement of reference databases could further advance the performance of MALDI-TOF MS.

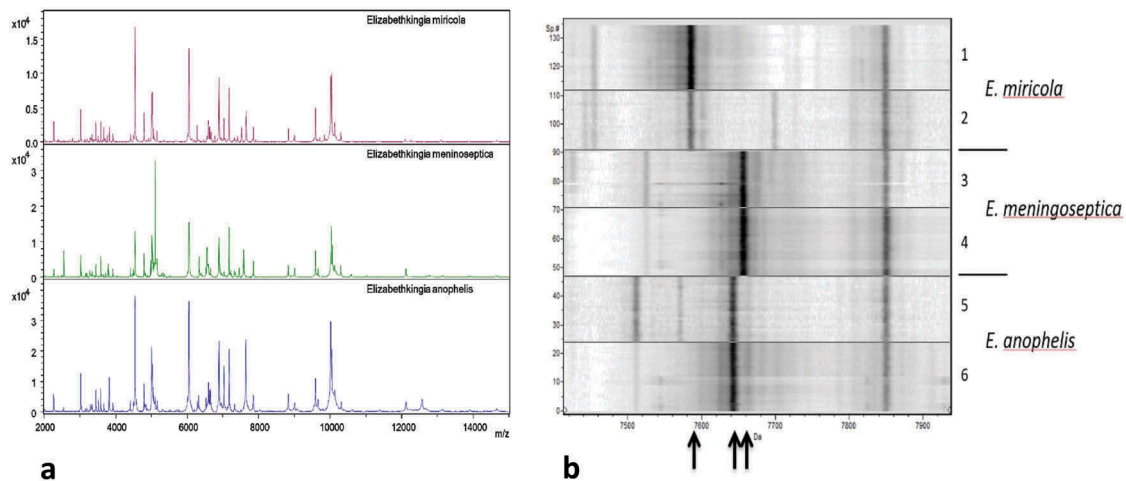
##### 3.1.1. Nonfermenters

Among the first groups that have been thoroughly investigated were gram-negative non-fermenting aerobic bacteria, a heterogeneous group of microbes which are difficult to identify by classical methods because of their lack of characteristic biochemical reactions. Nonfermenters can be ubiquitous in the environment and are responsible for a broad spectrum of (opportunistic) infections. Although the performance of MALDI-TOF MS was superior to biochemical systems [10] some closely related groups could not be differentiated and results ended at the group or even genus level. These limitations are also known for 16S rRNA gene sequencing. In addition, the permanent evolution of microbial taxonomy created new species which represented a new challenge for identification.

In 2011, 3 years after the first comprehensive studies for nonfermenting bacteria using MALDI-TOF MS, a new species *Elizabethkingia anophelis* was described. The type strain was isolated from the midgut of a mosquito and is closely related to *E. meningoseptica* and *E. miricola* [11]. Just shortly after its description, it was found that the new species could cause healthcare-associated outbreaks with high morbidity and high mortality [12]. Although commercial diagnostic systems at that

**Table 1.** Comparison of methods and technologies for identification of microorganisms in clinical microbiology laboratories with special regard to new or infrequently encountered species.

	Conventional phenotypic/biochemical ID	MALDI-TOF MS	Sanger sequencing (e.g. 16S/18S rRNA genes)	Next generation sequencing (NGS)
Availability for clinical routine	Available in all laboratories (commercial panels or in-house tests)	Available in a growing number of laboratories; First line ID method in most larger laboratories	Use limited to special cases. Mainly in reference laboratories and academic institutions.	No routine use. Usually limited to large reference laboratories and research institutions.
Primary material	Pure colony material	Pure colony material, positive blood cultures; Limited possibility of ID of a mixture of two microorganisms	Pure colony material. Special applications may allow purification/amplification and subsequent sequencing directly from sample material.	Pure colony material. Also allows parallel sequencing of (free) DNA of multiple (uncultured) microorganisms for metagenomic analysis directly from sample material.
Database/coverage of microorganisms	Coverage usually limited; different test panels have to be used for the various groups of microorganisms with different discriminatory characteristics.	Broad coverage by one or a combination of several databases. Some systems allow expansions of databases with own database references.	Very broad coverage in the commonly accessible databases. Matches with un-curated entries in open databases have to be critically reviewed and assessed.	(Very) broad coverage with commonly accessible databases. Matches with un-curated entries in open databases have to be critically reviewed and assessed.
Performance of ID for common microorganisms	Good. Limitations for slow growing organisms and organisms with a low number of characteristic phenotypic reactions.	Very good, permanently improving by database extension and quality control.	Gold standard. Performance may differ for different microorganism groups for the target used for sequencing.	Gold standard.
Performance of ID for infrequently encountered microorganisms in clinical routine	Restricted. Difficult if microorganism is not covered by the test panel used. (No identification result or probable misidentification) Good if corresponding panel exists and is used. No ID possible or misidentification.	Very good if covered by the database. If not covered by database, the obtained results may give a hint to related species but also may result in misidentifications with (very closely) related species. No ID possible. Database can be expanded with a reference to recognize the reoccurrence of the microorganism.	Gold standard. Several sequencing targets may be needed to distinguish closely related species.	Gold standard
Performance for yet undescribed microorganisms	No ID possible or misidentification.	Obtained results may give hints to species related. Database can be expanded with a reference to recognize the reoccurrence of the microorganism.	Matches with other entries in commonly accessible databases possible. Comparison to next best matches may show phylogenetic relationship.	Relationship to other species can be assessed by the position in the phylogenetic tree.
Time to result	8–48 h	Usually < 30 min. < 90 min with special extraction, e.g. mycobacteria or directly from positive blood cultures	Some days.	Several days – 2 weeks.
Operating expense and costs per analysis	Low to average	Very low	Medium to high	Very high



