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Original article

Identification of non-tuberculous *Mycobacteria* clinical isolates to species level using four different advanced diagnostic technologies

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ABSTRACT

Background: Infections by non- tuberculous mycobacteria (NTM) are of rising concern. Identification by phenotypic methods is time-consuming and challenging. In our work, we aimed to identify clinical NTM isolates to the species level using four different advanced diagnostic technologies. **Methods:** Clinical specimens underwent testing by Ziehl Neelsen (ZN) microscopy and GeneXpert®MTB/RIF assay. Suspected specimens for NTM were selected for inoculation on Lowenstein-Jensen medium (LJ) to obtain primary colonies. The isolated colonies were identified by matrix-assisted laser time-of-flight mass spectrometry (MALDI-TOF MS), and their DNA was extracted and tested by LCD-Microarray, 16SrRNA sequencing and whole genome sequencing (WGS). **Results:** Out of total 112 specimens, 6 were presumptively identified as NTM (5.36%). Whole genome sequencing identified NTM isolates as 2 *M. fortuitum, 2 M. abscessus, and 2 M. porcinum.* The 16SrRNA sequencing agreed, except for 2 *M. fortuitum isolates* identified as the closely related *M. neworleansense*. MALDI-TOF MS identified *M. fortuitum* (n=3), and one of each of *M. abscessus, M.porcinum, M. bacteremicum*. The Microarray identified *M. abscessus (n=2)*, Broad III group (n=3), however missed the identification of one isolate. An overall agreement of 66% was shown for 16SrRNA sequencing and MALDI-TOF, while 33% for LCD array with WGS in identification of NTM to the species level. **Conclusion:** The WGS remains the highest discriminatory identification method. The NTM isolates identified by the gold standard WGS were *M. fortuitum, M. abscessus, and M. porcinum.* The 16SrRNA sequencing and MALDI-TOF assay had the highest agreement with WGS in identification of NTM to species level.

Introduction

Non-tuberculous mycobacteria (NTM) are mycobacteria that can be isolated from environmental plants, air, soil, dust, and drinking water sources [1,2]. Runyon classified NTM according to growth properties in culture, into rapid and slow growers [3]. Non-tuberculous mycobacteria have caused human pulmonary and extra-pulmonary infections, especially in immunocompromised patients [2]. Some studies suggested an increased prevalence of NTM infections over the last four decades [4].

Laboratory diagnosis of NTM has been a challenge, requiring culture- dependent and

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advanced molecular laboratory diagnostic capacity [5, 6]. Culture and identification by biochemical reactions have been used, but were time-consuming, cumbersome and faced technical difficulties [7]. Old phenotypic methods used for NTM identification had a weak capability of differentiation between NTM with close genetic relatedness [8,9]. These methods have been substituted by molecular assays [8], which recognize a narrow range of NTM species, with unsuccessful differentiation between similar NTM strains [9].m Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) is a robust assay adapted for identification of NTM [10]. However, this assay is challenged by its poor discrimination of NTM strains with genetic similarities [8]. Currently, nucleic acid sequencing is the most reliable technique for accurate genetic description of NTM [11]. For many years, the 16SrRNA sequencing has been a cornerstone of genetic bacterial study. High-throughput entire 16SrRNA gene sequencing has become a feasible possibility, with enough precision to characterize bacteria at the strain and species levels [12].

Whole genome sequencing (WGS) is regarded as a reference method that plays an essential role in studying molecular characteristics and epidemiology of NTM. Whole genome sequencing can investigate outbreaks and events of transmission [11]. Additionally, WGS can uncover genetic determinants of pathogenicity and antimicrobial resistance of NTM [13, 14]. The high cost and the need for expertise remain the key obstacles in applying WGS [8]. Recently, updated protocols were introduced to facilitate the WGS procedures with online programs for analysis of the results [15].

We aimed in this work to identify NTM isolates from clinical samples to the species level using MALDI-TOF MS, LCD-Microarray, and 16SrRNA gene sequencing against the gold standard WGS.

