Video Article

Identification of Rare Bacterial Pathogens by 16S rRNA Gene Sequencing and MALDI-TOF MS

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Abstract

There are a number of rare and, therefore, insufficiently described bacterial pathogens which are reported to cause severe infections especially in immunocompromised patients. In most cases only few data, mostly published as case reports, are available which investigate the role of such pathogens as an infectious agent. Therefore, in order to clarify the pathogenic character of such microorganisms, it is necessary to conduct epidemiologic studies which include large numbers of these bacteria. The methods used in such a surveillance study have to meet the following criteria: the identification of the strains has to be accurate according to the valid nomenclature, they should be easy to handle (robustness), economical in routine diagnostics and they have to generate comparable results among different laboratories. Generally, there are three strategies for identifying bacterial strains in a routine setting: 1) phenotypic identification characterizing the biochemical and metabolic properties of the bacteria, 2) molecular techniques such as 16S rRNA gene sequencing and 3) mass spectrometry as a novel proteome based approach. Since mass spectrometry and molecular approaches are the most promising tools for identifying a large variety of bacterial species, these two methods are described. Advances, limitations and potential problems when using these techniques are discussed.

Video Link

The video component of this article can be found at https://www.jove.com/video/53176/

Introduction

Secure identification of rare pathogens in routine diagnostics is hampered by the fact that classical cultural and biochemical methods are cumbersome and sometimes questionable. Furthermore, a diagnostic microbiology laboratory has to process a large number of pathogens, ranging from a few hundred to several thousands, daily, which requires the use of automated systems. In addition to the management of a high daily throughput, the precise identification of bacterial species is needed. This is warranted since they differ in their antimicrobial susceptibility pattern and therefore correct identification provides the clinician with essential information to choose appropriate antibiotics (*e.g., Enterococcus* spp., *Acinetobacter* spp.) [12,43].

Automated microbial identification systems (aMIS) apply standardized sets of enzymatic reactions to characterize the metabolic properties of bacterial isolates [13,15,16,26,27]. Although the cartridges used in these systems utilize a large number of different biochemical reactions, *e.g.,* 47 in the GN card of the aMIS used in this study [52], this strategy permits secure identification only for a limited set of bacteria. Furthermore, the database, an advanced expert system, is clearly focused on detection of relevant and highly relevant bacteria of medical importance [13,15,16,36]. Two further systems, widely used in laboratories, also apply this biochemical approach for bacterial identification. Recent studies demonstrate a comparable identification accuracy between the aMIS used in this study and one of the competitors (93.7% and 93.0% respectively), while the 3rd aMIS has an identification accuracy of only 82.4% on species level [35]. Such discrepancies may be explained by the quality of the underlying identification data references, the versions of kits and software, differences in metabolism and proficiency of technical personnel [35,36].

Two automated MALDI-TOF MS systems (MALDI-TOF microbial identification system, mMIS) are mainly used. These systems allow for detection of a large number of bacterial species based on their protein fingerprint mass spectra. For instance, the database of the mMIS used contains 6,000 reference spectra. Identification systems based on mass spectrometry offer fast and reliable detection of a great variety of microorganisms including rare pathogens [11,48,51]. To date only a few direct comparisons are available between the mMIS used in this study and its competitor [19,33]. According to Daek *et al.* both systems provide a similar high rate of identification accuracy, but the mMIS used in this study seems to be more reliable in species identification [19].

Similarly, molecular techniques addressing well conserved but also distinct genes (*e.g.*, 16S rDNA or *rpoB*) permit a clear species identification [3,22,61]. Among these, the 16S rDNA is the most widely used housekeeping gene because of its presence in all bacteria [34]. Its function remains unchanged and finally, with roughly 1,500 bp, it is long enough to be suitable for bio-informatics [14,34]. Many researchers regard 16S rRNA gene analysis as the "gold-standard" for bacterial identification [21]. This is due to the fact that few laboratories use DNA-DNA hybridization techniques

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to date for identification of rare or new bacteria [14,34]. Additionally, more and more databases are available which can be used for 16S rRNA gene analysis [50]. However, it has to be taken into account that 16S rDNA based detection systems have a limited sensitivity compared to standard PCR protocols. Moreover, the molecular approach is sophisticated, time consuming and requires highly trained personnel as well as dedicated laboratory facilities and is, therefore, not easily implemented into routine diagnostics [55]. Furthermore, it has been shown that the combination of at least two different methods of bacterial identification leads to highly accurate strain identification. The combination of MALDI-TOF MS and 16S rDNA sequencing permits the identification of large numbers of different bacterial species with high accuracy. Recently the combination of MALDI-TOF MS and 16S rRNA gene analysis was presented for bacterial identification studying epidemiological questions and rare pathogens [56].

Protocol

1. Extraction of Bacterial DNA

1. Preparation of PBS Solution
    1. Weigh 1.65 g Na_2HPO_4 x $2H_2O$, 0.22 g NaH_2PO_4 x $2H_2O$ and 8.80 g NaCl in a flask and fill with distilled water to a final volume of 1,000 ml. Adjust the pH to 7.4. For final use filter the solution through a bacteria-proof (0.22 µm) filter.

