

# Comparison of MALDI-TOF MS, nucleic acid hybridization and the MPT64 immunochromatographic test for the identification of *M. tuberculosis* and non-tuberculosis Mycobacterium species

Asuman Şamli, Arzu İlki

Marmara University, Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkey

## SUMMARY

Mycobacteria are an important cause of morbidity in humans. Rapid and accurate mycobacterial identification is important for improving patient outcomes. However, identification of Mycobacterium species is not easy, due to the slow and fastidious growth of mycobacteria. Recently, biochemical, sequencing, and probing methods have come to be used for identification. This study compared the performance of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of *M. tuberculosis* and non-tuberculosis Mycobacteria (NTM) to those of nucleic acid hybridization (NAH) and the MPT64 immunochromatographic test.

A total of 69 isolates from Marmara University Hospital, Microbiology Laboratory obtained between 2012 and 2013 were included in our study. All strains were grown on Lowenstein-Jensen and Middlebrook 7H9 medium. Among the 69 isolates, 56 (81%) were isolated as *Mycobacterium tuberculosis complex* (MTC), and 13 (19%) were isolated as NTM by the MPT64 ICT. NAH was able to identify all isolates to the species level. The isolated NTM included *M. intracellulare* (n:5), *M. lentiflavum* (n:3), *M. xenopi* (n:2), *M. malmoense* (n:1), *M. abscessus* (n:1), and *M. avium* (n:1). MALDI-TOF MS identified 88% of the mycobacterial isolates. All *M. tuberculosis* strains were identified correctly, but the ratio was 38.5% for NTM. Mycobacterial identification using MALDI-TOF MS takes 45 minutes and costs 3 Euro/test, whereas mycobacterial identification using NAH takes 6-7 hours and costs 30 Euro/test.

In conclusion, MALDI-TOF MS has the potential to identify mycobacteria in the clinical laboratory setting by reducing identification turnaround time and laboratory costs for isolate referral.

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## INTRODUCTION

Tuberculosis (TB) is still a serious global public health concern. In 2014, an estimated 9.6 million people developed new TB, and 1.5 million people died from the disease (WHO, 2015).

Human tuberculosis is mainly caused by *M. tuberculosis* and less commonly by other species in the *M. tuberculosis* complex. Mycobacterial species other than *M. tuberculosis* complex (MTC) species are classified as non-tuberculosis mycobacteria (NTM). These bacteria are environmental microorganisms. In recent years, the incidence of diseases caused by NTM has been on the rise due to the increasing number of immunocompromised patients (McGrath and Anderson, 2007). Rapid and accurate diagnosis of mycobacterial infections is essential because the appropriate treatment for these infections differs according to the iso-

lated species (Griffith *et al.*, 2007). In addition, heightened awareness of these organisms as pathogens leads to increased testing, improved diagnostic methods for identification and sampling.

For the laboratory diagnosis of TB, smear microscopy is usually confirmed by culture which is the gold standard, however it requires 45 days of incubation time. Automated and semi-automated liquid culture systems have reduced the culture time and increased sensitivity. However, conventional methods used for the identification of TB still have low sensitivity and are time-consuming. Therefore, in recent years, molecular techniques have become important for the identification of mycobacteria. Nucleic acid amplification tests (NAA) are used for the identification of NTM species in epidemiologic studies. NAA are helpful for mycobacteria species identification with a specificity of 97% and a sensitivity of 85% and an identification time of 6-8 hours (Parsons and Somoskövi, 2011). NAA target 23S rRNA genes (Franco-Alvarez de Luna *et al.*, 2006, Kiraz *et al.*, 2010).

The TBc identification test (MGIT TBc identification test (TBc ID; Becton Dickinson, Sparks, MD, USA) is used in many laboratories and can differentiate MTC species from NTM species but cannot specifically identify dif-

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### Corresponding author:

Arzu İlki

E-mail: ailki@marmara.edu.tr, ailki@superonline.com

ferent NTM species (Parsons, 2011). The TBc ID test is a lateral-flow immunochromatographic assay that detects MPB64 in liquid culture using a MPT64-specific monoclonal antibody. MPB64 is a mycobacterial protein secreted by *M. tuberculosis* and certain strains of *M. bovis* (Yamaguchi *et al.*, 1989; Nagai *et al.*, 1991; Abe *et al.*, 1999).

