Infection, Genetics and Evolution xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Mass spectrometry based methods for the discrimination and typing of mycobacteria

E. Shitikov [a,*], E. Ilina [a], L. Chernousova [b], A. Borovskaya [a], I. Rukin [a], M. Afanas'ev [a], T. Smirnova [b], A. Vorobyeva [b], E. Larionova [b], S. Andreevskaya [b], M. Kostrzewa [c], V. Govorun [a]

[a] Research Institute for Physical–Chemical Medicine, Moscow, Russia
[b] Central TB Research Institute of RAMS, Moscow, Russia
[c] Bruker Daltonik GmbH, Bremen, Germany

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
MALDI
Mycobacterium
Tuberculosis
Direct bacterial profiling
Nontuberculous mycobacteria
Spoligotyping

ABSTRACT

Identification and typing of mycobacteria is very important for epidemiology, susceptibility testing and diagnostic purposes. This paper describes the development and validation of the alternative methods for species identification and typing of mycobacteria based on a matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-ToF MS). Altogether there were 383 clinical isolates analyzed which include 348 strains of *Mycobacterium tuberculosis* complex (MTBC) (342 strains of *M. tuberculosis* and 6 strains of *M. bovis*) and 35 strains of nontuberculous mycobacteria (NTM) represented by 16 different species. Direct bacterial profiling (DBP) by means of MALDI-ToF MS was carried out. Cluster analysis of DBP mass spectra divided them into two large separate groups corresponding to MTBC and NTM, and also demonstrated the possibility of isolate identification at the species level. Spoligotyping protocol based on mass spectrometry was developed and validated, it matched completely to classical spoligotyping data. Our results suggest that MALDI-ToF MS has potential as a rapid and reproducible platform for the identification and typing of *Mycobacterium* species.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

The genus *Mycobacterium* consists of approximately 140 heterogeneous species of rapid- and slow-growing bacilli. The number of infections caused by mycobacteria has increased over the past few decades. The *Mycobacterium tuberculosis* complex (MTBC) has become a major cause of death in many developing countries and continues to be a public health problem globally. Nontuberculous mycobacteria (NTM) are responsible for mycobacteriosis and many opportunistic infections in both immunocompromised and immunocompetent individuals (Glassroth, 2008). Accurate and definitive differentiation of MTBC and NTM has become important due to the rise of infections and antimicrobial resistance in this genus. Further molecular typing of MTBC isolates has greatly facilitated the understanding of epidemiology of these pathogens.

Identification of mycobacteria at the species level is important for epidemiological, public health and therapeutic reasons. Traditionally, mycobacterium is identified by its phenotypic traits, such as morphological features, growth rates and preferred growth temperature, pigmentation and biochemical profiles. These methods are well established and standardized. However, testing remains to be a laborious, difficult and time-consuming task, requiring as long as 12 weeks for positive identification of the organism, and its results are often not reported in a timely manner to guide clinical decisions. In the last decade advances in molecular methods have facilitated the cheap, rapid and reliable identification of many *Mycobacterium* species by molecular means (Cook et al., 2003; Devulder et al., 2005; Hance et al., 1989; Russo et al., 2006). However, these methods are still labor-intensive, narrowly focused and may be applicable only for a limited number of species. If further characterization of samples is required additional methods have to be applied, which usually means another instrument base.

The goal of this study was to demonstrate a complex solution based on a matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-ToF MS) suitable for a rapid and accurate species identification and typing of mycobacteria.

Recently, direct bacterial profiling (DBP) by means of MALDI-ToF MS has been suggested as an efficient approach for a rapid and accurate classification and species identification of microbes (Leuschner et al., 2004; Lynn et al., 1999; Mandrell et al., 2005; Mellmann et al., 2008) overcoming the existing limitations of classical biochemical methods. This technique based on analysis of unique mass spectra produced by proteins extracted from microbial

* Corresponding author. Address: Research Institute for Physical–Chemical Medicine, 119435 Malaya Pirogovskaya str., 1a, Moscow, Russia. Tel.: +7 095246 45 70; fax: +7 095246 45 01.
E-mail address: egorshtkv@gmail.com (E. Shitikov).

1567-1348/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2011.12.013

Please cite this article in press as: Shitikov, E., et al. Mass spectrometry based methods for the discrimination and typing of mycobacteria. Infect. Genet. Evol. (2012), doi:10.1016/j.meegid.2011.12.013

cells (Fox, 2006). Some authors have used MALDI-ToF MS for identification of mycobacteria (Hettick et al., 2006; Lotz et al., 2010; Pignone et al., 2006; Saleeb et al., 2011), but the global experimental data are still quite limited. We believe that in the future the development of the comprehensive DBP databases will give us a possibility for a rapid and reliable identification of *Mycobacterium* species.

For the other hand primer extension followed by MALDI-ToF MS detection approach can be applied for genetic markers identification. This method includes a PCR step followed by the dephosphorylation of excess nucleotides and primers, a primer extension step in the presence of dideoxynucleotides and, finally, purification of the samples for the mass-spectrometry analysis. It was used for genotyping (Honisch et al., 2010) of *M. tuberculosis* and analysis of genetic markers of drug resistance (Afanas'ev et al., 2007, 2011; Ikryannikova et al., 2007).

This paper describes the development and validation of the new methods for species identification and typing of mycobacteria combined on the same mass spectrometry platform. Both created techniques are open and easy-to-use and can be a useful tool for global epidemiological purposes.

This work was presented earlier on the 41st World Conference on Lung Health of the International Union Against Tuberculosis and Lung Disease in Germany in 2010.

2. Materials and methods

2.1. Strains

This study included 383 clinical isolates representing 2 species of MTBC (342 strains of *M. tuberculosis* and 6 strains of *M. bovis*) and 16 species of NTM (35 strains). The strains have been collected at the Microbiology Department of the Central Tuberculosis Research Institute of RAMS (Moscow, Russia) since 1993 and stored in the laboratory bank. Initial microbiological species' identification was performed as followed: Tween 80 hydrolysis (5 and 10 days), nitrate reduction, tolerance to 5% sodium chloride, growth in the presence of TCH (thiophene-2-carboxylic acid hydrazide), arylsulfatase (3 and 14 days), urease, heat-stable (68 °C) catalase, semiquantitative catalase, iron uptake, niacin production, tellurite reduction, pyrazinamidase (4 and 7 days), beta-glucosidase, acid phosphatase, citrate test, polymyxin B and ciprofloxacin inhibition (Holt et al., 1994; The Ministry of Public Health of Russian Federation, 2003). Growth at temperatures of 25, 28, 31, 37, and 42 °C; pigmentation; growth rates; and colony characteristics were also assessed. Detailed information on the strains is noted in Table 1.