2. DNA Extraction of Gram-negative Bacteria
    1. Streak the patient material on appropriate culture media (*e.g.*, Columbia blood agar), identify and isolate potential pathogens.
    2. Prepare pure culture and perform Gram-staining in order to determine morphotype and to confirm purity and of the culture [49].
    3. Pick a single bacterial colony and transfer it with a 1 µl single use inoculation loop into a sterile 2.0 ml reaction tube which contains 1 ml PBS solution.
    4. Incubate this suspension in a thermomixer at 95 °C for 10 min. After incubation, let the bacterial extract (containing the dissolved DNA) cool to room temperature and either use it directly for amplification or store at -20 °C for further use (**Figure 1a**).

3. DNA Extraction of Gram-positive Bacteria
    1. Streak the patient material on appropriate culture media (*e.g.,* Columbia blood agar), identify and isolate potential pathogens. Perform Gram-staining in order to confirm morphotype of the culture.
    2. Prepare pure culture and perform Gram-staining in order to determine morphotype and to confirm purity of the culture.
    3. Pick a single bacterial colony with a sterile 1 µl inoculation loop and suspend it in 500 µl PBS solution in a 2.0 ml reaction tube.
    4. Add 500 µl glass-beads and transfer the tube to an oscillating homogenizer, operated at maximum frequency and amplitude (50 Hz), for 5 min. Use glass-beads of 1.0 mm diameter to disrupt the bacterial cell walls.
    5. Incubate this tube for 10 min at 95 °C and cool to room temperature. Use the extract either directly for the PCR reaction or store at -20 °C for further use.

2. 16S rDNA PCR

1. Preparation of Amplification Primers
    1. Use the primers TPU1 (5´-AGA GTT TGA TCM TGG CTC AG-3' [M = A/C]) as forward primer and RTU4 (5´-TAC CAG GGT ATC TAA TCC TGT T-3´) as reverse primer [25]. Prepare a primer stock solution with DNAse and RNAse free water, having a concentration of 100 pmol/µl (100 µM) and dilute it further to a working solution at 10 pmol/µl. Store the primer stock and working solution at -20 °C or use directly.

2. Preparation of the PCR Reaction Mix
    1. Pipette 1 µl of each primer, 1 µl dNTP-mix (100 mM), 2.5 µl of the DNA extract, 5 µl 10x PCR buffer (containing 25 mM $MgCl_2$), 0.25 µl Taq-polymerase (5 units/µl) and 39.25 µl DNAse- and RNAse free PCR water into a sterile 200 µl reaction tube.

3. PCR Protocol
    1. Start the PCR program built as follows: First, an initial incubation step at 95 °C for 5 min which is necessary to activate the Taq-polymerase. Second, 35 amplification cycles, containing a 1 min. denaturation step at 95 °C, a 1 min. primer annealing step at 50 °C, and a 1.5 min primer extension step at 72 °C. Third, a final extension for 10 min at 72 °C. Finally, the PCR reaction is cooled to 4 °C. NOTE: *Staphylococcus aureus* and H_2O serve as positive and negative control (**Figure 1b**).
    2. Place the tube containing the PCR reaction mix in the thermocycler and start the program.

4. Purification of the PCR Product
   NOTE: In order to purify the PCR product, several protocols either enzyme based or with a silica adsorption matrix may be used. The single-step PCR cleanup used here utilizes two hydrolytic enzymatic reactions. While Exonuclease I (Exo I) removes single stranded DNA, shrimp alkaline phosphatase (SAP) hydrolyses unincorporated dNTPs. The second procedure is based on a silica membrane adsorption technique with a high salt fast binding - wash - low salt elution cycle.
    1. Add 1.5 µl of an enzymatic mix containing both Exo I and SAP to 25 µl PCR product, mix thoroughly and transfer to a thermocycler applying a simple protocol of first 15 min at 37 °C followed by 15 min at 80 °C. Store the purified PCR product either at -20 °C or use it directly as target for labeling PCR.

3. DNA Agarose Gel Electrophoresis

1. Preparation of the Electrophoresis Buffer (TBE-buffer)

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1. Titrate 54.0 g (445 mM) TRIS base, 27.5 g (445 mM) boric acid and 20 ml of a 0.5 M EDTA (10 mM) solution at pH 8.0 with NaOH in a flask and fill it with distilled water to a total volume of 1,000 ml. Then dilute this 5x buffer 1:10 with distilled water (0.5x TBE) for electrophoresis.

2. Preparation of the Loading Buffer
    1. Mix 250 mg bromophenol blue, 250 mg xylene cyanol FF and 15 g of polysucrose (**Materials Table**) into a flask. Then fill it with 0.5x TBE buffer to a total volume of 100 ml. Aliquot the loading dye to 1 ml portions and store at -20 °C for further usage.