**Figure 1.** Extremely similar MALDI-TOF mass spectra from three different *Elisabethkingia* species, *E. miricola*, *E. meningoseptica*, and *E. anophelis*. No differentiating features are obvious.

a) Pseudo-gel view of mass spectra from the three *Elisabethkingia* species, *E. miricola*, *E. meningoseptica*, and *E. anophelis*, zoom in the region of  $m/z$  7000–8000. Two strains of each species are depicted, at least 20 spectra per strain. Arrows point to potentially species-specific peaks.

time were not able to securely differentiate *Elisabethkingia* species, the new species *Elisabethkingia anophelis* could be identified by MALDI-TOF MS during an outbreak in Wisconsin using an in-house database at the Centers for Disease Control in Atlanta [13]. This demonstrates the possibility of rapid adaptation of a MALDI-TOF system for an emerging pathogen by expert users (see below). Supplementing the SARAMIS database of the Vitek MS research-use-only (RUO) system also enabled the correct identification of *E. anophelis* [14]. Figure 1 depicts the overall similarity of mass spectra acquired from strains of the three main species of *Elisabethkingia* and characteristic peak patterns which can be used for their differentiation. Nielsen and coworkers reported the limitations of current MALDI-TOF MS systems in the identification of *Elisabethkingia* species [15]. An update of the MALDI Biotyper database and a supporting subtyping functionality are in preparation for 2019 (own unpublished results). But meanwhile, three further species of the genus have been proposed which might require further updating of existing databases [16].

The genus *Acinetobacter* is an example of how microbial identification is being challenged by taxonomic evolution. It has undergone significant taxonomical changes during the recent years. Several new species have been described, which poses a challenge for all identification systems. The flexibility and high discriminative power of MALDI-TOF MS enable the rapid adoption of databases to offer an up-to-date identification result for diagnostics. Again, as nonfermenters, these species are common in the environment and also can be found as part of the normal flora of the human skin. The members of the *Acinetobacter baumannii* complex are especially clinically important as they may cause severe nosocomial infections. Furthermore, they tend to be epidemic in hospitals and are therefore important for questions of hospital hygiene and infection control.

Espinal and coworkers reported the capability of MALDI-TOF MS to differentiate species from the *A. baumannii* group, i.e. *A. baumannii*, *A. pittii*, and *A. nosocomialis* [17]. The authors concluded that the improvement in differentiation within this group might offer the chance to learn about their

epidemiology and allow for targeted therapeutic and infection control measures. Mari-Almirall and colleagues included further novel species, *A. seifertii* and *A. dijkschoorniae*, into the MALDI Biotyper database to extend the scope of identification inside the *A. baumannii* group [18]. To improve the differentiation of these closely related species they used the ClinProTools software (Bruker Daltonik, Germany) which enabled the utilization of species-specific peaks when the standard approach did not work. A later study investigated the performance of the MALDI Biotyper for the 18 *Acinetobacter* species described at that time [19]. As six of the novel species were not included in the commercially available database, additional reference spectra for those were added by a custom database. While this extension of the database only partially improved identification, use of an alternative MALDI matrix (ferulic acid) allowed the correct identification of nearly all problematic strains. Such an approach required a two-step or parallel preparative approach which could be too laborious for rapid routine diagnostics. It has to be investigated if a further search for differentiating mass peaks may lead to an alternative algorithm to overcome current limitations.

A previously underestimated pathogen, *Wohlfahrtiimonas chitiniclastica*, gained attention after introduction of MALDI-TOF MS into routine microbiology laboratories. Before 2014, the bacterium which lives in larvae of an obligate parasitic fly was only detected two times in human blood samples. As biochemical tests could not identify the microorganism, 16S rRNA gene sequencing had to be applied. With the spread of MALDI-TOF MS and the extension of its database with reference patterns of more rare microbes, the number of reports from several continents for this zoonotic pathogen as a cause of human diseases significantly increased [20]. Flies are potent vectors for the distribution of *W. chitiniclastica* causing local or systemic infections originating from wounds infected with fly larvae but also other transmission routes have been described. Infections in humans include wound infections, cellulitis, osteomyelitis, and sepsis [20].



Also, *Myroides odoratus* and *Myroides odoratimimus* are rare non-fermenting human pathogens. These bacteria have been regarded as non-pathogenic for humans for decades. But an increasing number of case reports now indicate that both species are human pathogens. Besides urinary tract infections, soft tissue infections (including cellulitis and necrotizing fasciitis) and sepsis have also been associated with *Myroides* spp. Mostly (but not only) immunocompromised patients are affected. To date, new species (*Myroides injenensis*, *Myroides pelagicus*, *Myroides profundi*, *Myroides marinus*, *Myroides phaeus*, *Myroides xuanwunensis*, *Myroides indicus*, and *Myroides gitamensis*) have been added to the genus *Myroides*. However, clinical cases are only related to *M. odoratus* and *M. odoratimimus*. Therefore, these two species need to be specially considered by both microbiologists working in diagnostic laboratories and clinicians. In this regard, it is essential to reliably identify to species level. For this reason, the suitability of MALDI-TOF MS (MALDI Biotyper, Bruker Daltonik, Germany), sequencing of the 16S rRNA gene and biochemical characterization by VITEK2 (bioMérieux, France) for identification of *M. odoratus* and *M. odoratimimus* have been compared. MALDI-TOF MS and sequencing could securely distinguish both species [21]. Applying VITEK2, it was only possible to obtain the genus *Myroides*, but not the distinct species.

Both *M. odoratus* and *M. odoratimimus* are highly resistant against multiple antibiotics, especially  $\beta$ -lactams and aminoglycosides [22]. Therefore, definitive identification allowed the study of the individual susceptibility profiles of *Myroides odoratus* and *Myroides odoratimimus* separately for the first time [22].

