Same-day identification and genotypic antibiotic resistance testing of *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria from positive blood culture bottles – a proof of principle assessment

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Summary

Background and Aims: patients suffering from systemic mycobacterial spread are at high risk of unfavorable clinical outcomes. Rapid microbiological diagnosis, however, is complicated due to the very slow cultural growth of mycobacteria. To facilitate the diagnostic workflow, we assessed a workflow allowing mass spectrometry-based identification and molecular resistance testing of mycobacteria directly from blood culture pellets.

Materials and Methods: for this study, 50 blood cultures spiked with n=10 *Mycobacterium tuberculosis* (MTC) isolates and n=40 isolates belonging to 12 other species of non-tuberculous mycobacteria (n=13 *M. abscessus* complex, n=5 *M. avium*, n=3 *M. chimaera*, n=5 *M. fortuitum*, n=5 *M. intracellulare*, n=2 *M. lentiflavum* and n=2 *M. mucogenicum*, n=1 for each one of the species *M. elephantis*, *M. hassiacum*, *M. marseillense*, *M. nebraskense* and *M. parascrofulaceum*) were used next to two clinical cases of a disseminated infection in HIV patients.

Results: the application of the MBT Sepsityper® kit allowed the correct species level identification by applying mass spectrometry as well as correct molecular resistance testing results as compared to the diagnostic reference approach applied with mycobacterial culture material.

Conclusions: in conclusion, the results provide a proof-of-principle of the suitability of the assessed potential workflow in order to achieve a shortened time-to-result for the diagnosis of systemic mycobacterial infections. Future studies in high endemicity settings are desirable to assess the clinical applicability and impact of such a shortened workflow.

Introduction

Reliable detection and resistance testing in the case of mycobacteremia is crucial, but phenotypic diagnostic strategies are limited by the slow growth characteristics of mycobacterial pathogens. Furthermore, the need for adequate diagnostic solutions has increased in the course of recent decades because the prevalence of disseminated mycobacterial infections has risen worldwide [3,12]. Disseminated Tuberculosis (TB) is an important cause of morbidity and mortality [6,16], particularly in Human Immunodeficiency Virus (HIV) patients. However, the

number of disseminated Non-Tuberculous Mycobacterial (NTM) infections has risen as well [1,11].

Those increases can be attributed to a rise in the number of patients living with immunosuppressive conditions because of underlying diseases like HIV infections / Acquired Immunodeficiency Syndrome (AIDS) or cancer, as well as because of iatrogenic interventions like the therapy with immunosuppressive drugs or hematologic and solid organ transplants. All those medical conditions have been associated with a higher risk even for the disseminated spread of NTM in the patients [1]. In addition, a previously unknown mycobacteria-associated emerging infectious threat has recently been reported. *Mycobacterium chimera*-associated bloodstream infections following cardiac surgery have been associated with contaminated heater-cooler units used during the surgical procedure [9].

An accurate and rapid diagnosis of disseminated mycobacteriosis is critical for the appropriate management of patients suffering from this life-threatening infection and could considerably reduce the mortality of both disseminated tuberculosis and disseminated NTM infections. Blood culture in specialized media is still a standard approach for the diagnosis of mycobacteremia [13,19]. Fully automated systems are commercially available (BACTEC FX system; Becton, Dickinson and Company, Sparks, USA), and provide a faster and more reliable method for the detection of mycobacteremia than non-automated approaches [19]. Nevertheless, an incubation time of several weeks is required, and once a bottle is flagged positive, a subculture on solid or liquid media is necessary to obtain a pure mycobacterial subculture suitable for identification and further downstream analysis purposes. Conventional genotypic methods like Line Probe Assays (LiPA) are commercially available and commonly applied for the identification of mycobacteria at the species level as well as for molecular resistance testing [4,18,20]. Unfortunately, however, this diagnostic approach is unfeasible directly from positive blood culture bottles, because they are unreliable if applied with samples containing blood as stated by the manufacturer.