3. Casting of the Agarose Gel
    1. Weigh 6 g standard electrophoresis agarose to 300 ml 0.5x TBE buffer (see section 3.1.1) in a flask, to prepare a 2% (w/v) agarose gel. Then put the agarose mixture in a microwave oven, heat it near-boiling point until the agarose is completely dissolved and the solution appears clear.
    2. Let the molten agarose cool sufficiently and add 10 µl of a 1% (w/v) ethidium bromide stock solution. Pour the solution into a cast and place a gel comb (allowing for 30 wells) in the cast. Remove the comb when the gel is completely solidified and put the gel into the electrophoresis chamber containing 0.5x TBE buffer.
    NOTE: Ethidium bromide is toxic and mutagenic. Therefore it should be handled with caution. It is advisable to use nitrile gloves. There are alternative intercalating nucleic acid stains which can be used as a non-toxic alternative.

4. Agarose Gel Electrophoresis of the PCR products
NOTE: In order to verify the correct size of the PCR amplicons and to estimate the DNA concentration the purified PCR product is separated in an agarose gel.
    1. Pipette 2 µl of the loading buffer into a PCR reaction tube and add 8 µl of the PCR product. Pipette this mix into the wells of the gel. Add 8 µl of a molecular-weight size marker (DNA ladder) in a separate well to estimate the size of the PCR product.
    2. Apply a constant voltage at 10 V/cm and stop the electrophoresis as soon as the bromophenol blue marker has reached about ¾ of the total length of the gel. Visualize the separated PCR fragments using an UV-transilluminator (wavelength 302 nm) and document them using a gel-documentation system. Estimate the quantity of DNA by visual comparison with the DNA ladder. Use approximately 10 ng as target for the labeling PCR.

4. Sanger Sequencing

1. Cycle Sequencing PCR
NOTE: Perform the PCR labeling in a 10 µl reaction using a commercial kit (**Materials Table**).
    1. Add 2 µl of 5x reaction mastermix, 2 µl of the DNA preparation, 1.5 µl of TPU1 or RTU4 working solution (10 pmol/µl) and 4.5 µl of DNAse and RNAse free water.
    2. Put this sequencing reaction mix in a thermocycler and run another PCR. In total, process 25 cycles consisting of a denaturation step (96 °C, 10 sec), an annealing step (45-60 °C, 5 sec) and an extension step (60 °C, 2 min). Finally, cool the reaction to 4 °C.

2. Purification of the Sequencing Reaction
    1. Purify the labeling reaction mix using commercial spin columns for PCR purification (**Materials Table**).
    2. Load 10 µl of the sequencing reaction onto the pre-hydrated gel-filtration matrix and perform a centrifugation step at 750 x g for 3 min.
    NOTE: By the use of this procedure unincorporated dye terminators are retained in the gel matrix.
    3. Then dry the aqueous eluate in a vacuum concentrator. Centrifuge at 2,500 x g for 20 to 30 min at 40 °C to complete dryness.
    4. Add 10 µl of highly deionized formamide then denature at 90 °C for 2 min and cool the mixture on ice. Pipette 10 µl of the denatured samples on a 96 well µl plate. Store the dried and cleaned PCR product at -20 °C if the analysis is to be carried out at a later time.

3. 16S rRNA Gene Sequencing and Analysis of the DNA Sequence
NOTE: Perform sequence analysis on an automated sequencer (**Materials Table**). The sequencer used here is an automated fluorescence-based capillary electrophoresis system that analyzes 4 samples simultaneously (50 cm 4-capillary array) with a liquid polymer [67] (**Figure 1c**).
    1. Place the microtiter plate in the sequencer. Write a sample sheet (plate record) as laid out in [2], save it and start the sequencing run/analysis following the steps given in [1].
    NOTE: Duration of a sequence run is 2 hr and provides data from 800 to 1,000 bp.
    2. Open the sequencing analysis software (**Materials Table**). Sequence data are given as a text file (.seq) and a file containing the electropherogram including quality values (QV) (.ab1).
    NOTE: Base calling is automatically performed by the sequencing analysis software and the underlying electropherogram is checked visually (*e.g.*, peak height, peak separation) before the sequence is used for further applications.
    3. Compare the stored DNA sequence file to the NCBI nr database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

5. MALDI-TOF MS

NOTE: The mass spectrometer uses a 337 nm N_2 laser and is operated by a specific control software (**Materials Table**). Spectra are recorded in linear mode and a mass range between 2,000 to 20,000 Da is covered. Data interpretation and allocation of scores to the samples is carried out in real time by an analysis software (**Materials Table**) (**Figure 1d**).

1. Preparation of Bacteria for MALDI-TOF MS Analysis
    1. Grow the bacteria of interest (*e.g.*, *Myroides odoratimimus*) on Columbia blood agar containing 5% horse blood at 37 °C for 18 to 24 hr, depending on the bacterial strain (**Figure 2a**). Perform Gram-staining to verify the morphotype of the organism under investigation as well as purity of the culture.