2.2. Direct bacterial profiling

Mycobacterium species were grown on Lowenstein–Jensen medium (Becton Dickinson, USA). Rapid- and slow-growing mycobacteria were being cultured for 1 and 2 weeks, respectively. After cultivation the cells were transferred from the tube with a 1.0 μl plastic loop into an extraction 1.5 ml tube (Eppendorf, Germany) with 300 μl pure water (Fluka, Germany) to wash them from the culture medium. After mixing at room temperature during 5 min, the suspension was centrifuged (5 min at 10000 g). The pellet was once more resuspended in the same volume of water. At the next step 900 μl of 96% ethanol was added. After centrifugation 20 μl of 70% formic acid (Sigma–Aldrich, Germany) was added to pellet and the solution was mixed intensively for 5 min. Then 20 μl pure acetonitrile (Sigma–Aldrich, Germany) was added. Solution was mixed and centrifuged again.

The supernatant was placed in quantity 2 μl onto MALDI target plate (MSP 96 target ground steel; Bruker Daltonics, Germany) in five replicates of each sample and was allowed to air dry at room temperature. A saturated solution of α-cyano-4-hydroxy cinnamic acid (CHCA) (Bruker Daltonics, Germany) was prepared in 1.0 ml of 50% acetonitrile, 2.5% trifluoracetic acid and used as a matrix. Finally, 2 μl of CHCA matrix solutions was deposited onto the dried supernatant and allowed to dry at room temperature. All preparation steps were performed in a 1.5 ml Eppendorf™ tube (Eppendorf, Germany). All the solvents utilized were MS grade.

Spectra were recorded in the mass range from 2000 to 20000 Da, but the majority of peaks were registered in the 2000–10000 Da intervals. Bruker bacterial standard was used for method calibration. A sufficient signal / noise ratio and resolution (average resolution was 400 in the 2000–10000 Da range) has been achieved for all spectra. Mass spectra were obtained on an AutoflexTM (Bruker Daltonics, Germany) MALDI-ToF MS. Thirty mass spectra (using five plate positions and six times collecting spectra on each position) were collected for each sample (strain) and then processed into one main spectrum using MALDI Biotyper v.2.0 (Bruker Daltonics, Germany) software. Additionally spectra processing for visual inspection was performed using the flexAnalysis 2.4 software (Bruker Daltonics, Germany).

2.3. DNA extraction

DNA extraction from the analyzed species was performed by "Polytube" DNA extraction kit (Lytech Ltd., Russia). If necessary, the prepared DNA samples were stored at the temperature of –20 °C.

2.4. GenoType Mycobacterium CM and AS assays

The GenoType Mycobacterium CM/AS (Hain Lifescience, Germany) assay was used for detection of MTBC (it does not distinguish *M. tuberculosis* from *M. bovis*) and identification of the most common NTM species. Assay was performed according to the manufacturer's instructions. The process involved PCR amplification, hybridization of PCR products to probes bound to test strips and detection of bound products. The test strips were fixed on a data sheet. The development of conjugate, universal and genus control lines were assessed carefully for each isolate. Reactions with probe lines were recorded. In accordance with the manufacturer's instructions, only bands with intensities as strong as or stronger than the universal control line were considered. Any isolates not identified by the GenoType CM assay were tested with the GenoType AS assay.

2.5. 16S rRNA gene and 16S–23S rDNA internal transcribed spacer (ITS) sequencing

PCR amplification was carried out in a reaction mixture containing 66 mM of Tris–HCl (pH 9.0), 16.6 mM of $(NH_4)_2SO_4$, 2.5 mM of $MgCl_2$, 250 μM of each dNTP, 1 U of Taq DNA polimerase (Promega, USA) and 5 pmol of each primers in final volume 10 μl using the thermocycler DNA Engine Tetrad 2 (MJ Research, USA). For the amplification of 16S rDNA, the forward primer was 271f (5′-CTTAACACATGCAAGTCGAAC-3′) and the reverse primer was 259r (5′-TTTCACGAACAACGCGACAA-3′). For the amplification of ITS1 rDNA, the forward primer was EC16S (5′-TTGTACACACCGCCCGTCA-3′) and the reverse primer was Mb23S (5′- TCTCGATGCCAAGGCATCCACC-3′) (Roth et al., 1998).

Then 10 μl of reaction mixture contained 66 mM of Tris–HCl (pH 9.0), 16.6 mM of $(NH_4)_2SO_4$, 2.5 mM of $MgCl_2$, 0.5 U of shrimp alkaline phosphatase (SAP) (Promega, USA), and 5 U Exonuclease I (Fermentas, EU) were added to each PCR product and incubated at

Please cite this article in press as: Shitikov, E., et al. Mass spectrometry based methods for the discrimination and typing of mycobacteria. Infect. Genet. Evol. (2012), doi:10.1016/j.meegid.2011.12.013

Table 1
Bacterial strains examined in current study.

Verified groups of mycobacteria	Initial species identification (N tested)	GenoType Mycobacterium CM/AS (N tested)	16S rRNA gene and ITS sequencing (N tested)
Rapidly growing mycobacteria			
M. abscessus	*M. abscessus* (3)	*M. abscessus* (3)	*M. abscessus* (3)
M. chelonae	*M. chelonae* (2)	*M. chelonae* (2)	*M. chelonae* sqv. I / Mche A[b] (2)
M. diernhoferi	*M. diernhoferi* (1)	*Mycobacterium* spp. (1)	*M. diernhoferi* (1)
M. fortuitum	*M. fortuitum* (5)	*M. fortuitum* (5)	*M. fortuitum* sqv. I[b] (5)
M. neoaurum	*M. neoaurum* (1)	*Mycobacterium* spp. (1)	*M. neoaurum* (1)
M. peregrinum	*M. peregrinum* (2)	*M. peregrinum* (2)	*M. peregrinum* (2)
M. phlei	*M. phlei* (1)	*M. phlei* (1)	*M. phlei* (1)
M. smegmatis	*M. smegmatis* (2) *M. phlei* (1)	*M. smegmatis* (3)	*M. smegmatis* (3)
Slowly growing mycobacteria			
M. avium	*M. avium* (5) *M. kansasii* (1)	*M. avium* (6)	*M. avium* sqv. I (3) and sqv. II[b] (3)
M. gastri	*M. gastri* (1)	*M. gastri* (1)	*M. gastri* Mga A[b] (1)
M. gordonae	*M. gordonae* (2)	*M. gordonae* (2)	*M. gordonae* sqv. II[b] (2)
M. intracellulare	*M. chelonae* (1) *M. smegmatis* (1)	*M. intracellulare* (2)	*M. intracellulare* sqv. V[b] (2)
M. kansasii	*M. kansasii* (3)	*M. kansasii* (3)	*M. kansasii* sqv. I / Mka A[b] (3)
M. simiae	*M. simiae* (1)	*M. simiae* (1)	*M. simiae* sqv. I[b] (1)
M. scrofulaceum	*M. scrofulaceum* (1)	*M. scrofulaceum* (1)	*M. scrofulaceum* (1)
M. xenopi	*M. xenopi* (1)	*M. xenopi* (1)	*M. xenopi* sqv. I[b] (1)
M. bovis	*M. bovis* (6)	[a]MTBC (6)	MTBC (6)
M. tuberculosis	*M. tuberculosis* (342)	MTBC (26)	MTBC (26)
Total	383	67	67