As shown by recent investigations [2,7,8], the MBT Sepsityper® kit (Bruker Daltonics GmbH & Co., Millerica, USA) allows the direct identification of pathogens on the species level from positive blood cultures. The technique is based on a lysis-centrifugation approach [14,17] and proved to be a robust and reliable method for the prompt diagnosis of bacteremia and sepsis [2,7,8,14]. For mycobacteria, however, this has not been validated by the manufacturer, and evaluation data have not yet been internationally published to the authors' best knowledge.

To close this information gap, a new approach enabling identification and subsequent genotypic antimicrobial resistance testing of mycobacteria directly from positive blood cultures applying the MBT Sepsityper® kit and thus saving the time required for the subculture step was assessed in this study. Thereby, the Sepsityper kit was used for the extraction of mycobacterial biomass directly from the positive blood culture bottles. After this preparation step, the material was used for direct identification and genotypic antimicrobial resistance testing. The aim of the study is to provide preliminary information on the reliability of this potential future diagnostic protocol.

Materials and Methods

Samples

A total of 50 spiked blood culture samples and two clinical cases were included in this study. The MBT Sepsityper® Kit (Bruker Daltonics GmbH & Co.) was applied to retrieve the bacterial biomass from the positive bottles once they turned positive. Bacterial biomass (pellet) was then used for species identification and antibiotic susceptibility testing by LiPA methodology.

Overall, n=10 *Mycobacterium tuberculosis* (MTC) isolates and 40 isolates belonging to 12 other species of NTM (n=13 *M. abscessus* complex, n=5 *M. avium*, n=*3 M. chimaera*, n=1 *M. elephantis,* n=5 *M. fortuitum*, n=1 *M. hassiacum,* n=5 *M. intracellulare*, n=2 *M. lentiflavum,* n=1 *M. marseillense,* n=2 *M. mucogenicum,* n=1 *M. nebraskense,* n=1 *M. parascrofulaceum*) were included in the study (Table 1).

The strains were prospectively collected from routine samples other than blood cultures, identified at the species level by MALDI-TOF MS (MALDI Biotyper; Bruker Daltonics GmbH & Co. KG) and/or LiPA (GenoType Mycobacterium CM and GenoType Mycobacterium AS; Bruker-Hain, Nehren, Germany). The bottles for mycobacterial culture (BACTEC™ Myco/F Lytic; Becton Dickinson and Company) were inoculated each one with 4.9 ml of human blood from volunteer donors and 100 µl of

Table 1. Dataset of mycobacteria clinical isolates (previously sequenced) included in this study, and results of identification by MALDI-TOF MS and Line Probe Assays (LiPA).

Non-Tuberculous Mycobacteria used (n)	MALDI-TOF MS Identification	Species identification and susceptibility testing by LiPA
M. abscessus subsp. abscessus (9)	*M. abscessus group*	*M. abscessus subsp. abscessus* (Genotype NTM-DR)
M. abscessus subsp. bolletii (2)	*M. abscessus group*	*M. abscessus subsp. bolletii* (Genotype NTM-DR)
M. abscessus subsp. massiliense (2)	*M. abscessus group*	*M. abscessus subsp. massiliense* (Genotype NTM-DR)
M. avium (5)	*M. avium*	*M. avium* (Genotype NTM-DR)
M. chimaera (3)	*M. chimaera-intracellulare group*	*M. chimaera* (Genotype NTM-DR)
M. elephantis (1)	*M. elephantis*	*M. spp.* (Genotype CM/AS)
M. fortuitum (5)	*M. fortuitum ssp. fortuitum*	*M. fortuitum* (Genotype CM)
M. hassiacum (1)	*M. hassiacum*	*M. spp.* (Genotype CM/AS)
M. intracellulare (5)	*M. chimaera-intracellulare group*	*M. intracellulare* (Genotype NTM-DR)
M. lentiflavum (2)	*M. lentiflavum*	*M. lentiflavum* (Genotype CM/AS)
M. marseillense (1)	*M. marseillense*	*M. intracellulare* (Genotype CM)
M. mucogenicum (2)	*M. mucogenicum*	*M. mucogenicum* (Genotype CM/AS)
M. nebraskense (1)	*M. nebraskense*	*M. spp.* (Genotype CM/AS)
M. parascrofulaceum (1)	*M. parascrofulaceum*	*M. scrofulaceum/M. parascrofulaceum* (Genotype CM)