2. Use a wooden toothpick for transferring a single bacterial colony to a well of a 96-well steel target (**Figure 2b** and **2c**). Spot 1 µl of 70 % formic acid on top of the air dried organism on the steel target and leave to dry for approximately 2-3 min.
3. Then, overlay the spot with 1 µl matrix solution, containing 50 mg/ml CHCA (α-Cyano-4-hydroxycinnamic acid) in an organic solvent (50% acetonitrile, 2.5% trifluoroacetic acid, 47.5% H_2O).
   NOTE: The acid overlay method (on plate preparation method) may be used to improve the quality of the mass spectra. It has been shown that for Gram-positive bacteria such an "acid attack" increases the score values of the measured spectra [45].
   NOTE: Alternatively an automated sample preparation system (**Materials Table**) may be used in which contact free spots of 1 µl 70% formic acid solution and, after a first drying cycle, 1 µl matrix solution are applied on the bacterial smear. The samples are then dried under standardized conditions at 60% relative humidity and are ready to use for analysis.
4. Let the smear with matrix overlaid air-dry again for about 5 min in a hood. This bacterial smear with the matrix applied is now stable for many hours.
   NOTE: Bacterial smears without matrix may not be stable due to protein degradation.

2. Performing the MALDI-TOF MS Analysis
   1. Introduction of samples
      1. Open the control software of our mMIS (**Materials Table**).
      2. Aerate the sample loading port of the mass spectrometer by pressing the IN/OUT button of the instrument.
      3. Open the loading port after a click and insert the MALDI-TOF MS target plate with the dried bacterial smear plus matrix.
      4. Close the port and evacuate again. Observe vacuum and when it reaches 4.5 x 10^{-6} mbar start measurement.

   2. Sample Analysis
      1. Open the analysis software of our mMIS (**Materials Table**) and click the "File" menu. Select "New classification" and a new window "MALDI biotyper real time classification wizard" opens. Type the project name, for instance "Myroides measurement project_1, in the field "Project name" and press the button "New". Under the new dialog box named "New project" check project name and proceed by pressing the button "OK".
      2. In the "MALDI biotyper real time classification wizard" observe the "Analyte placement" window open. Select target positions (*e.g.*, A1, A2, A3…), right-click any selected position (indicated by a blue square) and select "Add samples". In the automatically opened table view type in sample name, sample ID and optionally a comment. Click the button "Next" to proceed.
      3. In the "MALDI biotyper real time classification wizard" observe the "Selection of MALDI biotyper methods" window open. Select "Bruker Taxonomy" from "MSPs from taxonomy trees" and click "Next" to continue.
      4. In the "MALDI biotyper real time classification wizard" observe the "Project summary" window open. Check the inserted entries given above for the actual classification project. Start the classification run by pressing the button "Finish" (**Figure 2d**-**f**). Measurement starts automatically when the appropriate vacuum is reached.
      5. Follow the classification run until the measurement is successfully completed. View the results table in the "MALDI Biotyper Realtime Classification Project Myroides measurement project_1" open the menu "View" and click "Results".
      6. View the "Bruker Daltonics MALDI Biotyper Classification Results" as HTML file in the default web browser. Print and save the results.

   3. Measure a test standard daily prior to sample analysis to calibrate the instrument and perform instrument validation weekly (instrument self-test).
      NOTE: During measurement MALDI-TOF spectra are analyzed in real time by the analysis software (**Materials Table**). A list of ten species with their respective scores is given and is fed into the laboratory information system (LIS) for reporting.
   4. Critically evaluate the MALDI-TOF MS results (see representative results) and, if appropriate, include other tests, such as biochemical- and antibiotic susceptibility profiles or 16S rRNA gene sequencing and, if necessary, Gram-staining for assigning a bacterial species to the microbiological report.

Representative Results

MALDI-TOF MS is a novel, fast and inexpensive method for microbiological routine diagnostics. Bacterial species identification by MALDI-TOF MS produces spectra mainly composed of ribosomal proteins but also other "very conserved proteins with house-keeping functions affected to a minimal extent by environmental conditions" [17].The database of this mMIS contains a large set of reference spectra and even bacteria which are rarely found in clinical isolates can be securely identified [7,56,57]. Score values show the reliability of the identified species. Scores above 2.300 represent a highly probable species identification, a score between 2.000 and 2.300 indicates a secure species identification, a score between 1.700 and 2.000 stands for a probable species identification and a score below 1.700 is non-reliable. In the case of more than one species being identified with a score above 2.000, additional tests have to be applied and this is the reason why we have combined different methods such as 16S rDNA sequencing, API and techniques addressing the bacterial morpho- and/or phenotype such as Gram stain, motility *etc.* In the case where the score is below 1.700 the above mentioned tests have to be used to achieve reliable species identification. A combination of MALDI-TOF MS and 16S rDNA sequencing has been demonstrated recently [56-58]. It should be pointed out that clear guidelines for species determination based on 16S rDNA sequence homologies are still lacking [22,61]. For practical interpretation, homologies of ≥97%, as proposed by Stackebrandt and Göbel [61], are used [56]. Advances in DNA sequencing allow the determination of whole bacterial genomes by next generation techniques at relatively low cost and, therefore, in principle identification of bacteria based on their genome sequence. This technique has already been used in genome based outbreak management or in epidemiology [9,29]. However, the strongest limitation today is the lack of easy to use software packages for the end user [65].