### 3.1.2. HACEK group

Identification of bacteria from the so-called HACEK group, i.e. belonging to the genera *Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella*, and *Kingella*, has benefitted from the introduction of the MALDI-TOF MS technology [23,24]. These bacteria are involved in infective endocarditis and cause the majority of cases of gram-negative endocarditis [25]. In a recent nationwide population-based study of HACEK bacteremia in Denmark, the authors highlighted the benefit of the use of MALDI-TOF MS compared to traditional methods for identification [26]. MALDI-TOF MS was shown to be suitable for discrimination of *H. influenzae* from closely related *H. haemolyticus* which are considered to be much less pathogenic [27,28], and it even has the potential for capsular typing of *H. influenzae* [29].

### 3.1.3. Corynebacteria

Identification of species from the genus *Corynebacterium* and other coryneform-like bacteria has been considerably improved by the introduction of MALDI-TOF mass spectrometry [30]. Although these bacteria are frequently considered as contaminants as they are often part of the normal flora of the human skin, a study investigating 246 gram-positive rods isolated from blood cultures of 181 patients and identified by MALDI-TOF MS revealed that about one third had to be considered as pathogenic, highlighting the clinical significance of this improvement in diagnostics [31].

### 3.1.4. Nocardia

Another challenging group of bacteria for routine identification are *Nocardia* species. These gram-positive and partially acid-fast bacteria can be ubiquitous in the environment and are not part of the human flora. Some species may cause infections, primarily in immunocompromised patients. The clinical manifestations range from superficial infections occurring also in immunocompetent people, e.g. by inoculation into skin lesions, to pulmonary or systemic disease. Identification was initially performed using a large set of biochemical tests, but as many species are nonreactive reliable results were difficult to obtain and molecular methods have been proven to be more precise and rapid [32]. Now MALDI-TOF MS has also become a suitable alternative for routine use.

For identification of *Nocardia* species, different protocols exist for the different MALDI-TOF systems on the market. While the MALDI Biotyper uses the same method as recommended for other bacteria, the Vitek MS identification is based on an extraction using bead-beating of the cells. Both methods and systems have been shown to achieve high-quality results in multicenter studies [33,34]. A report of McTaggart and coworkers described that bead-beating has little impact on identification success by MALDI-TOF MS while analysis of early growth is crucial. The authors recommended culture media that promote quick growth of *Nocardia* [35]. But it might be that these findings are dependent on the MALDI-TOF MS system used and how the database references have been created.

### 3.1.5. Bite wound infections

Further aerobic bacteria which are rare and hard-to-identify by traditional methods have been reported to benefit from MALDI-TOF MS-based identification, and the number is still growing. As an example, among these are bacteria causing infections after a dog bite. Species from different genera can now be detected with a higher accuracy by MALDI-TOF MS compared to traditional biochemical tests, e.g. species from the genus *Pasteurella* [36], *Capnocytophaga* [37], or *Mycoplasma* [38].

## 3.2. Anaerobic bacteria

Anaerobes have always been challenging in medical microbiology. First, because until the mid-1960s proper anaerobic culture conditions for isolation of colonies on the surface of culture media were lacking in routine laboratories. Later, the isolated anaerobic bacteria (often causing mixed infections) were only reported as 'mixed anaerobes present' to the clinicians, as classical identification methods were too time-consuming due to the inactivity of the bacteria in automated or miniaturized biochemical tests. Another major drawback of conventional identification methods used is the amount of biomass needed, which is a particular problem with slow-growing anaerobes, producing tiny colonies even after 48 h of incubation in proper anaerobic conditions. Therefore, with regard to the different clinically relevant organism groups in medical microbiology, identification of anaerobic bacteria has probably benefitted most from the introduction of the protein profile-based approach by mass spectrometry.

Already the early studies with MALDI-TOF MS-based identification of frequently isolated gram-negative anaerobic bacteria, such as *Bacteroides*, *Prevotella*, *Porphyromonas* or *Fusobacterium* species proved the superiority of the new technology with results on species or genus level in a very timely manner even from limited amounts of biomass [39–42]. Furthermore, MALDI-TOF MS allowed separation of species like, e.g. *P. nigrescens* and *P. intermedia*, which previously could only be differentiated by sequencing and not with biochemical tests or gas-liquid chromatography [39,41].

The good performance in identification was also shown for the gram-positive anaerobic rods like clinical and environmental *Clostridium spp.*, again with the possibility to differentiate closely related species like *C. chauveii* and *C. septicum* [43], or for usually biochemically inactive gram-positive anaerobic cocci (GPAC) and *Actinomyces spp.* [44,45].

The convenience in the workflow and the robustness of identification of anaerobic bacteria by MALDI-TOF MS could also be proven by investigations of the pre-analytical conditions. Neither different culture media, which are essential for anaerobes, nor the short exposure to oxygen impaired the correct identification [43,46–48]. High-quality spectra for anaerobes can be acquired after 24–48 h of incubation, and direct spotting of biomass from suitable colonies followed by an on-target extraction with 70% formic acid is usually sufficient. Full extraction may only be important in routine for some groups such as GPAC and *Actinomyces spp.* [48,49]. As intensive sporulation may cause deviations in fingerprint patterns, extended incubation time may deteriorate species identification of clostridia [43,50].

Although the first studies showed good results, the quality of the identification was limited by the coverage of the available databases, as some frequently encountered species such as *C. hathewayi*, *Parabacteroides distasonis*, *Bacteroides eggerthii*, *B. goldsteinii*, *B. intestinalis*, and *Prevotella heparinolytica* were missing in the database and therefore could not be identified [3,40,42]. A review evaluating the performance of MALDI-TOF MS systems for identification of anaerobes comparing results published between 2010 and 2012 showed that correct species-level identification varied between 55% and 93.8% depending on the number of tested isolates and the number of different anaerobic species and genera investigated in the different studies [51]. This underlined the need for further development of databases for a broader applicability in a clinical routine which was also shown by a European ring trial with participants using different databases and yielding different performances based on the coverage of the databases [49]. In addition, an increasing number of publications about less frequently isolated or even newly described anaerobic species (e.g. *Solobacter moorei*, *Robinsoniella sp.*, *Sneathia sanguinegens*, *Eisenbergiella tayi*, *Collinsella aerofaciens*, *Atopobium rimaie*, and many others) causing severe infections and often leading to sepsis [52–56] aroused the interest of more laboratories for identification of anaerobic clinical isolates to species level. For this, MALDI-TOF MS seemed to be a suitable method in routine laboratories.