mycobacterial suspension. The bacterial inoculum, corresponding to a turbidity of 0.5 McFarland, were prepared starting from colonies grown on Middlebrook 7H10 agar for 3-15 days (depending on species), resuspended in 0.9% NaCl solution. The concentration of the bacterial suspension was chosen after a first step of optimization of the inoculum in relation to the time to positivity of the bottles, carried out using 9 serial dilutions 1:10 (data not shown). The inoculated bottles were incubated in a BACTEC™ FX instrument (Becton Dickinson) for up to 42 days.

In addition, two positive blood culture samples from 48 and 52-year-old women, both affected by HIV disease, with the suspicion of disseminated mycobacterial infection, were also included. Kinyoun staining confirmed the presence of acid-fast bacilli in the positive blood culture bottle thus the samples were subcultured onto solid (Middlebrook 7H10 agar; Becton Dickinson) and liquid (MGIT; Becton Dickinson) media. In addition to this routine standard protocol, the Sepsityper approach was also applied. The bacterial biomass obtained by the Sepsityper workflow was used to perform species identification by phenotypic (MALDI Biotyper Mycobacteria Module) and genotypic (GenoType Mycobacterium CM V2.0, Bruker-Hain) methods, as well as for genotypic susceptibility testing by LiPA using the Genotype NTM-DR V1.0 (Bruker-Hain). Identification results and antimicrobial susceptibility results obtained using the Sepsityper sample preparation procedure were compared with results obtained with the standard procedure (from liquid/solid subculture).

Sample preparation with the Sepsityper method

Once flagged positive, the blood culture bottles underwent sample preparation using the Sepsityper Kit. The manufacturer's procedure for bacteria and yeast was applied, with some modifications to adapt the method to mycobacterial peculiarities.

Briefly, two aliquots of 5 ml of positive blood culture broth were transferred to a 10 ml tube and centrifuged for 10 min at 3000 rpm. For each one of them, after removal of the supernatant, 0.5 ml of the pellet was transferred to a 1.5 ml Eppendorf tube, and 100 µl of Sepsityper lysis buffer was added. After a short vortexing step (10-15 sec) followed by a centrifugation step for 5 min at 13000 rpm, the supernatant was discarded, and 0.5 ml of Sepsityper washing buffer was added. After resuspension of the pellet by vortexing, centrifugation for 2 min at 13000 rpm and complete removal of the supernatant, two aliquots of mycobacterial biomass were obtained, suitable for downstream applications. One aliquot was used to perform species identification by MALDI Biotyper, while the other was used to perform genotypic analysis (Figure 1).

Species identification by MALDI Biotyper

For the ID by MALDI, 500 µl of 70% ethanol solution and a small amount of glass beads were added to the pellet. After vortexing for 15 min, followed by 10 min of incubation (for the inactivation of mycobacteria by ethanol) [10], the bacterial suspension was transferred to another 1.5 ml Eppendorf tube (to separate it from the beads), and it underwent another centrifugation step (2 min, 13000 rpm).

After removal of the supernatant, the pellet was air dried for 5-15 min, and then it underwent ethanol/formic acid extraction [10]. It was resuspended in 10-25 µl (in relation to the biomass amount) of 70% formic acid, and the same amount (10-25 µl) of acetonitrile was added. After vortexing and centrifuging (2 min, 13000 rpm), 1 µl of the supernatant was spotted in duplicate onto a MALDI Biotyper polished steel target plate, and once dried, covered by 1 µl of MBT HCCA matrix (Bruker Daltonics), and analyzed by the MALDI Biotyper system using a MALDI Biotyper equipped with MBT Compass software including the MBT Mycobacteria module and the mycobacteria library V2.0 (Bruker Daltonics).