37 °C for 30 min for dephosphorylation of the 5′-end phosphate groups of deoxynucleoside triphosphates and primer elimination. At the end, the enzymes were deactivated by heating at 85 °C for 10 min.

Sequencing was carried out using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI Prism 3100 Genetic Analyser (Applied Biosystems, USA; Hitachi, Japan). Alignment of nucleotide sequences was performed by means of Vector NTI Suite 9 software (Infomax Inc, USA). For species identification the sequenced data were compared with sequences uploaded in the GenBank of NCBI, RIDOM (Ribosomal Differentiation of Medical Microorganisms (http://rdna.ridom.de/)) and additionally verified with work of Roth et al. (1998).

2.6. Spoligotyping by hybridization

Spoligotyping was performed, as previously described by Kamerbeek et al. (1997), with a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands). The results were documented under the form of a binary code according to the results of hybridization for each spacer oligonucleotide probe and entered into Excel spreadsheet files.

2.7. Spoligotyping by MALDI-ToF MS

Amplification of DR region was done according to primers′ sets, which were described previously (Kamerbeek et al., 1997). Dephosphorylation of the 5′-end phosphate groups of deoxynucleoside triphosphates and primer elimination were carried out as described above. Internal primers for analysis of direct repeat (DR) region structure were designed using Oligo primer analysis software (version 6.31, Molecular Biology Insights, Inc.) (Table S1).

Primer extension reactions were performed in 66 mM of Tris–HCl (pH 9.0), 16.6 mM of $(NH_4)_2SO_4$, 2.5 mM of $MgCl_2$ and 2 U of TermiPol DNA Polymerase (Solis Biodyne, Estonia), 20 pmol of oligonucleotide primers, 2 mM of certain dideoxy nucleoside triphosphate (ddNTP) that was necessary for the reaction. The cycling parameters included 2 min at 94 °C, followed by 70 cycles of 20 s at 94 °C, 20 s at 60 °C, and 15 s at 72 °C.

A sample aliquot (0.2 μl) was spotted onto a matrix spot (saturated solution of 3-hydroxypicolinic acid (Fluka, Germany) in a 1:1 acetonitrile/water mixture (Merck, Germany) mixed with the 0.4 M dibasic ammonium citrate (Fluka, Germany) in 9:1 volume ratio), which was preliminarily dried on an AnchorChip™ (Bruker Daltonics, Germany) 400 μm target.

Mass spectra of extended oligonucleotide primers were collected using an Autoflex™ (Bruker Daltonics, Germany) MALDI-ToF MS. Registration and analysis of the obtained spectra were carried out using flexControl and flexAnalysis v.2.4 (Bruker Daltonics, Germany) software, respectively.

3. Results

In total, 67 clinical *Mycobacterium* spp. clinical strains were analyzed by DBP. This group represented 16 species from NTM (*M. abscessus, M. avium, M. chelonae, M. diernhoferi, M. fortuitum, M. gastri, M. gordonae, M. intracellulare, M. kansasii, M. neoaurum, M. peregrinum, M. phlei, M. scrofulaceum, M. simiae, M. smegmatis* and *M. xenopi)* and two species from MTBC (*M.* tuberculosis and *M. bovis).* For each strain belonging to this group the correctness of species identification was verified by GenoType Mycobacterium CM/AS assay (Hain Lifescience, Germany), and 16S rRNA gene and ITS sequencing (Table 1).

In case of mycobacterium the initial measuring protocol which was previously successfully applied by our working group in the study of *Neisseria* spp. (Ilina et al., 2009), *H. pylori* (Ilina et al., 2010) and non-fermenting bacteria (Mellmann et al., 2009) often failed. Presumably, the reason was the well known stability of the mycobacterium cell wall due to mycolic acids that require a more aggressive treatment for its destruction.

As a part of the study the sample preparation procedure was optimized in order to increase the effectiveness of protein extraction from bacterial cells. At every stage very intensive and longtime pipetting and mixing were introduced. Also two different ways of cell degradation were tried. In the first case bacterial cells suspended in pure water were periodically mixed well during 20 min at room temperature. In the second case cells suspension was heated ($T = 96$ °C) for the same period of time. It should be

Please cite this article in press as: Shitikov, E., et al. Mass spectrometry based methods for the discrimination and typing of mycobacteria. Infect. Genet. Evol. (2012), doi:10.1016/j.meegid.2011.12.013