The first database development studies were mainly conducted by reference laboratories for anaerobes. The manufacturer's databases were extended with missing anaerobic

species taken from culture collections or with well-characterized isolates from clinical routine whose species-level identification had been confirmed by 16S rRNA gene sequencing [40,42,44,45].

A more general approach for database extensions with regard to anaerobes was undergone by the ENRIA project (European Network for the Rapid Identification of Anaerobes) which was supported by the Study Group for Anaerobic Infections (ESGAI) by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). The main objective was the collection of clinically relevant anaerobic isolates in collaborating laboratories from different European countries for addition to the database of the Bruker MALDI Biotyper system.

In particular, species missing completely or which were represented with less than five strains in the reference database were targeted by the ENRIA collection [57,58]. To ensure a high-quality standard for the MALDI-TOF MS references, the collected strains were identified by classical methods as well as 16S rRNA gene sequencing. The newly created references were added to the database and finally also released for common use in routine laboratories.

The validation of the ENRIA database development was performed with 6309 independent anaerobic strains isolated from human clinical specimens over a period of 6 months. The validation collection comprised species from 61 different common and rare anaerobic genera [57,58]. Compared to the standard database the initial ENRIA database extension allowed an increase in identifications at the high confidence species level from 71.1% to 74.8%. In parallel, the number of isolates with low confidence identification decreased (16.9% to 14.9%) as well as the isolates without identification (12.0% to 10.4%). Adding all ENRIA MSPs to the database even further improved the identifications at high confidence level to 4999 of the 6309 anaerobic isolates (79.2%), and low confidence identification was achieved for 852 isolates (13.5%). Overall, 35.2% of the isolates yielded higher score values with the database extension. This could be accomplished not only for less frequent isolates (such as *Solobacter moorei*, *Bacteroides massiliensis*, *Bacteroides clarus*, *Bacteroides cellulosilyticus*, *Prevotella salivae*, or *Prevotella timonensis*), but also for more commonly occurring species of *Bacteroides*, *Prevotella*, *Fusobacterium*, or GPAC [58].

Moreover, the ENRIA database extension also enabled identification of several recently described and rare species from different genera, e.g. *Dielma fastidiosa*, *Moryella indoligenes*, *Fenollaria massiliensis*, *Filifactor alocis*, and *Odoribacter splanchnicus*, providing routine laboratories the capability to assess their potential clinical importance [57,58]. Nonetheless, there were still 458 (7.3%) strains which could not be identified even with the ENRIA database extension.

The coverage of the MALDI-TOF MS databases may be important to laboratories for their effectiveness in clinical routine. A recent paper [59] showed that at the beginning of 2018 both the FDA-cleared and the RUO database of the MALDI Biotyper comprised a considerably higher number of genus level (FDA 19/RUO 59) and species level (FDA 63/RUO 359) entries of anaerobes compared to the VITEK MS (FDA 11 and 26/RUO 24 and 103, respectively). Another study





NTM at various body sites has been observed [75] as well as a growing number of infections in immunocompetent individuals.

With the growing number of possibly clinically relevant species, DNA probe-based assays also reached their limitations in coverage and discriminatory power due to cross-reactions and not fully specific probes [75]. As a reference method for identification of mycobacteria sequencing of housekeeping genes (e.g. for 16S rRNA, hsp65, rpoB) is used [75]. With regard to the high technical requirements, this is mainly performed in specialized reference laboratories. The introduction of MALDI-TOF MS as a method for identification of mycobacteria offered new possibilities, and evaluations show good results [4,77]. The instruments are available in many laboratories now and databases today have a broad coverage of NTM species.

Unlike other bacteria, mycobacteria need special extraction methods as the amount of ribosomal proteins is low and the cell walls are more rigid so, for example, silica beads are used for mechanical disruption during the extraction of mycobacterial proteins [78].

The protein extraction is possible from biomass harvested from solid media as well as from cells grown in liquid media like MGIT which are broadly used for culture of mycobacteria. Especially the direct extraction from the liquid media of automated incubation systems allows timely identification of NTM species. Alcaide et al. have published overviews of sample processing [79].

The current databases for MALDI-TOF MS identification of mycobacteria comprise almost all currently known species of mycobacteria. The discriminatory power of MALDI-TOF MS for identification of mycobacteria seems to be sufficient for most of the species. There are limitations in differentiation between very closely related species, e.g. members of the *Mycobacterium tuberculosis* complex cannot be separated from each other. In other cases, subtyping by peak analysis of the mass spectra may allow differentiation of species with little genetic differences [80]. All in all, the broad coverage of the MALDI-TOF MS databases enables laboratories to identify even rare NTM species that could not have been identified or would have been misidentified with former routine methods. A more accurate species identification also offers the opportunity to learn more about the pathogenicity and prevalence of these mycobacteria.

A comprehensible example for the possible impact of precise identification even of assumed rare bacteria is *Mycobacterium chimaera*: Sax et al. published about an investigation in 2012 of a prolonged outbreak of infections with *M. chimaera* after open-chest heart surgery [81]. They identified six cases with prosthetic valve endocarditis or vascular graft infection due to *M. chimaera*, 1.5 to 3.6 years after surgery. As a source of the infection, the heater-cooler units used in open-heart surgery were suspected, as indicated by cultures from the water circuits of the devices as well as from air samples collected during usage of the units. It turned out that it was a global outbreak related to the contaminated heater-cooler units with more than 100 cases worldwide [82].