Species identification and susceptibility testing by Line Probe Assay

For genotypic analysis, the bacterial biomass aliquot obtained with the Sepsityper procedure was washed twice with 100 µl of distilled water and centrifuged for 2 min at 13000rpm. The DNA extraction was performed with the Genolyse kit (Bruker-Hain), following the manufacturer's instructions. All samples underwent species ID by GenoType CM and/or GenoType AS (Bruker-Hain). Genotypic susceptibility testing was performed by GenoType NTM-DR (for *M. abscessus* complex and *M. avium* complex) and by GenoType MTBDR*plus* and MTBDR*sl* (for mycobacterium tuberculosis complex), according to the manufacturer's instructions.

Results

For all samples, it was possible to obtain the species identification using MALDI Biotyper within two hours of flagging posi-

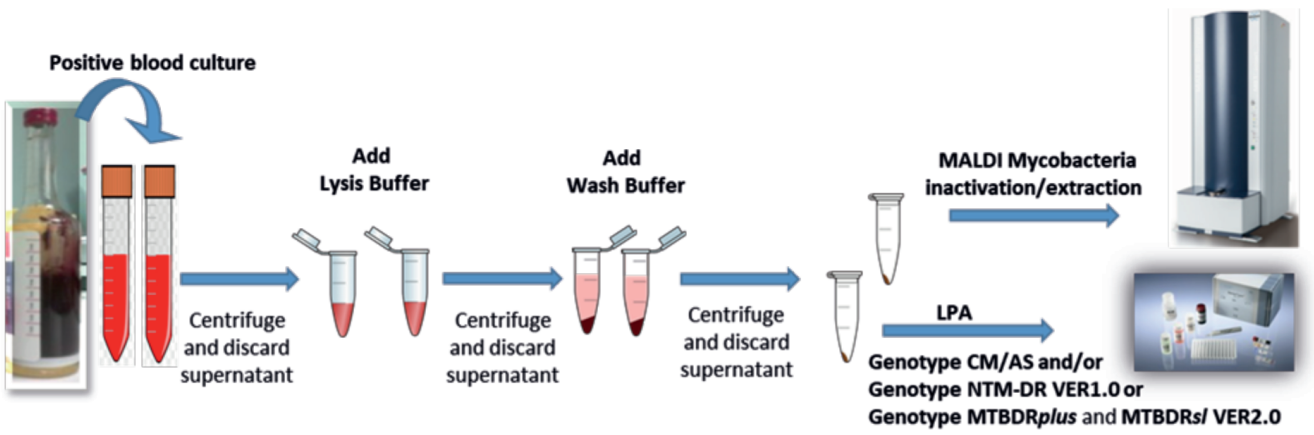

Figure 1. Workflow of the approach used in this study applying the Sepsityper preparation method for the fast species identification and antibiotic susceptibility testing directly from positive blood culture bottles.

Table 2. First- and second-line antimicrobial resistance results of the *M. tuberculosis* complex isolates were performed using the Sepsityper pellet and the MGIT liquid cultures (laboratory standard procedure). No discrepancies were observed.

M. tuberculosis used	GenoType MTBDR*plus* mutations	Genotype MTBDR*sl* mutations	Discrepant result
Drug sensible (6)	No mutations detected	No mutations detected	None
Isoniazid-resistance (2)	katG MUT1 S315T1	No mutations detected	None

tive. Further, and also without exemptions, the Sepsityper preparation method enabled the achievement of a correct identification at the species level with highly reliable log(score) values between 1.8 and 2.4, and concordant in all cases with the result of Genotype CM/AS (Table 1). Of note, *M. fortuitum* complex, *M. abscessus* complex and *M. chimaera*/*M. intracellulare* group mass spectra were not differentiated by the MBT Mycobacteria Module approach. The subtyping functionality of the MBT Mycobacteria module for differentiation of *M. chimaera* and *M. intracellulare* was not available in the Novara laboratory at the time of the study.