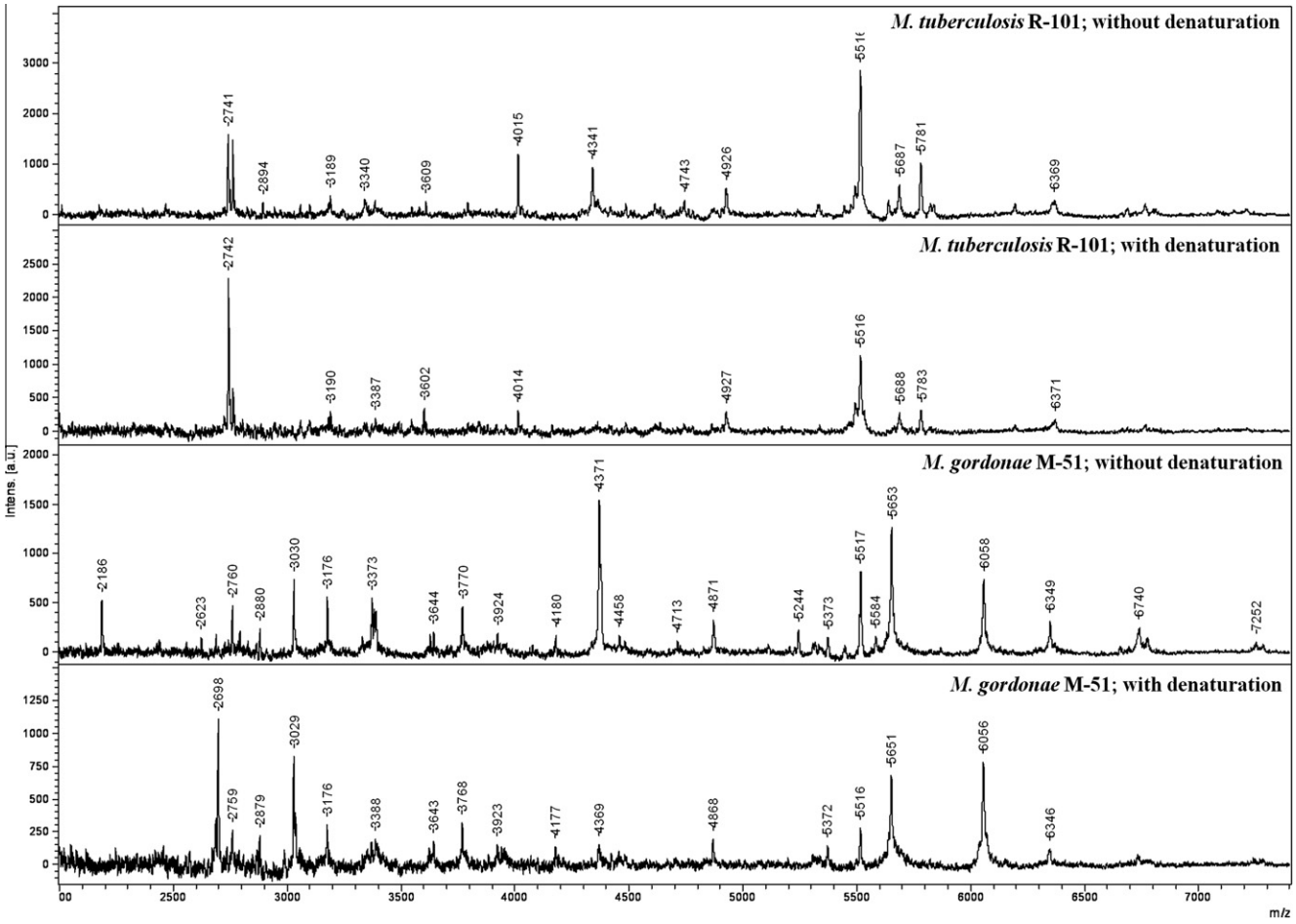

Fig. 1. Comparison of the mass spectra collected for *M. tuberculosis* and *M. gordonae* after different ways of cell degradation, with and without denaturation. The relative intensities of the ions are shown on the *Y* axis, and the mass to charge ratios are shown on the *X* axis.

noted that the quality of spectra decreased when 20 min of thermal denaturation at 96 °C had been performed (Fig. 1).

For some species (e.g. *M. scrofulaceum)* the quality of mass spectra based on signal to noise rate, intensity, the amount of peaks and their resolutions changed from spot to spot that resulted in the decrease in quantity of collected spectra.

When an agreeable method of protein extraction had been developed, we could achieve reproducible and sufficiently resolved spectra. At last it became possible to start automatic runs for mass spectra acquisition.

Thirty spectra (using five plate positions and six times shooting) were obtained for each sample. The comparison of MALDI-ToF mass spectra collected for different *Mycobacterum* species revealed numerous significant differences in peak positions (Figure S1).

For further cluster analysis MALDI Biotyper 2.0 was used. A main spectrum based on the comparative analysis of 30 raw spectra was created for each strain using the dedicated data processing algorithms of the software. Further cluster analysis of all main spectra divided them into two large separate groups corresponding to MTBC and NTM, and also demonstrated the possibility of isolates identification at the species level (Fig. 2). It should be noted that the only minor differences were caught within MTBC between *M. tuberculosis* and *M. bovis*.

Then respective main spectra were introduced into Biotyper library and single mass spectra collected for every strain were matched against a newly created library. In result, we could undoubtedly identify all species inside the NTM group, except for *M. bovis* that had not been securely differentiated from *M. tuberculosis*.

An extended group of *M. tuberculosis* strains (n = 342) was used for developing and validation of spoligotyping protocol based on mass spectrometry. We adapted the primer extension reaction with the following MALDI-ToF MS analysis for rapid determination of DR-region structure. Eight multiplexed primer extension spoligotyping systems (5-6-plex assays) were designed on the basis of all 43 spacer sequences in the informative DR region of MTBC (Table S1). Extension primers were carefully designed and multiplexed to avoid primer cross-hybridization. Upon binding to the target region, the oligonucleotides were elongated by single-base extension. At the same time, in each reaction mixture the only ddNTP was added to reduce the possibility of nonspecific annealing.

The assays were designed in such a way that the non-extended oligonucleotides and their subsequent extension products occupied unique separated masses in the resulting spectrum. The concentrations of the extension primers were adjusted to compensate for the differences in sequences and molecular weights and to equalize the signal intensities in the spectrum. A schematic diagram of primer extension reaction and mass spectra of exemplary products of spacers 1–6 is presented in Fig. 3. As depicted, the chain extension occurs only if the spacer and ddATP appeared in the reaction mixture. Other spacers of the locus were detected similarly.

The whole group of *M. tuberculosis* strains (n = 342) was tested in parallel by the classical spoligotyping method and by the newly

Please cite this article in press as: Shitikov, E., et al. Mass spectrometry based methods for the discrimination and typing of mycobacteria. Infect. Genet. Evol. (2012), doi:10.1016/j.meegid.2011.12.013