*M. chimaera* is a member of the *Mycobacterium avium* complex (MAC), and separation from *M. intracellulare* is especially challenging. Even molecular identification of *M. chimaera*

is difficult, and different genes were proposed for differentiation by DNA-based method: 16S rRNA gene sequencing was suggested for accurate differentiation by Sax et al. [81] while Schweickert and coworkers found the ITS sequence more suitable for this purpose [83]. Ben Salah et al. used 16S rRNA, ITS, rpoB and hsp65 to distinguish within the MAC [84]. The widely used DNA probe-based assays identified *M. chimaera* as *M. intracellulare* and although MALDI-TOF MS showed very good discriminatory power with its broad database, there were limitations with *M. intracellulare/M. chimaera* as well. This was finally overcome by analysis of the MALDI-TOF mass spectra for distinct peaks specific for *M. chimaera* and *M. intracellulare* [80]. Now, this has also been automated in subtyping modules for the MALDI-TOF MS systems that can automatically differentiate between the two species.

Usage of these new possibilities in clinical routine shows that *M. chimaera* is the prevalent species in comparison to *M. intracellulare*. With DNA probe-based assays these isolates were usually (mis)identified as *M. intracellulare* (Pranada, unpublished data). The more accurate identification will provide future knowledge of the clinical relevance of these two species. Therefore, identification methods used in clinical routine like MALDI-TOF MS and also molecular methods have to be re-evaluated continuously for their discriminatory power, especially when the microorganisms are rare in general, and when new species are described. Due to the awareness for *M. chimaera*, for example, the current molecular assays have also been adapted to differentiate accurately between *M. chimaera* and *M. intracellulare*. A recent study by Epperson et al. evaluating the differentiation of *M. intracellulare/M. chimaera* by MALDI-TOF MS by comparing to sequencing results from different gene loci and even data from whole-genome sequencing shows that the phylogeny might be more complex as certain strains identified by MALDI-TOF MS as *M. intracellulare* cluster more closely with *M. chimaera* in WGS although they are relatively distant from any available reference [85]. This again underlines the need to include new knowledge about taxonomy and phylogenetic relations of references and clinical strains into the identification methods available for routine diagnostics.

### 3.4. Highly pathogenic bacteria

A general problem for routine laboratories is the unforeseeable but occasional appearance of highly pathogenic bacteria. This is not a new problem arising with MALDI-TOF MS but there is a residual risk of infection during the sample preparation process for MALDI-TOF MS, in particular, if good laboratory practice is not applied. This risk should be considerably lower compared with the risk arising from aerosols during sample preparation for automated biochemical identification when appropriate McFarland suspension densities are adjusted. On the other hand, the rapid identification of a highly pathogenic microorganism by mass spectrometry enables the rapid recognition and then proper handling of the respective organisms in the remaining workflow, reducing the time of possible exposure to laboratory staff [86]. Nevertheless, laboratories should be aware that not all highly pathogenic bacteria can be differentiated from less pathogenic closely related species [87] and that databases

comprising references for highly pathogenic bacteria have to be implemented and included explicitly in routine. In a recent study about misidentification of highly pathogenic microorganisms by MALDI-TOF MS in Canada in the time from November 2015 to October 2017, at least six of eight reported misidentifications occurred on systems which were not equipped with a respective database or where the available database was not used [88].

#### 4. Culturomics

The increasing awareness of the importance of the human microbiome for health and diseases has induced a growing number of studies. The investigation of microbiome composition, its dynamics, and activity today are mainly based on genomic technologies like 16S rDNA sequencing or metagenomic shotgun sequencing in combination with powerful bioinformatic approaches and computational tools [89]. These approaches enable the detection and quantification even of non-cultivable microorganisms; however, they are hampered by biases introduced by experimental protocols and data-cleaning approaches [90]. In 2012, a strategy for the investigation of complex microbiota called 'culturomics' was described as an alternative and complementary approach to metagenomics [91]. The principle proceeding in culturomics is the cultivation of microorganisms on a broad variety of solid media to obtain as many different microbial colonies as possible. Subsequently, the obtained colonies are used as starting material for identification of the respective microorganisms. As tens of thousands of single colonies have to be investigated in such a study, here MALDI-TOF MS as a very rapid, accurate technology with low costs per identification plays a key role. The comprehensive databases which are available today already enable the identification of a large number of these colonies. But the utility of MALDI-TOF MS is not limited to the identification of known microorganisms in this approach. Colonies which cannot be identified using existing databases undergo 16S rRNA gene sequencing for identification. In parallel, new database entries are created for these strains. Identification by DNA sequencing enables the species designation of the new references in the extended database.

Sometimes DNA sequence analysis does not result in the identification of a known species, but a yet unnamed taxon is detected [92,93]. The MALDI-TOF MS reference created from this unknown species can nevertheless be used for further simple screening of this new undescribed microorganism. An iterative approach of screening of more colonies and addition of new reference entries allows the detection of rare as well as of novel species which to date have no name. The drawback of this method is the enormous effort in sample preparation, in particular, the cultivation of microorganisms under many different conditions and the manual picking of many thousands of colonies. DNA-based methods have the advantage to detect microorganisms which cannot currently be cultivated and thereby are missed by culture-dependent techniques. Culturomics studies have proven to detect a significant number of bacterial species which are missed by culture-free techniques [94,95].

While the very complex gut microbiome has been investigated initially and most intensely, the analysis of other microbiota also offers new insights into bacterial composition which might impact future diagnostics or therapy. The investigation of the microbial flora of the vagina revealed several species which had not been described before [96].

In a study that aimed to evaluate the bacterial richness and diversity of the skin in ICU patients, it was found that daily bathing with chlorhexidine was associated with substantial shifts of skin microbiota and a reduction in richness and diversity of bacteria, in particular gram-negatives [97]. Also in this study, several novel bacteria were detected.