Seven examples of rare occurring bacteria, isolated from patient samples during routine diagnostics and analyzed by both MALDI-TOF MS and 16s rRNA gene sequencing are listed in **Table 1**. MALDI-TOF spectra of strain #1 to #6 are shown in **Figure 3** and a spectrum of strain #7, *Sphingobacterium spiritivorum*, is shown in **Figure 1e**. All isolates show high scores in MALDI-TOF MS analysis and 99 to 100% 16S rDNA sequence homologies. Thus, both techniques lead to secure genus and species identification. However, as pointed out in a recent publication on comparing identification methods of *Myroides* sp., the combination of MALDI-TOF MS and 16S rRNA gene sequencing led to more reliable results [56]. These data illustrate that a single method used may not always be sufficient to achieve a reliable identification result. The combination of two independent methods leads to a higher accuracy and expands the commercial MALDI-TOF MS database with in-house entries resulted in an unequivocal species identification [56].

Strain #1 was identified as *Chryseobacterium gleum* (MALDI-TOF MS score 2.490, sequence homology 99%). Chryseobacteria are Gram-negative, non-fermenting rods and related to *Myroides* spp. *Chryseobacterium* spp. are regarded as emerging pathogens, associated with septicemia, pneumonia and urinary tract infections. The genus *Chryseobacterium* comprises a large number of species and the best studied species is *Chryseobacterium indologenes*. Similar to infections caused by *Myroides* sp., mostly immunocompromised patients are affected [6,10,60]. Strain #2 was identified as *Myroides odoratimimus* (MALDI-TOF MS score 2.436, sequence homology 99%) and strain #3 as *Myroides odoratus* (MALDI-TOF MS score 2.237, sequence homology 99%) *Myroides* sp. are Gram-negative, nonfermenting rods which are associated with severe diseases such as sepsis, cellulitis or pneumonia. Mostly immunocompromised patients with underlying hematologic or oncologic diseases are affected [8,18,42]. Strain #4 was identified as *Sphingobacterium multivorum*[32,71] (MALDI-TOF MS score 2.092, sequence homology 99%).

The bacteria are Gram-negative non-fermenting rods, which may cause septicemia in immunocompromised patients [5,24,44,53]. In addition, a case of fatal meningoencephalitis has been reported [70]. The stains #5 (MALDI-TOF MS score 2.282, sequence homology 100%) and #6 (MALDI-TOF MS score 2.289, sequence homology 100%) were identified as *Wohlfahrtiimonas chitiniclastica.* These bacteria are short, non-motile, Gram-negative rods which were first isolated from larvae of the parasitic fly *Wohlfahrtia magnifica* and described in 2008 [66]. These zoonotic bacteria are regarded as emerging pathogens and causative agents for bacteremia, septicemia or soft tissue infections [4,40,64]. Interestingly, most patients reported were either homeless and/or suffered from alcoholism and, therefore, the occurrence of these rare pathogens may be explained by the higher rate of ectoparasites in the homeless population [4,54]. Strain #7, *Sphingobacterium spiritivorum* (MALDI-TOF MS score 2.269, sequence homology 100%), is a rare pathogen. It may cause infections in immunocompromised patients and first human isolates were described in 1982 [31,71]. A recent report identifies *Sphingobacterium spiritivorum* as causative agent for a fatal case of bacteremia and sepsis in a patient with acute myeloid leukemia [38].