Materials and Methods

The study was carried out from January to December 2022 in the Mycobacteriology and Sequencing Laboratories in Cairo University Hospital, in Egypt. Specimens for patients clinically suspected of mycobacterial infections, were subjected to decontamination and concomitant examination by Ziehl-Neelsen (ZN) stain, and GeneXpert®MTB/RIF. Specimens positive by ZN and negative by GeneXpert®MTB/RIF were presumptively suspected of having NTM [2] and were selected for culture on Lowenstein- Jensen medium (LJ) to obtain primary colonies for further species identification. The isolated colonies were tested by MALDI-TOF for identification to the species rank, and their DNA was extracted and tested by DNA-Microarray, 16SrRNA gene sequencing and WGS, as described briefly below.

Collection and preparation of samples

Clinical specimens were collected in consideration of the required biosafety measures to avoid the risk of contamination. At least three sputum specimens were collected on different occasions. Aseptic technique was followed for sampling of extra-pulmonary specimens [8]. Non-sterile specimens were decontaminated by shaking with NALC/ NaOH solution (1% N-acetyl-L-cysteine, 4% NaOH, and 2.9% sodium citrate), in screw- capped centrifuge tubes, then centrifuged at 15,000xg for 5 minutes for concentration. After aseptically decanting the supernatant, sterile phosphate buffer (1.5 ml) was added to resuspend the sediment. The sediment was used for microscopic examination of direct smears and culture on LJ medium slants [16].

ZN-stain microscopy and GeneXpert®MTB/RIF assay

Microscopic examination for acid-fast bacilli was done by preparing ZN smears. [17]. All samples were subjected to testing by GeneXpertMTB/RIF assay (Cepheid, Sunnyvale, USA) for automated concomitant rapid detection of *Mycobacterium tuberculosis* (MTB) and rifampicin resistance. [18]. As GeneXpert®MTB/RIF identified only MTB, ZN +ve/ Xpert -ve specimens were presumptively suspected of the presence of NTM, and were cultured on LJ medium [2, 19].

Culture of presumptive NTM specimens

We cultured the specimens with presumptive NTM (ZN+ve/Xpert-ve) on LJ slants that were manually prepared to obtain primary colonies for further identification . LJ slants were inoculated with a volume of 0.2 ml of the prepared specimens, incubated at 37^{o}C and were inspected weekly over a period of 8 weeks for mycobacterial growth [20, 21]

Phenotypic identification by MALDI-TOF assay

The recovered NTM isolates were identified by means of MALDI-TOF MS- VITEK®

MS (Biomerieux, France), following the protocol of the manufacturer. The MALDI-TOF procedure includes steps in the form of inactivation, extraction, and analysis. An aliquot is added onto a steel plate and coated with a chemical matrix after extraction of proteins. The plate is placed into the device that uses laser to ionize the proteins, and the ions are dissociated depending on the ratio of the mass/charge ratio [22]. The resulting protein signatures were analyzed as per Mycobacteria Library Software v3.0 (VITEK® MS, Biomerieux) [23].

Genomic DNA extraction for molecular assays

Fresh colonies of NTM were used to extract genomic DNA using QIAamp® DNA Mini kit [24]. Briefly, a loopful of mycobacterial culture was added to an equal amount of TE buffer and heated to 80oC for 20 minutes. Then 100 mg/ml lysozyme (10 µl) was added with overnight shaking incubation at 37ºC. All subsequent steps were done following manufacturer's instructions, and the DNA quality was checked using fluorometer (Denovix).

LCD- microarray

We used the DNA hybridization kit LCD Array MycoDirect 1.7 kit (CHIPRON GmbH, Berlin, Germany), which is predesigned for molecular detection and differentiation of MTB and NTM. It encompasses two combined PCR reactions in one array, before hybridization [25]. All procedures were carried out in accordance with the manufacturer guidelines. The Chip Scanner PF 2700 was used to scan the LCD chip and the Slide Reader V7.00.01 (CHIPRON GmbH, Berlin, Germany) was used to interpret the data.

16SrRNA sequencing

The library of 16S genomic sequencing was made following the working protocol of Illumina (Illumina, San Diego, CA, USA) [26]. The MiSeq Reagent Kit v3 (600-cycle format; Illumina) was used to perform paired-end 300 bp sequencing. Sequences were automatically analyzed by the Miseq reporter once the sequencing run has finished. Metagenomics can characterize microorganisms at different taxonomic ranks, through the amplification of certain regions in the 16S rRNA [27].