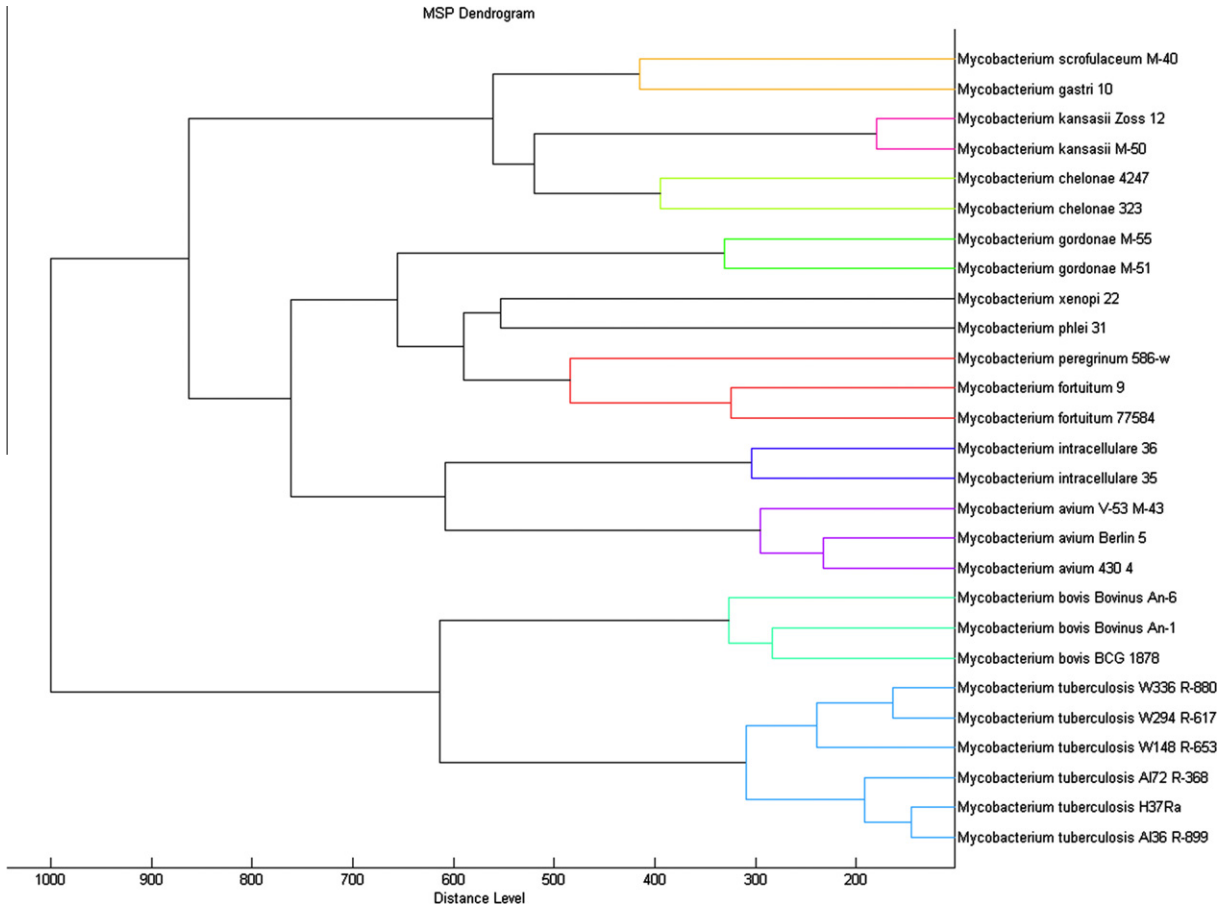

Fig. 2. A score oriented dendrogram of MALDI-ToF mass spectra profiles illustrated the relationship of tested strains. Each main spectrum of the dataset was compared with each other main spectrum resulting in a matrix of cross-wise identification scores. This matrix was used to calculate distance values for each pair of main spectra. Based on these values a dendrogram was generated using the according function of the statistical toolbox of Matlab 7.1 (The MathWorks Inc.) integrated in the MALDI Biotyper software (distance measure was set at Euclidian and linkage set at complete).

developed spoligotyping protocol based on mass spectrometry. The spoligotype designation was attributed by comparing to those included in an international spoligotype database SpolDB4 (Brudey et al., 2006). The most dominant genotype found, was Beijing (74.9%), followed by LAM9 (8.2%), T1 (3.5%), Haarlem3 (2.6%), T4 (2,4%) and T1_RUS2 (1.7%) strains. We also received 16 additional spoligotypes, but the occurrence of each of them was less than 1%. The results obtained by spoligotyping protocol based on mass spectrometry completely matched to classical spoligotyping data (Table 2).

4. Discussion

Here we represent the development and introduction of an open and easy-to-use system based on MALDI-ToF MS platform suitable for identification and typing of mycobacteria. To date, the MALDI-ToF MS is an attractive analytical instrument with a lot of capabilities in measuring different biomolecules such as proteins, lipids, nucleic acids. Using this single device one can perform investigations in various fields of scientific research.

Thus the advantages of MALDI-ToF MS can be used for the rapid identification of bacteria based on the simultaneous detection of a mixture of cellular components, mainly proteins and peptides, directly from the "whole" cells. In the present study, we applied a MALDI-ToF MS based technique for species identification of closely related bacteria from the genus *Mycobacterium*.

In contrast to the previously reported studies (Hettick et al., 2006; Pignone et al., 2006) we mainly analyzed clinical *Mycobacterium* spp. isolates instead of reference strains. Though this allowed us to study the natural variability of these bacterial pathogens, it also forced us to use additional methods for proper species identification of each sample. Expectedly, the initial species identification performed by traditional bacteriological testing was corrected for some specimen when the molecular tests were applied.

Since the aim of our study was to identify species of mycobacteria, there was no need to use the full array of MTBC, and therefore, there were taken all of 35 NTB strains, all of 6 *M. bovis* strains, and 26 randomly selected strains of *M. tuberculosis* (Table 1). In total 67 clinical strains belonging to 18 species from the genus *Mycobacterium* were analyzed by DBP based on MALDI-ToF MS.

To date, there are only four papers describing the strategy of MALDI-ToF MS protein fingerprinting for *Mycobacterium* species identification. Pignone et al. (2006) were the first who declared that unique mass spectra were obtained for 37 ATCC (American Type Culture Collection) strains representing 13 species of genus *Mycobacterium*. According to their measuring protocol, the analyzed mass range was set between 500 and 4000 Da, and the majority of ions detected were less than 1000 Da. In that study, the root mean square values were reported both for the comparisons of different profiles and for construction of a dendrogram which could also show the relationship of microorganism basis

Please cite this article in press as: Shitikov, E., et al. Mass spectrometry based methods for the discrimination and typing of mycobacteria. Infect. Genet. Evol. (2012), doi:10.1016/j.meegid.2011.12.013