In recent years, several reports have shown the feasibility of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for the identification of microorganisms in routine clinical microbiology laboratories (Sauer and Kliem, 2010). The detection of protein mass patterns has become a convenient tool for the rapid analysis of bacteria (Chen *et al.*, 2013). MALDI-TOF MS is a proteomic method that analyzes the profiles of proteins that are extracted from whole bacteria. A MALDI mass spectrometer can efficiently detect numerous molecules simultaneously. Protein mass patterns can be used for identification of bacteria at the genus and species levels. Qualified spectra for the identification of various bacteria have been determined, but the methods for identifying mycobacteria species are new (Lonmann *et al.*, 2013; Martiny *et al.*, 2012).

This study evaluated the performance of MALDI TOF MS (Vitek MS; BioMerieux, France) for the identification of mycobacteria from MGIT (BD) liquid cultures and Löwenstein Jensen (LJ) solid cultures. We compared the identification results of MALDI TOF MS with those of the MPT64 immunochromatographic test and NAA.

## MATERIALS AND METHODS

We studied samples obtained between January 2012 and December 2013 at the Mycobacteriology Laboratory at Marmara University Hospital, Istanbul, Turkey.

### Isolates

A total of 69 clinical isolates, 56 identified as MTC and 13 identified as NTM, were included in the study. The isolates were subcultured in both Löwenstein-Jensen medium (LJ) as solid media and mycobacterium growth indicator tubes (MGIT, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) as liquid media. Samples were Gram-stained and Ziehl-Neelsen (EZN)-stained and were evaluated for acid-fast bacilli and contamination. Mixed samples were decontaminated using the N-acetyl cysteine and NaOH (NALC-NAOH) method. All samples were identified by MPT64 immunochromatographic test, NAA and MALDI TOF MS.

### Identification Methods

#### TBc identification test (BD MGIT TBc identification test, TBc ID, Becton Dickinson, Sparks, MD)

TBc ID is an immunochromatographic assay for the detection of MPT64 Ag secreted from *M. tuberculosis* complex isolates. MPT64 is a mycobacterial protein that is only secreted by MTBC and has been shown to differentiate MTBC from NTM.

The presence of mycobacteria indicated by positive signaling in MGIT tubes was confirmed by EZN staining. One hundred microliters of liquid media from the positive MGIT tubes was added to the TBc ID card. All inoculated cards were incubated for 15 min at room temperature. The results were visually assessed. The test results were either positive or negative. A pink test line indicated a positive test result, and there was a visible control line.

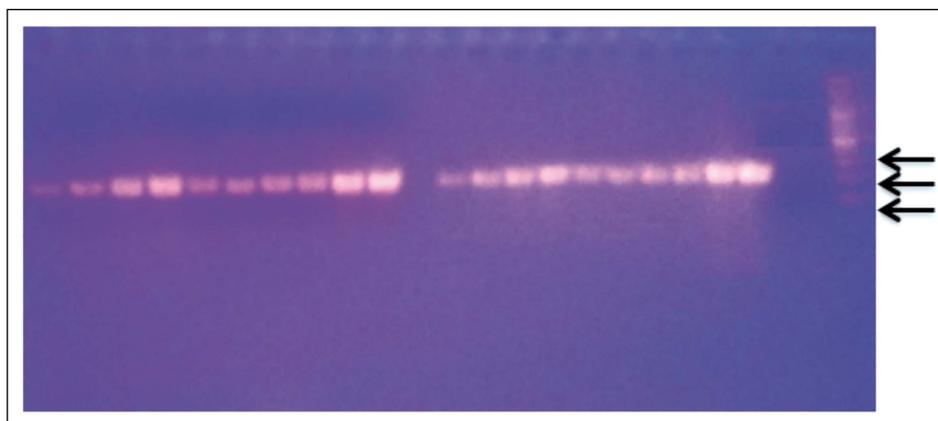
#### Nucleic Acid Hybridization Test

a) DNA Isolation: For the positive liquid samples, 100 µL was vortexed and centrifuged (X10000 g). The supernatant was discarded, and the pellet was resuspended in lysis buffer and boiled at 100°C for 5 min. Then, 100 µL neutralizing buffer was added and the sample was centrifuged for 5 min. The supernatant was collected into a new sterile Eppendorf tube.