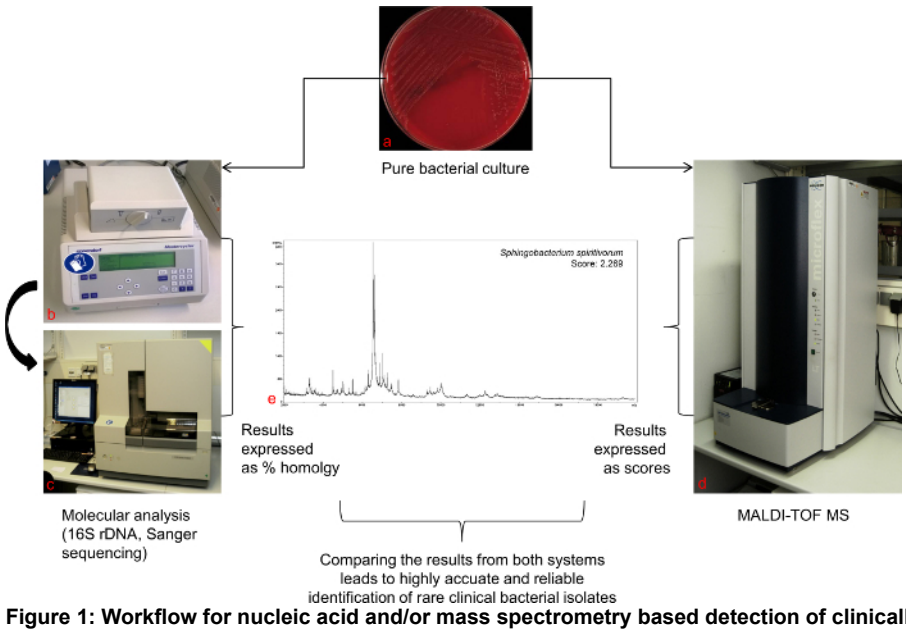

Figure 1: Workflow for nucleic acid and/or mass spectrometry based detection of clinically relevant bacteria in the medical microbiology laboratory. Starting from a pure culture (**a**), target DNA is amplified in a thermocycler (**b**). PCR amplicons are sequenced in a four capillary sequencer using Sanger technology (**c**). Sequencing results are determined as percent homologies of query sequences to database entries by computer assisted comparison. A second, proteomics based method, uses mass spectrometry (**d**) and in the center a mass spectroscopic result of strain #1 in **Table 1**, *Sphingobacterium spiritivorum*, including its MALDI-TOF MS score, is given (**e**). A combination of mass spectrometry and 16S rRNA gene sequencing may be necessary, in order to achieve a more reliable and accurate species identification. Please click here to view a larger version of this figure.

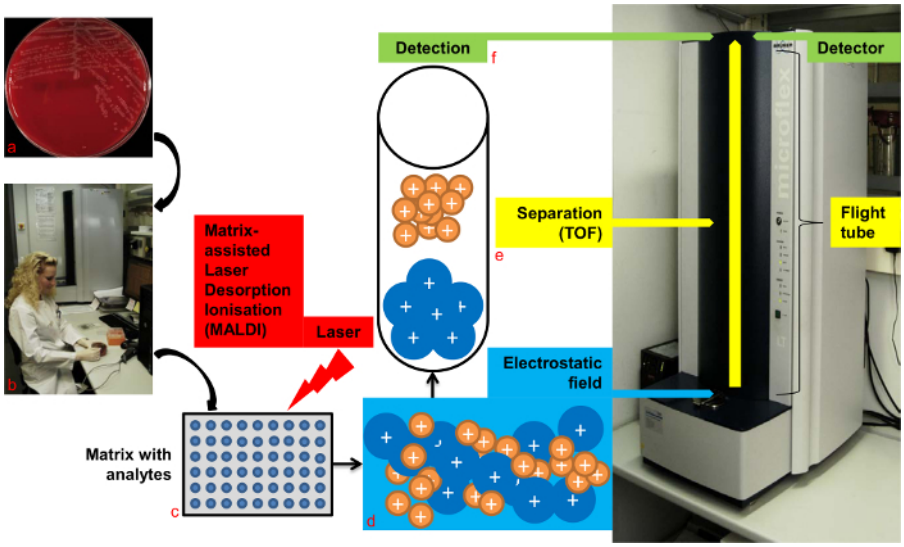

Figure 2: Analysis procedure using MALDI-TOF MS for identification of clinically relevant bacteria. An operator takes whole cell bacterial material (**a**) with a toothpick from a pure culture (**b**) and places bacterial smears on a steel target (**c**). The steel target is inserted into the loading port of the mass spectrometer. When the proper vacuum of 5 x 10^{-6} mbar is reached, the target will be moved to the ionization chamber. Using an N_2-laser, ions are created by soft desorption which are then accelerated in an electrostatic field (**d**) and separated in the flight tube (**e**). The time of flight (TOF) needed for the ions to reach the detector of the flight tube (**f**) is directly related to their mass and forms the basis of subsequent calculations of mass peaks. Specific identification software (**Materials Table**) then assigns score values to the mass spectrum fingerprint of ionized bacterial proteins. Please click here to view a larger version of this figure.