Whole genome sequencing of NTM isolates

The extracted genomic DNA was used for whole genome bacterial sequencing using Illumina MiSeq Next Generation Sequencing. Following manufacturer guidelines, a total of 1ng of bacterial DNA was utilized for library preparation with use of Nextera XT DNA Library Preparation Kit (FC-131-1096, Illumina, San Diego, CA, USA). The 600 v3 MiSeq reagent kit (Illumina, San Diego, CA, USA) was used for sequencing, producing 301 base pair paired-end reads on average [28]. Genome sequences were uploaded to the NCBI database under Bioproject ID PRJNA1095647. For bioinformatics analysis, fastQ files were submitted to Mykrobe analysis tool for NTM identification [29].

Ethical approval

The Research Ethics Committee at Cairo University's Faculty of medicine, Egypt granted approval for the study (N-53-2019).

Statistical analysis

The statistical program statistical package for Social Sciences - SPSS v.21 (SPSS Ins. Chicago, IL, USA) was used to do statistical analysis. The kappa test was used to analyze the agreement between several tests

Results

Demographic and clinical data

Out of 112 clinical specimens, we identified 85 (75.89%) as *M. tuberculosis*. Six specimens (5.36%) were ZN- positive and GeneXpert-negative, and all grew NTM in culture. The demographic and clinical data of the NTM culture +ve patients (n=6) are illustrated in **table (1)**. Regarding patients' comorbidities, one had uncontrolled diabetes mellitus, one with cystic fibrosis disease (16.6%), and one had systemic lupus (16.6%), but no one was HIV positive. Three were heavy smokers (50%). Non-tuberculous mycobacteria were isolated from one skin biopsy of a non- healing lacerated traumatic wound, one sputum sample of patient with cystic fibrosis, from 3 pus specimens from non- healing surgical site infection and one post- mesotherapy injection.

Identification of NTM by advanced diagnostic assays

As shown in **table (2)**, WGS identified the NTM as 2 *M. fortuitum*, 2 *M. abscessus*, and 2 *M. porcinum* (supplementary files 1 and 2). The 16SrRNA gene sequencing agreed, except for 2 *M. fortuitum* isolates identified as the closely related M. neworleansense (supplementary file 3). MALDI-TOF MS identified 3 NTM as *M. fortuitum*, and identified each of *M. abscessus*, *M. porcinum*, *M. bacteremicum* once **(Figure 1)**. The Microarray identified 2 NTM as *M. abcessus*, and 4 NTM as Broad III group complex that includes *M. fortuitum*

and *M. porcinum* **(Figure 2)**. It is notable that *M. neworleansense* and *M. porcinum* are included in the *M. fortuitum* complex, while *M. bacteremicum* belongs to *M. neoaurum*-like group, which are among the pigmented, rapidly growing mycobacterial isolates. The results of mycobacteriology work up in our study are summarized as demonstrated in **figure (3)**.

As regard NTM identification to the species rank, in comparison to WGS, the16S gene sequencing identified all the isolates to species level except for 2 isolates to a closely related species, with an overall categorical agreement (66%, kappa value: 0.66). MALDI-TOF-MS identified 4 isolates to species level, one isolate to the complex level and misidentified one *M. abscessus* isolate as *M. bacteremicum*. The LCD-Microarray identified 4 NTM to complex level (broad III group) and 2 NTM to species level (*M. abscessus*). The overall categorical agreement for MALDI-TOF and LCD-microarray with WGS was accounted as 66% (kappa value: 0.66) and 33% (kappa value: 0.33), respectively in identification of NTM to the species level **(Figure 2)**. As per each of the WGS- identified NTM species, MALDI-TOF showed substantial agreement (Kappa value: 0.66), while LCD array and 16SrRNA sequencing showed no agreement in identification of *M. fortuitum*. 16SrRNA sequencing and MALDI-TOF showed perfect (kappa: 1), and moderate agreement (kappa value: 0.57), respectively with WGS in identification of *M. porcinum*, unlike LCD array that showed absent agreement. For *M. abscessus*, each of 16SrRNA sequencing and LCD array perfectly agreed (Kappa value: 1), while MALDI_TOF moderately agreed with WGS (Kappa value: 0.57) **(Table 3)**