Further microbiota investigated by culturomics were obtained, e.g. from diabetic foot [98], human oral cavity and respiratory tract [99], and the mosquito gut [100]. In all these studies, novel species or species which had not been associated with the respective habitats before were found.

It is obvious that these huge culturomics studies extend our knowledge about microbiological diversity, in particular of the human microbiota which could be extended by 400 species, 288 of those newly described [101]. Yet, it is not clear to what extent the approach will improve the understanding of human diseases, diagnostics, and treatment of infections. It has been speculated that through the knowledge gained by such studies, keeping and reconstructing an optimized gut microbiome might prevent the occurrence of human diseases [102]. As an example, irreversible disruption of gut microbiota is suspected in most severe cases of malnutrition, leading to persistent impaired development or death. The alimentation with selected probiotic microorganisms detected by culturomics studies might improve the treatment of such cases through the re-establishment of a healthy gut microbiota [103].

On the other hand, the direct involvement of newly detected species has to be verified in most cases. In contrast to metagenomic studies, the culturomics approach has a significant advantage here as the novel species are available as cultures and can be thoroughly investigated. Reference profiles can be incorporated into MALDI-TOF MS databases for further studies. After export of the references and their transfer to other laboratories, they can be utilized also at other institutions for investigation of a broad variety of routine samples from clinical microbiology. As the mass spectra of MALDI-TOF identification in routine are stored electronically, even retrospective analyses for the occurrence of novel species in microbial diagnostics are possible. Thereby, new data and insights from MALDI-TOF MS-based culturomics studies can directly feed into microbial diagnostics using MALDI-TOF mass spectrometry.

#### 5. Library expansion for rare bacteria

The manufacturers of the currently available MALDI-TOF MS systems have continuously extended the databases and libraries with new reference spectra for clinical use (as example, the development of the Bruker MALDI Biotyper databases for bacteria is depicted in Table 2). With growing species diversity in the databases, the total number of different genera

**Table 2.** Development of the MALDI Biotyper databases during the recent years, number of included species (a) General IVD-CE labeled database/bacteria & yeast.

Database version	Release year	No. species	Gram +	Gram -
(a) General IVD-CE labeled database/bacteria & yeast				
DB 4613*	2012	2144	1111	923
DB 5627*	2013	2257	1138	946
DB 5989*	2015	2329	1164	973
DB 6763	2016	2380	1172	1015
DB 7171	2017	2428	1205	1030
IVD 7712	2018	2665	1274	1189
IVD 8326	2019	2887	1411	1266
(b) Mycobacteria database				
Mycobacteria db v1.0	2012	94		
Mycobacteria db v2.0	2014	131		
Mycobacteria db v3.0	2015	149		
Mycobacteria db v4.0	2016	159		
Mycobacteria db v5.0	2017	164		
Mycobacteria db v6.0**	2019	178		

\*These versions were including several MSPs of mycobacteria and molds which were removed in 2016.

\*\*First IVD-CE labeled mycobacteria database.

and species effectively reported in a routine laboratory also increases significantly [78].

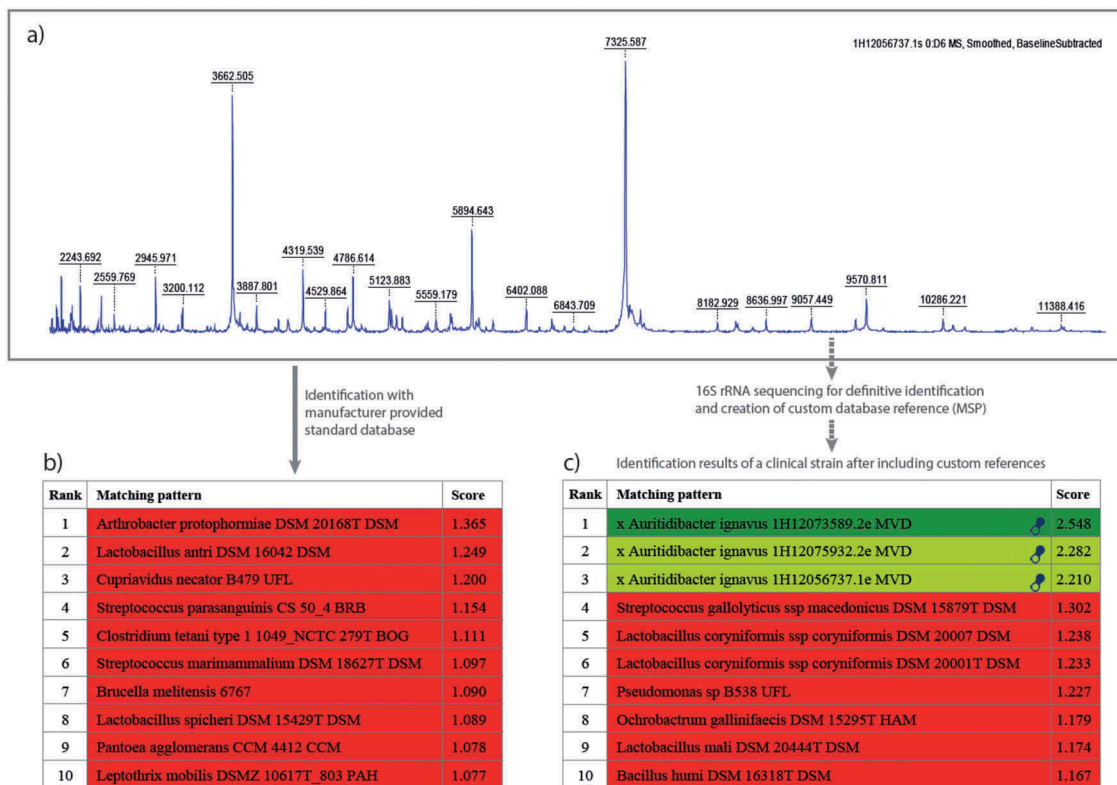
The extensions of databases usually also include rare bacteria. An example for this is *Actinotignum schaalii* (formerly: *Actinobaculum schaalii*) where MALDI-TOF MS with the corresponding reference spectra was the first routine suitable

method that enabled correct identification. Now evidence shows that this species can be found quite often in clinical samples and is recognized as a relevant pathogen [104]. This underlines the value of extensions of the databases as it may also add to the future knowledge in medical microbiology.