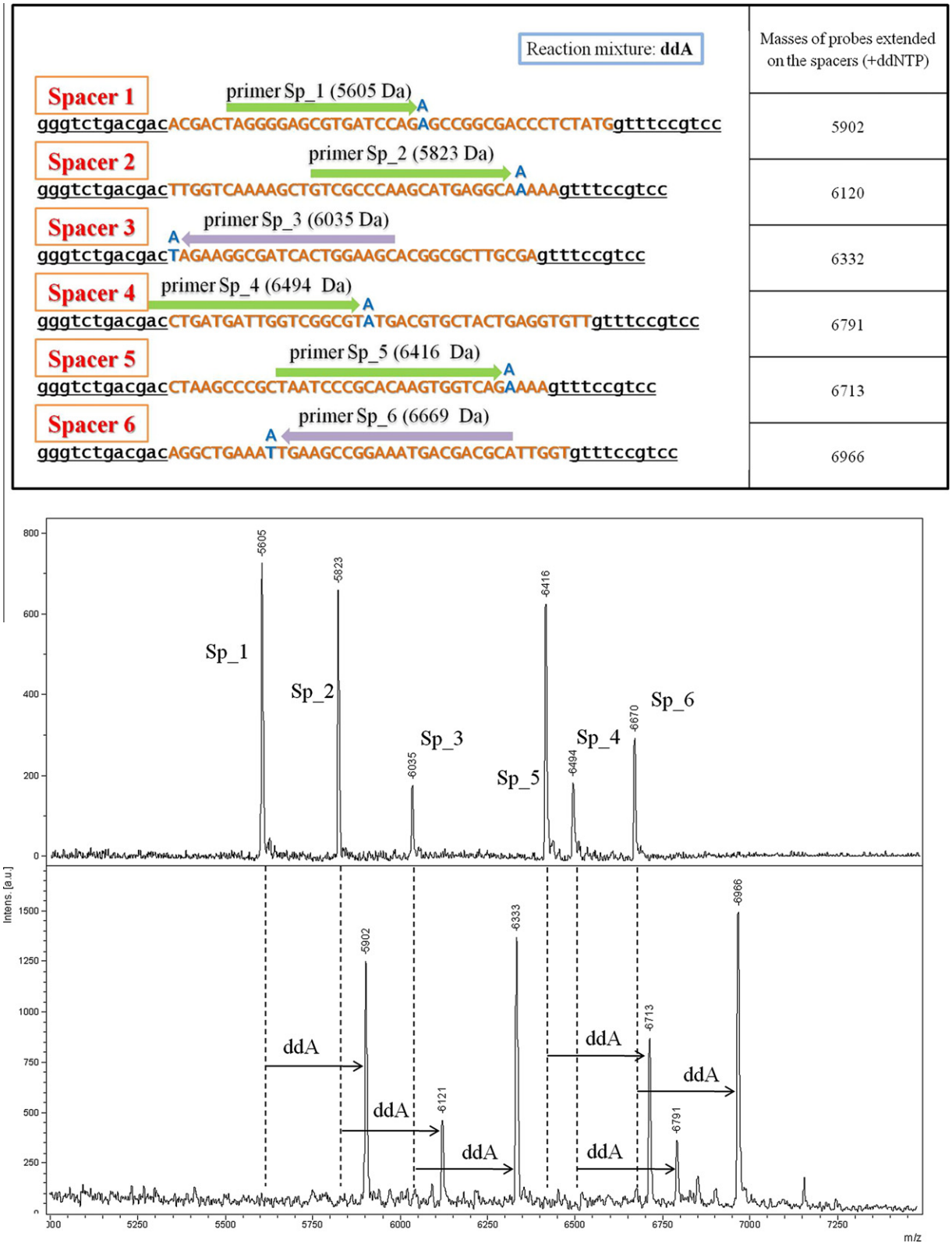

Fig. 3. Schematic diagram of chain extension reaction on the example of spacer 1-6 detecting. Probes Sp_1, Sp_2, Sp_4 и Sp_5 are forward directed, probes Sp_3 and Sp_6 are reverse. Mass spectra of original probes (upper part) and products of reaction (lower part) are displayed. The picture presents the shift of all original probes by ddA, which shows the presence of spacers in the analyzed locus.

on their spectral profile. The other investigation published the same year was also performed based on ATCC strains (n = 16) from four species of mycobacterium (*M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*) (Hettick et al., 2006). Authors have also reported a possibility to unambiguously identify strains within a species based on MALDI-ToF MS data. Additionally, they have communicated opportunity to correctly classify bacterial strains as either *M. tuberculosis* or non-tuberculosis with 100% accuracy on

Table 2
Spoligotyping results for 342 DNA samples of *M. tuberculosis* obtained by the spoligotyping protocol based on mass spectrometry and reverse line blot hybridization.

Spoligotype	No. of isolates with spoligotype according to:	
	Spoligotyping protocol based on mass spectrometry	Reverse line blot hybridization
Beijing	256	256
LAM9	28	28
T1	12	12
Haarlem3	9	9
T4	8	8
T1_RUS2	5	5
Unknown	3	3
Family33	2	2
Haarlem4	2	2
LAM9 (252)	2	2
T1 0.22 Haarlem3 0.77	2	2
T4, H37Rv	2	2
T5_RUS1	2	2
Family36	1	1
Haarlem1	1	1
Haarlem2	1	1
LAM3_S (conver)	1	1
LAM9 (42)	1	1
T2	1	1
T2 (U (likely H3))	1	1
T2-T3	1	1
U	1	1

the basis of unique m/z values observed in the fingerprint spectrum using random forest analysis.

In our study as well as in the recently published investigations (Lotz et al., 2010; Saleeb et al, 2011) we focused mainly on the analysis of clinical *Mycobacterium* spp. strains by means of MALDI-ToF MS protein fingerprinting. For characterization and classification of mass spectra, we used commercially available MALDI Biotyper software (Bruker Daltonics, Germany) that opens a perspective of utilization of our data by other MALDI Biotyper users.

Collected mass spectra can be exchanged between users, but they are dependent on the MALDI platform of Bruker, as any mass spectra are specific for the respective manufacturer. In an upcoming version of the software, different users will even be able to distribute libraries of main spectra which they have created themselves, thereby absolutely identical databases (not only same spectra but also same data processing applied) can be used among different users.

A new development protocol of cell destruction and protein extraction was applied, which allowed to collect mass spectra from all *Mycobacterium* spp. clinical strains investigated. The great advantage of this method is the application of the same automatic measuring protocol based on the strong criteria of mass spectra collection (high spectra reproducibility, little noise and a flat baseline, presenting as many peaks as possible and medium resolution of 600 within the range of m/z selected for analysis) which has earlier been sufficiently optimized for analysis of other bacterial pathogens (Ilina et al., 2009, 2010).

In general, the MALDI mass spectra produced by our method were conserved for each specimen and slightly different within the same species. Moreover, most differences observed while comparing individual measurements of one strain appeared in intensities, and only in few cases in absence or presence of peaks. These measurement variations, which are intrinsic to the MALDI-ToF MS technology, are reduced significantly by merging 30 raw spectra for each sample into one main spectrum, which displays the most reproducible peaks.