Table 1. The demographic and clinical criteria of the patients infected with NTM

Cases	Age	sex	Diagnosis	Sample type	Department
1	34	male	Post-traumatic lacerating wound	skin biopsy	inpatient
2	19	female	Cystic fibrosis	sputum	inpatient
3	31	male	Post- mesotherapy injection	pus	outpatient
4	30	male	Post- abdominal surgery	pus	outpatient
5	42	female	Post-abdominal surgery	pus	outpatient
6	39	female	post laparotomy surgical site infection	pus	outpatient

Table 2. NTM results with interpretative level of identification by MALDI-TOF, LCD-microarray and 16SrRNA gene sequencing compared to the WGS.

Isolates	WGS- species	MALDI-TOF		LCD-microarray		16SrRNA sequencing	
		NTM	ID level	NTM	ID level	NTM	ID level
1	*M. fortuitum*	*M. fortuitum*	species	Broad III[b]	complex	*M. neworleansense**	complex
2	*M. fortuitum*	*M. fortuitum*	species	Broad III[b]	complex	*M. neworleansense**	complex
3	*M. abscessus*	*M. abscessus*	species	*M. abscessus*	species	*M. abscessus*	species
4	*M. porcinum**	*M. porcinum**	species	Broad III[b]	complex	*M. porcinum*	species
5	*M. porcinum**	*M. fortuitum*	complex	Broad III[b]	complex	*M. porcinum*	species
6	*M. abscessus*	*M .bacteremicum*[a]	(-)	*M. abscessus*	species	*M. abscessus*	species

ID: identification rank either to complex or species level
(*) *M. neworleansense* and *M. porcinum* are included in the *M. fortuitum* complex.
(a) *M. bacteremicum* belongs to *M. neoaurum*-like group, which are among the pigmented, rapidly growing mycobacterial isolates.
(b) Broad III group comprises different NTM species including *M. fortuitum* and *M. porcinum*, according to the manufacturer
(-): discrepant identification,

Table 3. Species-specific analytical agreement of different assays with WGS in identification of NTM .

	Agreement with WGS (*kappa value*) in identification of NTM			
	Confidently identified NTM by WGS	**MALDI-TOF**	**LCD-array**	**16SrRNA sequencing**
WGS	*M. fortuitum*	0.66	0	0
	M. abcessus	0.57	1	1
	M. porcinum	0.57	0	1

Kappa values: ≤0 (no agreement); 0.01- 0.2 (slight); 0.21- 0.4 (fair); 0.41- 0.6 (moderate); 0.61- 0.8 (substantial); 0.81- 1 (perfect agreement)

Figure 1. Identified *M. porcinum* and *M. fortuitum* NTM isolates by MALDI-TOF assay displaying species-specific protein spikes.