For RUO, laboratories can even create custom reference spectra (main spectra, MSP) and expand the existing databases with new microorganisms or for special applications. In Pubmed, a tremendous amount of publications about MALDI-TOF MS employing custom databases are listed. Other publications, especially about newly described species, provide reference spectra or MSP libraries for inclusion into custom databases. There are also platforms for the exchange of reference spectra between users (e.g. MALDI-UP) [105]. A fundamental prerequisite of such database expansions is the secure species identification by a reference method or even a combination of suitable methods and a rigorous quality control during reference establishment.

Current software versions of the Bruker Biotyper system allow export and import of custom MSPs for easy direct exchange of MSPs between users.

With these possibilities, laboratories can also detect new or rare bacteria that are not yet included in the officially released database libraries. Figure 2(a) shows an example from a routine laboratory (MVZ Dr. Eberhard & Partner Dortmund, Dortmund, Germany) of a mass spectrum acquired from a bacterium cultured from an ear swab in April 2012.



**Figure 2.** Benefit of the creation of new database references for the detection of rare pathogens. (a) MALDI-TOF mass spectrum of a clinical strain recovered from an ear swab; (b) Despite the good quality of the spectrum with many distinct peaks, the identification software yields no good matches with the standard database; (c) Identification results of other clinical strains some months later after the creation of custom database references (MSPs).

Although the spectrum showed a lot of clearly defined peaks the software could not find any matching reference spectra (Figure 2(b); log(score) value = 1.365; for identification at high confidence level a log(score)  $\geq$  2.000 is expected). Conventional methods were not successful either.

Definitive identification could be achieved by 16S rRNA sequencing and resulted in *Auritidibacter ignavus*, a gram-positive rod-shaped bacterium that had been described about one year earlier [106]. The type strain of this species had been isolated from an ear swab of a man with otitis externa. From the bacterial biomass, a new custom MSP was created for the local MALDI-TOF MS system. With this new MSP, two other isolates could be identified from routine samples within the following month. These were also confirmed as *A. ignavus* by sequencing and were used for MSP creation. This enabled the detection of this until-then unseen species (Figure 2(c)). In the following years until March 2019, these new reference spectra were hit in isolates from routine more than 200 additional times, mostly from cultures from ear swabs. This example shows the strength of MALDI-TOF MS for finding new (rare) bacteria again from clinical samples (unpublished data). This also contributes to the growing knowledge in medical microbiology as the clinical significance of microorganisms can now be easily assessed.

This approach, with the creation of new MSPs whenever good spectra from routine isolates are found with no match in the reference libraries, can also be used to find yet unnamed microorganisms. Sometimes the sequences can already be found in public databases, but there have not been any valid publications for these new species. In the laboratory in Dortmund, for isolates that cannot be identified even after sequencing, MSPs with generic names will be created. Using this method it is possible to track the reoccurrence of these unnamed species in clinical routine. With more than 25 of these MSPs included in the database for research purposes the regular reoccurrence of these species in clinical samples can be seen (Table 3).

Again, this example underlines the power of MALDI-TOF MS for the detection and description of new and rare bacteria together with the opportunity to gain information about clinical significance in daily routine.

**Table 3.** The 10 most frequently hit MSPs of presumably not yet described bacterial species in the laboratory in Dortmund (MVZ Dr. Eberhard & Partner Dortmund). According to the manufacturer log(score) values  $\geq$  2.000 are regarded as identification at a high confidence level.

MSP	[n]	Average log(score)
NONAME <i>Corynebacterium</i> sp.	312	2.137
NONAME <i>Haemophilus</i> sp.	185	2.248
NONAME <i>Pseudomonas</i> sp.	158	2.366
NONAME <i>Pantoea</i> sp.	156	2.366
NONAME Pseudoclavibacter-like bacterium	101	2.140
NONAME <i>Peptoniphilus</i> sp. (1)	97	2.144
NONAME <i>Peptoniphilus</i> sp. (2)	39	2.196
NONAME <i>Enterobacter</i> sp.	38	2.320
NONAME <i>Acinetobacter</i> sp.	24	2.022
NONAME Actinomycetaceae bacterium	23	2.033

## 6. Rapid adaption of MALDI-TOF MS identification to emerging pathogens

The ability of current MALDI-TOF MS systems to easily extend databases also enables the possibility to rapidly include emerging pathogens. This is possible because users are able to create own references with high quality for their in-house database if the manufacturer of a system gives clear guidance for the procedure and quality control, as the quality of a database has to be strictly controlled from sample preparation to quality control [107]. In 2016 the Centers for Disease Control (CDC) investigated outbreaks with *Elizabethkingia anophelis* causing serious infections with fatalities in the midwest of the United States (Nicholson 2016). Usually, *Elizabethkingia* species can be found in the environment and rarely cause infections. The CDC could quickly react and supplement their own MALDI Biotyper database. Subsequently, the commercial databases could be extended for easy identification of this new pathogen. The CDC hosted resource MicrobeNet [108] can play a key role in the rapid reaction on emerging infectious agents. As the CDC is collecting difficult to identify organisms in the USA nationwide, it has the capability to create references for its MALDI-TOF MS database very soon after a pathogen's appearance. Thereby, these references can be made available to MALDI Biotyper users via the MicrobeNet embedded library before commercial suppliers expand their own databases.