For the LJ positive samples, 1-2 colonies were suspended in lysis buffer, and the rest of the nucleic acid hybridization test procedure was similar to that used for the liquid samples (Genotype, Hain Lifescience, Germany).

b) Amplification: 35 µL PNM (primer nucleotide mix), 5 µL 10x PCR buffer (15 mM MgCl<sub>2</sub>), 2 µL MgCl<sub>2</sub> (25 mM MgCl<sub>2</sub>), 3 µL H<sub>2</sub>O, 0.2 µL Taq polymerase (1 unit), and 5 µL of DNA solution were combined to generate a 50 µL reaction volume for amplification. The following thermal cycler settings were used: 95°C for 5 min; followed by 10 cycles of 95°C for 30 s and 58°C for 2 min; 20 cycles of 95°C for 25 s, 53°C for 40 s, and 70°C for 40 s; and a final step of 70°C for 8 min. The amplification products were inoculated into 2% agarose gels with 1.2% ethidium bromide. The amplification products were evaluated under UV light (Fig. 1).

c) Hybridization: HYB (hybridization buffer) was added to the wells, and the DNA strips were placed in the wells. The plates were mixed for 30 min at 45°C in a TwinCubator. The HYB was aspirated, and all wells were washed with washing buffer. Then, substrate solution was added to the wells and subsequently aspirated. The DNA strips were transferred to drying paper. The results of the nucleic acid



**Figure 1** - PCR products of Mycobacteria isolates.

**Table 1** - Comparison of the three methods for the Identification of *M. tuberculosis* and NTM.

Methods	<i>M. tuberculosis</i> n (%)	Nontuberculous mycobacteria n (%)	Total n (%)
MPT 64 Immunochromatographic Test	56 (81)	13 (19)	69 (100)
NAH	56 (81)	13 (19)	69 (100)
MALDI TOF	56 (81)	5 (7)	61 (88)

hybridization test were evaluated according to the identification card (Genotype, Hain Lifescience, Germany).

### MALDI TOF MS

Isolation from liquid media: a 1.8 mL aliquot of the culture liquid in each positive MGIT bottle (Middlebrook 7H9 broth) was transferred to a 2 mL microtube and centrifuged for 10 min at 8000 rpm. The supernatant was discarded, and the pellet was resuspended in 500  $\mu$ L of 70% ethanol; then, 0.5 mm glass beads were added to the solution and it was vortexed for 15 min. The solution was incubated at room temperature for 10 min. The supernatant was added to a new tube and was centrifuged for 2 min at 14000 rpm. The supernatant was discarded, and the pellet was resuspended in 10  $\mu$ L of 70% formic acid. Then, 10  $\mu$ L of 100% acetonitrile was added to the suspension, and it was centrifuged for 2 min at 14000 rpm. Then, 1  $\mu$ L of the supernatant was spotted on a MS target slide and allowed to dry. Immediately after drying, the spot was overlaid with 1  $\mu$ L CHCA ( $\alpha$ -cyano-4-hydroxycinnamic acid) matrix solution and allowed to air dry. The slide was then processed using the Vitek MS instrument. The obtained mass spectra were analyzed using the automatic database within MYLA software (Biomerieux, France). If no spectra were obtained or the mycobacterial species was not identified, a second target spot was analyzed.

Isolation from solid media: A 1  $\mu$ L loop of L-J colonies was suspended in 500  $\mu$ L of 70% ethanol, and the suspension was placed in a 2 ml Eppendorf tube with glass beads and vortexed; the remainder of the procedure was similar to that used for the liquid cultures.