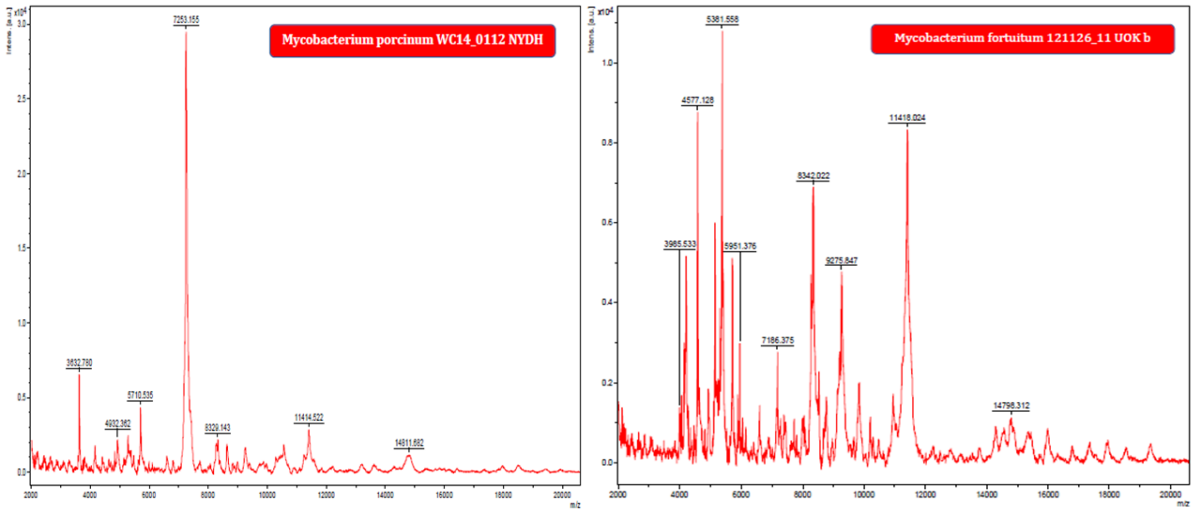


Figure 2. LCD chip array showing identification of NTM as *M. abscessus*, b) Broad group III (Complex group of NTM includes *M. fortuitum* and *M. porcinum*)

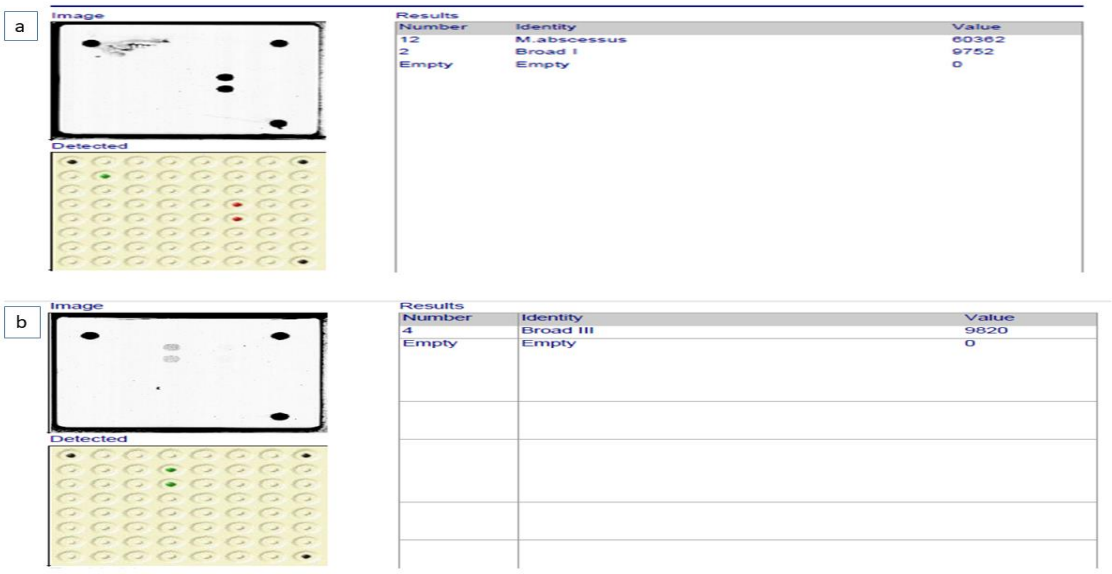
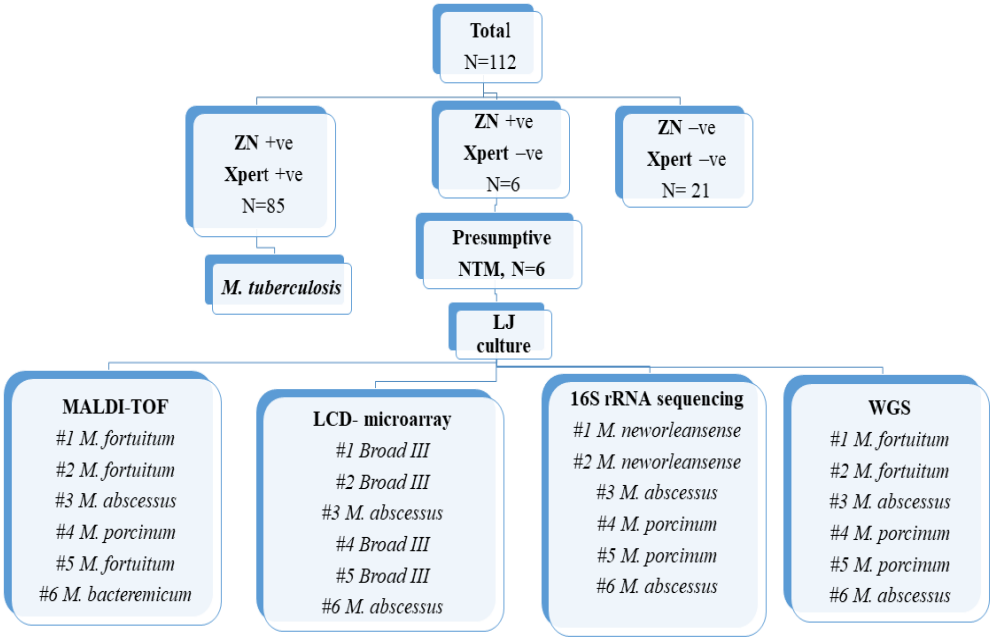