The mycobacteria resistance results obtained using the Sepsityper pellet were congruent with those obtained from the solid media cultures of the isolate used for the spiked samples or present in the patient-derived blood culture (Table 2).

In the patient-derived blood culture samples, the MALDI identification (*M. avium* and *M. chimaera*/*M. intracellulare*), as well as the susceptibility profile, delivered by the sample obtained using the Sepsityper method were lately confirmed by testing using the subcultures. The *M. chimaera/intracellulare*_group MALDI identification result was better discriminated as *M. chimaera* with GenoType NTM-DR. Later, this case appeared to be associated with a North Italy cluster of *Mycobacterium chimaera*-associated bloodstream infections following cardiac surgery.

Thus, these cases exemplarily confirmed the potential diagnostic real-life applicability of the proof-of-principle assessment.

Discussion

The study was performed to propose a potential future diagnostic protocol for the rapid identification and molecular resistance testing for patients with disseminated, systemic mycobacteriosis. As such, the disease is associated with poor prognosis, in particular in case of incorrect diagnosis and associated inappropriate therapy [3,12]; respective protocols with the aim of an early optimization of patient therapy are desirable and potentially life-saving in the diagnostic routine.

The diagnostic strategy proposed in this research study would allow a combined differentiation and molecular resistance testing of mycobacteria grown in blood culture in case of systemic dissemination directly from the positively tested blood culture material. By the application of the MBT Sepsityper® kit, not only the mass-spectrum-based identification of mycobacteria was successfully demonstrated, but the so prepared sample material also became suitable for molecular resistance testing of the mycobacteria applying the Bruker-Hain PCR platform. While the MBT Sepsityper® kit has already been successfully applied and validated for the diagnostic identification of other bacterial and fungal pathogens [2,7,8,14], its suitability for the differentiation of mycobacteria has not been validated by the manufacturer or internationally published so far to the authors' best knowledge. In addition, the applicability of the MBT Sepsityper® Kit-prepared sample material from positive mycobacteria-containing blood cultures would be an achievement with the potential for a considerable reduction of time until the first resistance results become available. This may be highly beneficial for the therapeutic management, in particular in areas where high resistance rates need to be considered [5].

The need for rapid adequate therapy of disseminated mycobacteriosis is stressed by the fact that this disease particularly affects most vulnerable patients with underlying conditions like HIV infection and immunosuppressive treatments, which have been described to be associated with mycobacteriemia [1,6]. Patients undergoing cardiac surgery are further endangered by *Mycobacterium chimaera*-associated bloodstream infections which may occur associated with contaminated heater cooler units [9].

The clinical case description included in this study indicates that these considerations are more than just hypothetical. Indeed, the application of the Sepsityper® approach was shown to be beneficial for the accelerating of the diagnostics of mycobacterial bloodstream infections (identification and resistance profile achieved in a few hours), resulting in an optimization of the antimicrobial therapy.

The study has a number of limitations. In its present stage, the work just provides a proof-of-principle, and sufficiently powered studies in high-endemicity settings seem desirable to assess both the procedural reliability of the technique and the clinical benefit associated with its application. Secondly, regulation (EU) 2017/746 makes a full validation of the whole diagnostic workflow prior to its diagnostic use necessary, a requirement which also needs to be met by future multicentric studies.

Conclusions

In spite of the limitations mentioned above, the provided proof-of-principle assessment indicated that the proposed diagnostic workflow, including the MBT Sepsityper® kit, represents a promising method both for the identification of mycobacteria to the species level and for providing genotypic antibiotic resistance detection directly from positive blood culture material. So, the time-consuming subculture can be circumvented to provide first preliminary results to facilitate clinical management, thus relevantly shortening the turnaround time. If the provided promising results are confirmed by future studies, the application of the Sepsityper kit for the direct identification of mycobacteria from positive blood cultures might become a substantial help in the microbiological diagnostic workflow in case of suspected disseminated mycobacterial infections, which might drastically reduce the time to preliminary results by 5 to 15 days depending on mycobacteria species.

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