According to comparative analysis of mass spectra, the members of MTBC formed a unique cluster distinct from the NTM (Fig. 2). It is significant observation since it suggests that MALDI-ToF MS has the potential to effectively identify these important pathogens and unambiguously distinguish them from their less harmful relatives. When the main spectra of NTM were introduced into the MALDI Biotyper library undoubted species identification was achieved for each strain.

As far as members of MTBC are concerned one can see three different groups based on their mass spectra comparison. The first group was formed by *M. bovis* strains, the other two by *M. tuberculosis* ones. Although we fixed the divergence of *M. tuberculosis* strains and the analysis of mass spectra has shown minor differences, we were unable to determine their origin on this stage. Unfortunately, when we introduced respective main spectra into MALDI Biotyper library, we could not discriminate species within MTBC by standard identification procedure, although some differences in the main spectra were found. Apparently, this has to be further evaluated with a higher number of strains and based on detailed mass spectra analysis. It might be possible to differentiate the different species inside the MTBC then by the adopted algorithm.

At the same time, this approach requiring a small amount of biological material and very easy sample preparation offers highly accurate discrimination of MTBC from NTM and species identification within NTM group. The comparative price of this method is lower than the price of most common approaches (Seng et al., 2009).

To demonstrate the potential of MALDI-ToF MS for mycobacteria differentiation at the strain level, we have adopted this platform for spoligotype detection. Spoligotyping method has been very successful as a tool for the rapid acquisition of MTBC genotyping information and for the establishment of a global picture of MTB diversity (Supply et al., 2006). The classical assay, carried out about 10 years ago, based on reverse line blot hybridization has proven to be suboptimal, mainly due to variations in the intensities of the hybridization signals for particular spacers and a large number of time-consuming procedures (Kremer et al., 1999). Recently, an improved assay format of PCR and a multiplexed primer extension assay followed by MALDI-ToF MS detection on a MassARRAY system (Sequenom, Inc.) have been suggested for spoligotyping (Honisch et al., 2010).

In our work, we present a slightly different methodology, which includes an easily reproducible protocol of PCR followed by eight multiplexed primer extension reactions and MALDI-ToF MS measurement. In their study Honisch et al. (2010) used a complete and closed commercially available solution for high throughput MALDI-based SNP genotyping by primer extension termed MassARRAY. Here we have reported the creation of our own spoligotyping scheme combined with DBP on a single technological platform. We increased the number of primer extension reactions and thereby reduced the complexity of single reactions to simplify the sample preparation in routine practice. In general, 3–5-plex assays can be routinely done in parallel (Sauer, 2006).

We have utilized an established spoligotyping protocol based on mass spectrometry and applied it to the routine typing of *M. tuberculosis* strains. Results for each sample were recorded as binary characters. Each binary character denotes the presence or the absence of a spacer in the DR locus of the *M. tuberculosis*. The final spoligotypes can thus be easily reported by simple Excel spreadsheet calculation, which makes the results readily shareable among laboratories.

We demonstrate that the new approach produces results fully concordant with those derived from the traditional membrane-based method. Twenty two different spoligotypes were found. Although the most dominant spoligotype was Beijing, a set of all the spacers was present in the samples studied. Unfortunately, we didn't find any associations between spoligotype and groups of *M. tuberculosis* after DBP.

The use of spoligotyping protocol based on mass spectrometry enables the analysis of 48 samples to be carried out within about

Please cite this article in press as: Shitikov, E., et al. Mass spectrometry based methods for the discrimination and typing of mycobacteria. Infect. Genet. Evol. (2012), doi:10.1016/j.meegid.2011.12.013

8 h. In comparison, a spoligotyping analysis of 45 samples under optimal conditions by reverse line blot hybridization requires more than 24 h of labor. At the same time, MALDI-ToF MS is an open platform and additional assays such as ones with additional spacer sequences or antibiotic resistance assays (Afanas'ev et al., 2007; Ikryannikova et al., 2007) can be incorporated.

Based on the described experiments we can conclude that the DBP technique works very well for species identification inside the closely related genus *Mycobacterium*. Moreover, in case of *M. tuberculosis* the other MALDI-ToF MS based method can be applied for spoligotyping. The illustrated capability of using the same device for identification and typing of *M. tuberculosis* emphasizes the functionality and universality of presented solutions as a useful tool for molecular epidemiology purposes.

Acknowledgements

Authors sincerely thank Dr. L. N. Ikraynnikova for useful discussion and invaluable assistance in preparation of this article.

We also thank Prof. Tomotada Iwamoto for expert conclusion regarding our manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.12.013.

References

A decree of the Ministry of Public Health of Russian Federation No 109 "About the procedures of microbiology experiments in treatment of tuberculosis"- Russian Federation, 2003.

Afanas'ev, M.V., Ikryannikova, L.N., Il'ina, E.N., Sidorenko, S.V., Kuz'min, A.V., Larionova, E.E., Smirnova, T.G., Chernousova, L.N., Kamaev, E.Y., Skorniakov, S.N., Kinsht, V.N., Cherednichenko, A.G., Govorun, V.M., 2007. Molecular characteristics of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates from the russian federation. J Antimicrob Chemother 59, 1057–1064.

Afanas'ev, M.V., Ikryannikova, L.N., Il'ina, E.N., Kuz'min, A.V., Larionova, E.E., Smirnova, T.G., Chernousova, L.N., Govorun, V.M., 2011. Molecular typing of *Mycobacterium tuberculosis* circulated in moscow, russian federation. Eur J Clin Microbiol Infect Dis 30, 181–191.

Brudey, K., Driscoll, J.R., Rigouts, L., Prodinger, W.M., Gori, A., Al-Hajoj, S.A., Allix, C., Aristimuno, L., Arora, J., Baumanis, V., Binder, L., Cafrune, P., Cataldi, A., Cheong, S., Diel, R., Ellermeier, C., Evans, J.T., Fauville-Dufaux, M., Ferdinand, S., Garcia de Viedma, D., Garzelli, C., Gazzola, L., Gomes, H.M., Guttierez, M.C., Hawkey, P.M., van Helden, P.D., Kadival, G.V., Kreiswirth, B.N., Kremer, K., Kubin, M., Kulkarni, S.P., Liens, B., Lillebaek, T., Ho, M.L., Martin, C., Mokrousov, I., Narvskaia, O., Ngeow, Y.F., Naumann, L., Niemann, S., Parwati, I., Rahim, Z., Rasolofo-Razanamparany, V., Rasolonavalona, T., Rossetti, M.L., Rusch-Gerdes, S., Sajduda, A., Samper, S., Shemyakin, I.G., Singh, U.B., Somoskovi, A., Skuce, R.A., van Soolingen, D., Streicher, E.M., Suffys, P.N., Tortoli, E., Tracevska, T., Vincent, V., Victor, T.C., Warren, R.M., Yap, S.F., Zaman, K., Portaels, F., Rastogi, N., Sola, C., 2006. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 6, 23.