Similarly, new reference entries in (commercial) MALDI-TOF MS databases can help to recognize emerging pathogens. An example for this is *Klebsiella pneumoniae* which initially was divided into three phylogroups, Kpl-III, and then phylogenetic analyses led to the differentiation as distinct species *K. pneumoniae*, *K. quasipneumoniae* and *K. variicola* [109]. Differentiation with conventional methods was difficult and special molecular analysis was needed. MALDI-TOF MS can easily distinguish these new species that formerly all have been identified just as *K. pneumoniae* if the database is properly established, as shown for the MALDI Biotyper system, recently [110]. Now there are reports stating, that *K. variicola* might be more virulent as it is a frequent cause of bloodstream infections [111].

## 7. Expert opinion

Before MALDI-TOF mass spectrometry was introduced into clinical microbiology laboratories, diagnostics in bacteriology were expected to move towards culture-free molecular technologies. The long turnaround times, limited detectable microbe spectrum, and frequently low precision of culture-dependent techniques seemed to justify the acceptance of the drawbacks of methods based on molecular biology, i.e. high costs and mainly targeted approaches.

With MALDI-TOF MS and broad reference databases as a powerful diagnostic tool, this picture changed dramatically. It did not only improve the present diagnostic but also enables knowledge generation for future diagnostics. The observation of such microbes during infections expands the knowledge about their relation to diseases as well as resistance trends. This will enable a better diagnosis and treatment of such infections in the future, e.g. adopted antibiotic susceptibility tests can be developed. The diagnostic gap which



appears with emerging pathogens can be closed quickly by an integration of specific references into diagnostic MALDI-TOF MS systems, as shown for *E. anophelis*. As also yet unknown pathogenic bacteria can be quickly integrated into databases, their occurrence and relevance in infectious diseases can be observed soon after their first detection.

In culturomics studies, this knowledge generation can be performed on a large scale with affordable workload and costs, not as a substitute but as a complement to metagenomic studies. The advantage of such an approach compared to culture-free, molecular studies is the existence of viable organisms which can be further analyzed after their detection. This revival of culture-based diagnostics is also reflected by the introduction of extensive laboratory automation of microbial culture in an increasing number of laboratories today. It can be speculated that this would not have happened without the introduction of MALDI-TOF MS.

Now novel applications of MALDI-TOF MS are being developed, e.g. subspecies differentiation, typing, and antibiotic resistance testing. It has yet to be seen which of these applications will further expand the utility of the technology in microbiology laboratories. Their success is not certain as such novel applications have to survive the competition of other upcoming technologies. The success of MALDI-TOF MS in the last 10 years, from a technology unknown to nearly all clinical microbiologists, to a workhorse technology accepted as standard worldwide, now has lowered the hurdles of acceptance of novel technologies. This catalyzed the introduction of new promising tools into microbial diagnostics. Thereby, MALDI-TOF MS helped to improve diagnostic practice not only by itself but also indirectly, acting as a pioneering technology.

In the upcoming years, it can be expected that the trend to use MALDI-TOF MS for identification and further microbiological analyses will continue. Although the technology has been in routine use now for about ten years, still the development to improve speed and accuracy, as well as the addition of novel applications, is ongoing. International studies using shared databases and large-scale culturomics analyses will broaden the knowledge about microbial diversity. Recently disregarded pathogens will be detected and investigated regarding their involvement in diseases. The MALDI-TOF MS databases for microorganisms will further grow, and differentiation of very closely related microorganisms by the novel, improved bioinformatic algorithms will have the effect that 'hard-to-identify' will be a term only rarely attributed to bacteria in the future.

Automation within the laboratories will be a strong driver in the development of this technology. An increasing number of diagnostic microbiology laboratories are introducing partial or total automation of microbial culture workflows. The lack of well-trained and experienced technical staff will accelerate this transformation. As a consequence, the sample preparation for subsequent MALDI-TOF MS analysis is also being automated, i.e. the automated transfer of colony material to the MALDI target plate and the addition of matrix. Thus, the decision to analyze a bacterial colony will be less dependent on the expertise of a human specialist, but more in the direction of 'in doubt – better analyze'. Throughput ability and low costs of MALDI measurements will allow such a change of routine.

The introduction of MALDI-TOF MS has already increased the number of microbial identifications in routine diagnostics (observation in our own laboratories and from the feedback of Bruker customers). This will further avoid the missing of rare, yet unrecognized pathogens. In a final step, the automated transfer of a prepared target into a mass spectrometer for subsequent measurement and bioinformatic analysis will create microbial identification workflows with minimal human intervention for the majority of samples. MALDI-TOF MS will not only deliver precise identification for an increasing number of pathogens but also additional information about virulence and/or resistance.

MALDI-TOF MS-based resistance/susceptibility tests may find their place in the laboratory, in particular for samples that require a rapid phenotypical susceptibility information, e.g. in the case of positive blood cultures [112].

In particular, slow-growing and biochemically inactive organisms like anaerobes might benefit from such accelerated phenotypic MALDI-TOF MS tests. Also, recently described methods for direct detection of antibiotic resistance-causing proteins [113] or analysis of bacterial lipid modifications [114] might find their way into routine diagnostics. Such developments will partially require different MALDI matrices and different measurement modes. More analyses directly from clinical samples are intended, but here the limited sensitivity of MALDI-TOF MS and its low performance in the analysis of mixtures will be limiting factors.

Such developments are partially in competition with rapid molecular tests or other fast phenotypic approaches. The advantage of mass spectrometry will be that there are already several thousand instruments for such analyses available in diagnostic laboratories, and the increasing speed of measurement of modern systems enables the transfer of additional tests.

It is difficult to estimate to what extent MALDI-TOF MS will take over additional parts of microbial diagnostics, but for sure it will keep and probably strengthen its position as the laboratory standard for broad routine identification of pathogens.

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