### Statistical Methods

The MALDI TOF MS and nucleic acid hybridization (NAH) results were evaluated according to species identification using the Chi square method. The sensitivity of the MALDI TOF MS method was evaluated based on the results of the NAH and Tbc identification tests.

**Table 2** - Evaluation of MALDI TOF MS and NAH Methods for the identification of NTM species.

Isolates	NAH n (%)	MALDI TOF MS n (%)
<i>M. intracellulare</i>	5 (38.5)	5 (38.5)
<i>M. lentiflavum</i>	3 (23)	-
<i>M. xenopi</i>	2 (15.5)	-
<i>M. avium</i>	1 (7.6)	-
<i>M. abscessus</i>	1 (7.6)	-
<i>M. malmoense</i>	1 (7.6)	-
Total	13 (100)	5 (38)

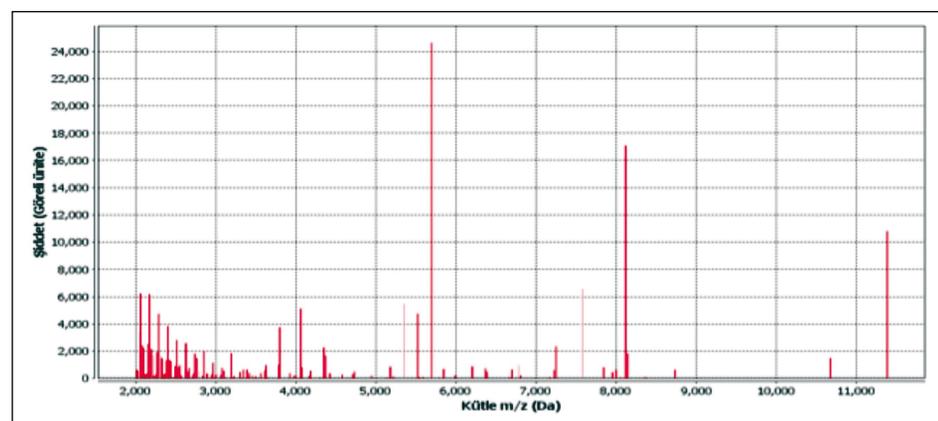
## RESULTS

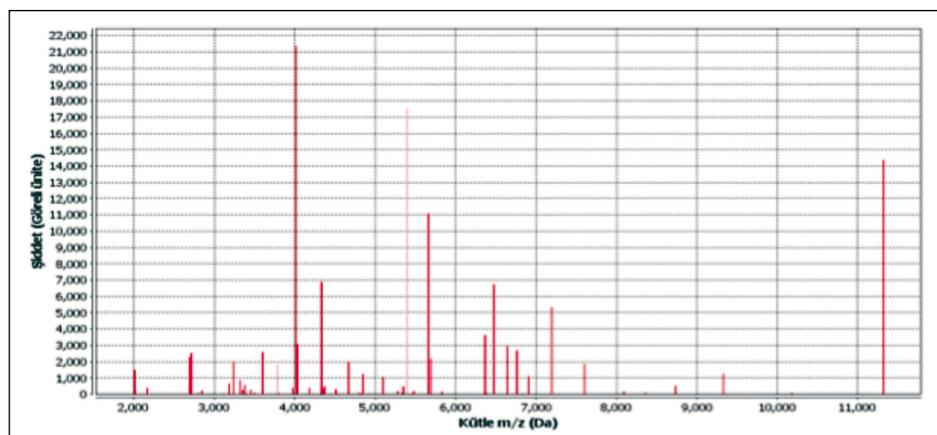
Of the 69 isolates included in this study, 56 (81%) were identified as *M. tuberculosis* by all three methods (Table 1). The Tbc identification test identified the remaining 13 (19%) isolates as NTM species. In contrast, NAH was able to identify all isolates to the species level. The following NTM species were isolated: *M. intracellulare* (n:5), *M. lentiflavum* (n:3), *M. xenopi* (n:2), *M. malmoense* (n:1), *M. abscessus* (n:1), and *M. avium* (n:1) (Table 2).