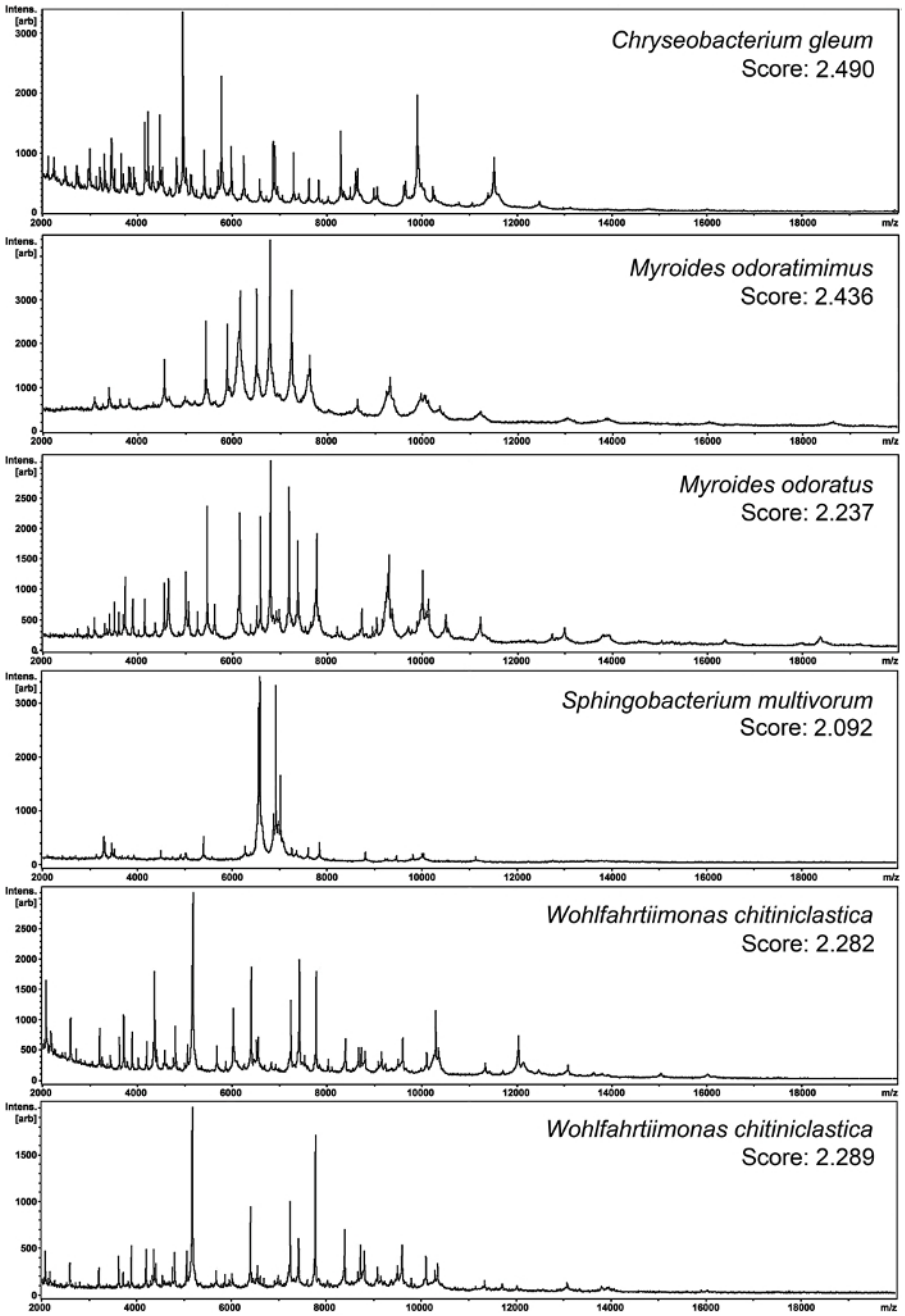

Figure 3: MALDI-TOF spectra and assigned scores of rare pathogens. In this figure, representative MALDI-TOF spectra of the first six rare pathogens listed in **Table 1** are shown. Species name and corresponding MALDI-TOF MS score are noted in each spectrum. Please click here to view a larger version of this figure.

Strain	MALDI identification	MALDI score	16s rDNA result	BLAST homology
#1	***Chryseobacterium gleum***	2.211	***Chryseobacterium gleum***	99% identity
#2	***Myroides odoratimimus***	2.397	***Myroides odoratimimus***	99% identity
#3	***Myroides odoratus***	2.237	***Myroides odoratus***	99% identity
#4	***Sphingobacterium multivorum***	2.093	***Sphingobacterium multivorum***	99% identity
#5	***Wohlfahrtiimonas chitiniclastica***	2.282	***Wohlfahrtiimonas chitiniclastica***	100% identity
#6	***Wohlfahrtiimonas chitiniclastica***	2.289	***Wohlfahrtiimonas chitiniclastica***	100% identity
#7	***Sphingobacterium spiritivorum***	2.269	***Sphingobacterium spiritivorum***	100% identity

Table 1: Representative MALDI-TOFs results of rare occurring bacteria in comparison to 16s rRNA gene sequencing.

Discussion

Both MALDI-TOF MS and 16S rRNA gene sequencing offer the possibility to identify large numbers of different bacteria. MALDI-TOF MS is a fast and inexpensive method, which is easy to handle and large databases of bacterial mass spectra are available. For this reason, MALDI-TOF MS is a rapid, cost effective and reliable method to conduct screening studies focused on rare bacterial pathogens [17,20,39,51]. In a prospective study comparing MALDI-TOF MS with other phenotypic identification methods, Seng *et al.* demonstrated cost effectiveness and speed of MALDI-TOF MS [59] and Tan *et al.* reported a reduction of reagent and labor costs for bacterial identification in their laboratory setting by 56.9% annually [62]. Such cost reductions are supported by a note of Gaillot *et al.* [28]

On the other hand, 16S rDNA sequencing is more time consuming, laborious and warrants specialized personnel to perform the analysis [34]. Nevertheless, both approaches are suitable for routine diagnostics and it could be demonstrated that the combination of these two methods leads to a higher reliability and more secure identification accuracy, which is especially beneficial in case of doubtful results [56]. Therefore, we propose the combination of both methods as the best approach to verify the identification of rare occurring bacteria in routine diagnostics. For reliable species identification, it is absolutely imperative to use pure bacterial cultures because mixed cultures prevent species determination. As a plausibility check, species identification obtained with either MALDI TOF MS or 16S rDNA sequencing has to be in accordance with the results from Gram-staining, biochemical characterization and the clinical presentation [49].