Figure 3. Results of the mycobacteriology work up of all specimens



Discussion

In our study, specimens positive by microscopic examination of ZN-stained smears and negative by GeneXpert®MTB/RIF were presumptively identified as NTM, and were cultured on LJ medium. Six out of 112 patients' specimens (5.36%) fulfilled the presumptive identification and all grew NTM by culture. Smear microscopy done in conjunction with GeneXpert may have immense diagnostic value in differentiating MTB and NTM infections in resource- limited settings, as GeneXpert can detect only *M. tuberculosis* [19].

DNA sequencing managed to discover about two hundred species of NTM, that vary according to different climates and geographical regions, which is reflected on the types of human exposure, and explains the variation in the rates and sites of infection by NTM [2]. In our study, cutaneous infections by NTM had the upper hand in the form of chronic infected wounds in the abdominal wall, after trauma or abdominal surgeries, and the isolated NTM were all rapid growers, dominated by *M. fortuitum* complex. In concordance, a previous study underscored infections with NTM after trauma, especially in the limbs [30]. **Sharma et al.** reported NTM at a lower rate of 1.2% of 1042 processed specimens, mostly from extrapulmonary sources (62%), and among high age groups 41-60 years (69%), and *M. intracellulare* was the commonest species isolated [31]. On the contrary, **Huang et al.,** 2018 noted a higher rate of NTM (37.3%), mainly, isolated from respiratory samples (86.2%), while 3.6% and 3.1% were recovered from lymph nodes and pus, respectively [32]. A previous study from Egypt reported NTM in the form of *M. marinum* (84.8%), M. fortuitum (10.9%) and *M. kansasii* (4.3%) [33]. In keeping with our results, **El-Khalawany** reported that skin trauma is a main driver for cutaneous NTM infection in Egypt (91.3%) [33]. In one of our patients the infection followed mesotherapy. This is in keeping with **Veraldi et al.** 2020 and **Machado et al.** 2014, who identified mesotherapy as a risk for infection by NTM., reporting cases infected by *M. chelonae* (n=2), and *M. abscessus* (n=45), respectively [34, 35]. Laparoscopic procedures were another risk factor for NTM infections, probably due to contamination from disinfectant solutions or water used to clean the catheters, equipment, and scopes used for the procedures [36]. The exposure of trauma or surgical sites to polluted water sources can seed the wounds with NTM [37]. The influencing role of host immunity in predisposing to NTM infections has been well established. Although immunocompromised patients are more prone to NTM infection, individuals with competent

immunity may contract infections. Immunosuppression is among the risk factors for NTM infections, such as cystic fibrosis, autoimmune diseases, malignancy or immunosuppressive therapy [37]. Regarding our patients' comorbidities, one had uncontrolled diabetes mellitus, one with cystic fibrosis disease (16.6%), and one had systemic lupus (16.6%), 3 were heavy smokers (50%); these factors could have impaired the immunity [21]. In accordance, **Sharma et al.** (2018) found that the presence of underlying lung disorder may predispose to mycobacterial pulmonary infections, however they identified the infection in immunocompetent patients as well [31].