MALDI TOF MS (Vitek MS, bioMérieux, France) was able to identify all *M. tuberculosis* and *M. intracellulare* isolates. The protein profiles of the two species isolated are presented in Figures 1 and 2.

For those two Mycobacteria species, the results of MALDI TOF MS and NAH were concordant. MALDI TOF MS did not identify any isolates as *M. lentiflavum*, *M. xenopi*, *M. malmoense*, *M. abscessus*, or *M. avium* (Table 2). The total identification rate of MALDI TOF MS was 88%.

MTC and *M. intracellulare* isolates from LJ media were correctly identified by MALDI TOF MS. However, the percentage of isolates in liquid media identified by MALDI

**Figure 2** - Protein Profiles of MTC by MALDI TOF MS.



**Figure 3** - Protein Profiles of *M.intracellulare* by MALDI TOF MS.

TOF MS was 85.2% (n:52). Of the isolates studied here, 49 (87,5%) and 3 (23,5%) were identified by MALDI TOF MS as MTC and NTM (*M. intracellulare*) species, respectively. Nine strains could not be identified by MALDI TOF MS. Of those, 7 were identified as MTC and 2 were identified as NTM species by NAH and the TBc identification test. The MALDI TOF MS and NAH results were concordant for 61 (56 MTC and 5 NTM isolates; 88%) of the 69 positive cultures. Identification of *M. tuberculosis* by MALDI TOF MS (Vitek MS) is 100% concordant with NAH and the TBc identification test. When MALDI TOF MS (Vitek MS) results were analyzed according to the NAH results using chi-square test no significant difference was obtained ( $p < 0.05$ ).

## DISCUSSION

Tuberculosis remains a major public health problem worldwide, and immediate diagnosis and appropriate treatment are important. However, conventional methods used for the identification of tuberculosis infection have low sensitivity and are time-consuming, and rapid, easy and cheap methods for the identification of tuberculosis infection are needed.

The TBc identification test is a rapid, easy and cheap immunochromatographic assay. It can differentiate MTC from NTM isolates using an MPT64-specific monoclonal antibody, but it cannot differentiate between different NTM species (Martiny *et al.*, 2012). MPT64 is a major culture filtrate protein (24 kDa) encoded by the RD2 region genes and has been shown to be a specific antigen that differentiates the *M. tuberculosis* complex from non-tuberculosis mycobacteria species (Shenoy and Mukhopadhyay, 2014).

While *M. tuberculosis* is the main pathogen of tuberculosis, infections by NTM are also problematic because they affect immunocompromised patients and patients with underlying diseases.

Molecular methods for the identification of mycobacteria species have improved. Nucleic acid amplification tests (NAAT) are new tests used for the identification of NTM species. Commercial hybridization methods are available, but they are generally expensive and require thorough personnel training.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was developed in recent years and is a rapid method for the identification of microorganisms. MALDI-TOF MS seems to rapidly and

effectively identify different species of mycobacteria from positive cultures (Bader, 2013). Compared to DNA hybridization-based tests, MALDI-TOF MS is more cost effective, provides faster identification of mycobacterial isolates to the species level, and often results in patients being started on more appropriate therapies earlier than with the previously used identification methods.

Most of the microbiology laboratories use simple immunochromatographic tests based on the presence of MPT-64 antigen in *M. tuberculosis*, but not in NTM. Our study is the first to compare MALDI TOF MS with MPT 64 Ag-TB Identification test and NAAT.