Cook, V.J., Turenne, C.Y., Wolfe, J., Pauls, R., Kabani, A., 2003. Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. J Clin Microbiol 41, 1010–1015.

Devulder, G., Perouse de Montclos, M., Flandrois, J.P., 2005. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. Int J Syst Evol Microbiol 55, 293–302.

Fox, A., 2006. Mass spectrometry for species or strain identification after culture or without culture: past, present, and future. J Clin Microbiol 44, 2677–2680.

Glassroth, J., 2008. Pulmonary disease due to nontuberculous mycobacteria. Chest 133, 243–251.

Hance, A.J., Grandchamp, B., Levy-Frebault, V., Lecossier, D., Rauzier, J., Bocart, D., Gicquel, B., 1989. Detection and identification of mycobacteria by amplification of mycobacterial DNA. Mol Microbiol 3, 843–849.

Hettick, J.M., Kashon, M.L., Slaven, J.E., Ma, Y., Simpson, J.P., Siegel, P.D., Mazurek, G.N., Weissman, D.N., 2006. Discrimination of intact mycobacteria at the strain level: a combined MALDI-ToF MS and biostatistical analysis. Proteomics 6, 6416–6425.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., 1994. Bergey's Manual of Determinative Bacteriology, ninth ed. Williams and Wilkins, Baltimore, London.

Honisch, C., Mosko, M., Arnold, C., Gharbia, S.E., Diel, R., Niemann, S., 2010. Replacing reverse line blot hybridization spoligotyping of the *Mycobacterium tuberculosis* complex. J Clin Microbiol 48, 1520–1526.

Ikryannikova, L.N., Afanas'ev, M.V., Akopian, T.A., Il'ina, E.N., Kuz'min, A.V., Larionova, E.E., Smirnova, T.G., Chernousova, L.N., Govorun, V.M., 2007. Mass-spectrometry based minisequencing method for the rapid detection of drug resistance in *Mycobacterium tuberculosis*. J Microbiol Methods 70, 395–405.

Ilina, E.N., Borovskaya, A.D., Malakhova, M.M., Vereshchagin, V.A., Kubanova, A.A., Kruglov, A.N., Svistunova, T.S., Gazarian, A.O., Maier, T., Kostrzewa, M., Govorun, V.M., 2009. Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic *Neisseria*. J Mol Diagn 11, 75–86.

Ilina, E.N., Borovskaya, A.D., Serebryakova, M.V., Chelysheva, V.V., Momynaliev, K.T., Maier, T., Kostrzewa, M., Govorun, V.M., 2010. Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the study of *Helicobacter pylori*. Rapid Commun Mass Spectrom 24, 328–334.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35, 907–914.

Kremer, K., van Soolingen, D., Frothingham, R., Haas, W.H., Hermans, P.W., Martin, C., Palittapongarnpim, P., Plikaytis, B.B., Riley, L.W., Yakrus, M.A., Musser, J.M., van Embden, J.D., 1999. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol 37, 2607–2618.

Leuschner, R.G., Beresford-Jones, N., Robinson, C., 2004. Difference and consensus of whole cell *Salmonella enterica* subsp. *Enterica serovars* matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectra. Lett Appl Microbiol 38, 24–31.

Lotz, A., Ferroni, A., Beretti, J.L., Dauphin, B., Carbonnelle, E., Guet-Revillet, H., Veziris, N., Heym, B., Jarlier, V., Gaillard, J.L., Pierre-Audigier, C., Frapy, E., Berche, P., Nassif, X., Bille, E., 2010. Rapid identification of mycobacterial whole cells in solid and liquid culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 48, 4481–4486.

Lynn, E.C., Chung, M.C., Tsai, W.C., Han, C.C., 1999. Identification of *Enterobacteriaceae* bacteria by direct matrix-assisted laser desorptiom/ionization mass spectrometric analysis of whole cells. Rapid Commun Mass Spectrom 13, 2022–2027.

Mandrell, R.E., Harden, L.A., Bates, A., Miller, W.G., Haddon, W.F., Fagerquist, C.K., 2005. Speciation of *Campylobacter coli*, *C jejuni*, *C. helveticus*, *C. lari*, *C. sputorum*, and *C. upsaliensis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 71, 6292–6307.

Mellmann, A., Bimet, F., Bizet, C., Borovskaya, A.D., Drake, R.R., Eigner, U., Fahr, A.M., He, Y., Ilina, E.N., Kostrzewa, M., Maier, T., Mancinelli, L., Moussaoui, W., Prevost, G., Putignani, L., Seachord, C.L., Tang, Y.W., Harmsen, D., 2009. High interlaboratory reproducibility of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria. J Clin Microbiol 47, 3732–3734.

Mellmann, A., Cloud, J., Maier, T., Keckevoet, U., Ramminger, I., Iwen, P., Dunn, J., Hall, G., Wilson, D., Lasala, P., Kostrzewa, M., Harmsen, D., 2008. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J Clin Microbiol 46, 1946–1954.

Pignone, M., Greth, K.M., Cooper, J., Emerson, D., Tang, J., 2006. Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. J Clin Microbiol 44, 1963–1970.

Roth, A., Fischer, M., Hamid, M.E., Michalke, S., Ludwig, W., Mauch, H., 1998. Differentiation of phylogenetically related slowly growing mycobacteria based on 16S–23S rRNA gene internal transcribed spacer sequences. J Clin Microbiol 36, 139–147.

Russo, C., Tortoli, E., Menichella, D., 2006. Evaluation of the new GenoType *Mycobacterium* assay for identification of mycobacterial species. J Clin Microbiol 44, 334–339.

Saleeb, P.G., Drake, S.K., Murray, P.R., Zelazny, A.M., 2011. Identification of mycobacteria from solid culture media by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Clin Microbiol 2011, 16.

Sauer, S., 2006. Typing of single nucleotide polymorphisms by MALDI mass spectrometry: principles and diagnostic applications. Clin Chim Acta 363, 95–105.

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.E., Rolain, J.M., Raoult, D., 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49, 543–551.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C., van Soolingen, D., 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44, 4498–4510.