Our work investigated the recovered six NTM isolates using MALDI-TOF, LCD-array and 16SrRNA sequencing, against the gold standard WGS, which identified the NTM as 2 *M. fortuitum*, 2 *M. abscessus*, and 2 *M. porcinum*. We noticed that 16SrRNA gene sequencing showed agreement with WGS, except for the 2 *M. fortuitum* isolates, that were identified as a closely related M. neworleansense. MALDI-TOF MS identified 3 NTM as *M. fortuitum*, and the other 3 NTM as *M. abscessus*, *M. porcinum*, *M. bacteremicum*. The Microarray identified 3 *M. fortuitum*, one *M. abscessus*, and missed the identification of one isolate. It is worth to note that *M. fortuitum* complex encompases several species including *M. fortuitum*, *M. porcinum*, *M. neworleansense* and *M. alvei*; while *M. abscessus* complex includes *M. abscessus* subspecies of *abscessus*, *massiliense*, *Bolletii. M. bacteremicum* species is known as a rapid grower and shows close relatedness to *M. neoaurum* [38].

We noticed by comparing the performed diagnostic assays with WGS that 16S gene sequencing identified all the isolates to species level except for 2 isolates that were identified to a closely related species. This is because targeted sequencing of single gene may not discriminate genetically linked strains [11]. MALDI-TOF managed to identify 4 isolates to species rank, one isolate to the complex level, and misidentified one *M. abscessus* isolate as *M. bacteremicum*. There has been previous reports about the misidentification by MALDI-TOF, which can be related to the quality of the databases and optimizing protein extraction procedures [8, 39]. For identification of NTM, a European multicenter study evaluated the performance of MALDI-TOF in fifteen laboratories showed 100% correct identification of NTM to species level and complex level in 8 and 12 centers, respectively. In most cases, the misidentifications obtained were associated with closely related species [39]. The LCD-Microarray identified 4 NTM isolates to complex level as Broad III group which encompasses species of *M. fortuitum* and *M. porcinum*, and managed to identify 2 NTM isolates to species level (*M. abscessus*), which could be due to the low sensitivity of the kits. Two commercial kits of PCR used to identify MTB and NTM were previously evaluated, where both kits revealed sensitivities of 100% in identifying MTB, while sensitivities of 77.1% and 94.9% for NTM [40].

Traditionally, phenotypic culture and biochemical characteristics have been used to identify NTM, however, most of these are time-consuming and unable to characterize many of the species. Recently, cutting-edge technology have been adapted to identify NTM [21]. The use of MALDI-TOF for identification of mycobacteria has been gradually increased, and is achieved by detecting proteins according to mass/charge ratio and generating spectra that are matched to databases for interpretation [23]. The performance of MALDI-TOF relies on the accuracy of databases and the quality of generated spectra and which needs procedures prior to extraction, that must be carried out in a BSL-3 laboratory [21]. DNA-Microarray is a broadly used identification method of mycobacteria. As previously reported, the use of LCD- microarray has improved the differentiation of NTM [25]. At present, among available advanced technologies, WGS remains the superior comprehensive technology for characterization and discrimination of NTM species, enabling better study of molecular epidemiology and investigation of outbreaks and incidents of transmission, added to the detailed genetic profiling of microbial virulence and resistance [11].

Limitations

The low incidence of recovered NTM in our study hindered the ability to conduct a detailed performance evaluation for the used diagnostic assay. Larger-scale researches are recommended to better emphasize the differences in analytical performance between the available assays in the diagnostic field for identification of NTM

Conclusion

Our study recovered six NTM isolates from pulmonary samples identified as M. fortuitum, M. abscessus, and M. porcinum by the gold standard WGS. As for the NTM identification to the species level, 16SrRNA and MALDI-TOF showed correct

identification in 66%, while LCD array correctly identified NTM in 33% of isolates. Species-specific agreement for different assays with WGS ranged from no to perfect agreement.

Conflicts of interest: All authors declare no conflicts of interest

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