In this study, our objective was to evaluate the performance of MALDI TOF MS (Vitek MS) in a routine clinical mycobacteriology laboratory. We analyzed 69 mycobacterial isolates from positive cultures obtained from consecutive clinical samples. Overall, 88% of the isolates were identified by both NAA and MALDI-TOF. Previous studies showed a correct identification rate between 77% and 88%. This can be explained by different extraction protocols and culture media (Hettick *et al.*, 2004, Pignone *et al.*, 2006, Lotz *et al.*, 2010, El Khéchine *et al.*, 2011, Machen *et al.*, 2013). In our study, the identification of mycobacteria from solid media was 100% and that from liquid medium was 85%. Similarly, Lotz *et al.* (2010) correctly identified 97% of solid culture isolates and 77% of liquid culture isolates.

All these tests; immunochromatographic, MALDI TOF and NAAT can be applied only after a significant growth has taken place in the medium which can take several days. This is the limitation of the study.

The specificities and sensitivities of the TBc identification method are 97.9% and 98.6%, respectively, but this method is limited in distinguishing *M. tuberculosis* with NTM (Lohmann *et al.*, 2013).

MALDI TOF MS correctly identified all *M. tuberculosis* isolates, but only 38.5% of NTM isolates. As shown in Table 2, NAH identified the following NTM species: *M. intracellulare* (n:5), *M. lentiflavum* (n:3), *M. xenopi* (n:2), *M. malmoense* (n:1), *M. abscessus* (n:1), and *M. avium* (n:1). Only, *M. intracellulare* NTM isolates were correctly identified by MALDI TOF MS. The NTM identification failures of MALDI TOF MS can be explained by the incompleteness of the database (VITEK MS V2.0 (bioMérieux)). Vitek MS 2.0 contains data for *Mycobacterium africanum*, *M. bovis*, *M. tuberculosis*, *M. avium*, *M. fortuitum*, *M. intracellulare*, *M. kansasii*, *M. malmoense*, *M. scrofulaceum*, and *M. smegmatis*. Although *M. avium* and *M. malmoense* are included in

the data, they were unidentified probably because of the less amount of the inoculum concentration.

None of the NTM isolates were misidentified as MTC isolates or vice versa. These results indicate the potential role of MALDI TOF MS for the accurate initial differentiation of MTC from NTM isolates. This is vital in terms of clinical therapeutic management and infection control.

This study also evaluated the cost effectiveness of the three methods. All species were identified to the level of MTC and NTM by the TBc identification test, which cost 3 € per strain and took 15 min to perform. All isolates were identified to the species level by NAH, but the cost was 30 € per strain, and the time needed was 6-7 hours. MALDI TOF MS provided 88% correct identification. Out of 13 NTM isolates, 5 (38.5%) were identified to the species level. The cost of MALDI TOF MS was 1 € per strain, and it required 45 min to perform.

In conclusion, MALDI TOF MS is a cost effective, rapid, suitable technique for identifying TB from NTM which is important for early initiation of TB therapy, stewardship, infection control/prevention, etc.