Mass spectrometry as an analytical tool for bacterial identification was first proposed in 1975 [51]. However, it took until the late 1980s to establish a practicable approach for protein analysis. The "soft desorption ionization" technology was introduced by Koichi Tanaka in 1985 [63], who received the Nobel Prize for chemistry in 2002, allowing analysis of intact proteins. At the same time the matrix-assisted ionization time of flight mass spectrometry was introduced by Hillenkamp and Karas as they were the first to use an organic acid to analyze biomolecules [37] and this is the method which is still used today. Although the MALDI technology has been used sporadically [23,30,41], it took until 2004 when Bruker Daltonics introduced its microflex MALDI Biotyper system (Pittcon Conference & Expo, 2004, press release), that MALDI-TOF MS based microbial identification became a routine diagnostic technique [46]. Recent approaches in MALDI-TOF MS techniques gave the opportunity to identify yeasts, detect multiresistant bacteria and perform antimicrobial susceptibility testing [17,51]. Moreover, certain procedures offer the possibility to directly analyze bacteria from primary samples, such as urine or blood cultures [17,51].

Although the use of this system is principally easy, there are some pitfalls which may influence the results. The age of the microorganisms for instance affects the bacterial protein expression and therefore the reproducibility of the results. First, growth conditions may be a problem. As an example, enterobacteria such as *Escherichia coli* grow faster than non-fermenting bacteria (*e.g., Pseudomonas aeruginosa*) and consequently have to be analyzed earlier [17]. Second, the matrix used for MALDI-TOF MS consists of small organic acid molecules that have a strong laser optical absorption for the wavelength of the laser used. Prior to analysis the matrix is added to the sample and both components undergo a crystallization process forming a solid solution.

This explains why changes in the matrix may affect the accuracy of the bacterial identification [17]. Therefore, freshly prepared matrix solutions, not older than 7 days, should be used. Third, the medium from which bacteria are picked may influence the identification results [17,68]. For instance, crystal violet, which is a component of MacConkey agar, interferes with mass spectra [17]. Additionally, too many bacteria applied on the steel target will lead to lower scores and therefore affect the identification accuracy. Therefore, it is advisable to define clear criteria as to how an analysis is carried out. However, misleading results performed by MALDI-TOF MS are mostly caused by insufficient reference spectra contained in the database. (Currently the data base contains >6,000 entries.) This is especially still the case for the identification of anaerobic bacteria [17]. However, additional spectra can be added by the user. MALDI-TOF MS library for instance contains 98 additional reference spectra of isolates which are not adequately addressed by the original database, such as *Mycoplasma* sp., *Myroides* sp., *Legionella* sp., *Roseomonas* sp., *Comamonas* sp. and *Chryseobacterium* sp. Consequently, additional reference spectra will lead to a higher identification accuracy [59,69]. Finally, in some cases the spectra of different species are very similar. This can lead to misidentification in cases such as the discrimination of *Escherichia coli* and *Shigella* spp. or *Streptococcus pneumoniae* and *Streptococcus mitis* [17,51].

Sequencing of 16S rDNA and additional genes such as *rpoB* have simplified the molecular identification of rare or unknown bacteria. These genes are common in most bacteria and their individual function is identical. However, since they possess enough genetic variability to produce results which permit a differentiation on genus and species level, they can be used for bacterial identification [34]. According to Stackebrandt and

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Göbel, homologies <97% represent different species [61]. However, homologies >97% do not necessarily lead to a secure species identification [34,47]. These uncertainties have several reasons.

The quality of the databases which are used to calculate the homologies is sometimes questionable [34]. In cases where high homologies at the 16S rDNA level exist, uncertainties may result in species identification. A combination of different genetic regions may therefore lead to more secure results. Moreover, there are no general guidelines for the interpretation of 16S rDNA sequencing data [22,34,61]. However, increasing the reliability of the existing databases will lead to a higher accuracy for bacterial identification using this molecular approach [34]. Regarding the sequencing reaction we need to mention that the accuracy of the sequencing results mostly relies on the quality of the underlying PCR. Furthermore, since the results are being gained by comparing them to the entries listed in public databases, quality of sequence entries and maintenance of the data is of crucial importance.

In general, although both approaches, MALDI-TOF MS and 16S rRNA gene sequencing, have advantages they have also weaknesses. The combination of both methods, however, leads to a high accuracy for bacterial identification. For instance, in a previous study we could demonstrate that MALDI-TOF MS is able to distinguish between *Myroides odoratimimus* and *Myroides odoratus* [56]. In a few cases the MALDI score suggested unreliable species identification, while the results obtained by 16S rDNA sequencing could confirm the species identity. Mass spectral fingerprints of these isolates were then created and introduced into our MALDI reference spectra database. Future research focusing on other rare bacteria may demonstrate the applicability of the proposed strategy.

Disclosures

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