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### References

- Abe C., K. Hirano, T. Tomiyama. (1999). Simple and rapid identification of the Mycobacterium tuberculosis complex by immunochromatographic assay using anti-MPB64 monoclonal antibodies. *J Clin Microbiol.* **37**, 3693-3697.
- Bader O. (2013). MALDI-TOF-MS Based Species Identification and Typing Approaches in Medical Mycology. *Proteomics*; 788-799.
- Balada M., Kamboj K., Pancholi P. (2013). Identification of Mycobacteria from Solid and Liquid Media by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in the Clinical Laboratory. *J Clin Microbiol.* **51**, 2875-2879.
- Chen J.H., Yam W.C., Ngan A.H., Fung A.M., Woo W.L., Yan M.K., et al. (2013). Advantages of using matrix-assisted laser desorption ionization-time of flight mass spectrometry as a rapid diagnostic tool for identification of yeasts and mycobacteria in the clinical microbiological laboratory. *J Clin Microbiol.* **51**, 3981-3987.
- El Khéchine A., Couderc C., Flaudrops C. (2011). Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Identification of Mycobacteria in Routine Clinical Practice. *Université de la Méditerranée et Pôle de Maladies Infectieuses.* **6**, 24720.
- Franco-Alvarez de Luna F, Ruiz P, Gultierrez J, Casal M. (2006). Evaluation of the Geno Type Mycobacteria Direct assay for detection of Mycobacterium tuberculosis complex and four atypical mycobacterial species in clinical samples. *J.Clin.Microbiol.* **44**, 3025-3027.
- Griffith D.E., Aksamit T., Brown-Elliott B.A. (2007). An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med.* **175**, 367-416.
- Hettick J., Kashon M., Simpson J. (2004). Proteomic Profiling of Intact Mycobacteria by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Anal. Chem. Cilt.* **76**, 5769-5776.
- Kiraz N., Saglik I., Kiremitçi A., Kasifoglu N., Akgun Y., (2010). Evaluation of the GenoType Mycobacteria Direct assay for direct detection of the Mycobacterium tuberculosis complex obtained from sputum samples, *J. Med Microbiol.* **59**, 930-934.
- Lohmann C., Sabou M., Moussaoui W., et al. (2013). Comparison Between the Biflex III-Biotyper and the Axima-SARAMIS Systems for Yeast Identification by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *J Clin Microbiol.* **6**, 1231-1236.
- Lotz A., Ferroni, Beretti J. (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.
- Machen A., Kobayashi M., Connley R. (2013). Comparison of Heat Inactivation and Cell Desorption Ionization Time of Flight Mass Use of Vitek Matrix-Assisted Laser Mycobacteria from Solid Culture Media by Disruption Protocols for Identification of Spectrometry. *J Clin Microbiol.* **51**, 4226.
- Martiny D., Busson L., Wybo I. (2012). Comparison of the Microflex LT and Vitek MS Systems for Routine Identification of Bacteria by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry *J Clin Microbiol.* 1313-1325.
- Mather C., Ivera F., Butler S. (2013). Comparison of the Bruker Biotyper and Vitek MS Matrix - Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry Systems for Identification of Mycobacteria Using Simplified Protein Extraction Protocols. *J Clin Microbiol.* **52**, 130.
- McGrath E.E., Anderson P.B. (2007). Increased prevalence of non-tuberculous mycobacteria infection. *Lancet.* **370**, 28.
- Nagai S., Wiker H.G., Harboe M., Kinomoto M. (1991). Isolation and partial characterization of major protein antigens in the culture fluid of Mycobacterium tuberculosis. *J Clin Microbiol.* **37**, 3693-3697.
- Parsons M., Somoskövi Á. (2011). Laboratory Diagnosis of Tuberculosis in Resource-Poor Countries. *Clin. Mic. Rev.* **24**, 314-350.
- Paul S., Drake S., Murray P. (2011). Identification of Mycobacteria in Solid-Culture Media by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry *J Clin Microbiol.* 1790-1794.
- Pignone M.I., Kimberly M., Cooper J. (2006). Identification of Mycobacteria by Ionization-Time-of-Flight Mass Matrix-Assisted Laser Desorption Spectrometry. *Clin Microbiol.* **44**, 1963.
- Quinlan P., Phelan E., Doyle M. (2015). Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) for the identification of mycobacteria from MBBact ALERT 3D liquid cultures and Lowenstein-Jensen (LJ) solid cultures *J Clin Pathol.* **68**, 229-235.
- Sauer S., Kliem M. (2010). Mass spectrometry tools for the classification and identification of bacteria. *Nat. Rev. Microbiol.* **8**, 74-82.
- Shenoy V.P., Mukhopadhyay C. (2014). Rapid Immunochromatographic Test for the Identification and Discrimination of Mycobacterium tuberculosis Complex Isolates from Non-tuberculous Mycobacteria. *J Clin Diagn Res.* **8**, 13-15.
- WHO, Global Tuberculosis Report. 2015. [www.who.int/tb/publications/global\\_report](http://www.who.int/tb/publications/global_report).
- Yamaguchi R., Matsuo K., Yamazaki A., Abe C., Nagai S., Terasaka K., Yamada T. (1989). Cloning and characterization of the gene for immunogenic protein MPB64 of Mycobacterium bovis BCG. *Infect Immun.* **57**, 283-288.