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Simultaneous Detection of *Mycobacterium tuberculosis* and Atypical Mycobacteria by DNA-Microarray in Egypt

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Highlights of the Study

- DNA-microarray is a useful technique for the detection of typical and atypical mycobacterial species.
- Immunocompromised patients are prone to typical/atypical mycobacterial coinfection.

Keywords

Mycobacterium tuberculosis complex · Atypical mycobacteria · DNA-microarray · Immunocompromised patients · Nontuberculous mycobacteria

Abstract

Background and Objectives: Immunocompromised patients are a high-risk group for developing mycobacterial infections with either pulmonary and/or extrapulmonary diseases. Low-cost/density DNA-microarray is considered an easy and efficient method for the detection of typical and atypical mycobacterial species. ***Materials and Methods:*** Thirty immunocompromised patients were recruited to provide their clinical specimens (sputum, serum, urine, and lymph node aspirates). Real-time polymerase chain reaction (PCR) and DNA-microarray techniques were performed and compared to the conventional methods of Ziehl-Neelsen staining and Lowenstein Jensen culturing. ***Results:*** *Mycobacterium tuberculosis* complex was detected in all 30 clinical specimens (100% sensitivity) by real-time PCR and DNA-microarray. Additionally, coinfection with 4 atypical species belonging to nontuberculous mycobacteria was identified in 7 sputum specimens. These atypical mycobacterial species were identified as *M. kansasii* 10% ($n = 3$), *M. avium* complex 6.6% ($n = 2$), *M. gordanae* 3.3% ($n = 1$), and *M. peregrinum* 3.3% ($n = 1$). ***Conclusion:*** This study documents the presence of certain species of atypical mycobacteria among immunocompromised patients in Egypt.

Introduction

Tuberculosis (TB) remains a global public health threat, especially in developing countries. Worldwide, human TB, which is caused mainly by *Mycobacterium tuberculosis*, is associated with one million deaths annually [1]. On the other hand, nontuberculous mycobacteria (NTM) have been recognized as a diverse group of patho-

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genic organisms that are abundant in nature [2]. Exposure to NTM can cause serious infections in both immunocompetent and immunocompromised patients [3]. Culture of mycobacterial isolates is the classical method used to confirm the diagnosis of TB; however, it is time-consuming. While direct staining and microscopic examination of clinical specimens can yield quick results, this method lacks sensitivity and specificity [4]. Accurate diagnosis is important to avoid infection spread and drug resistance due to excessive exposure [5]. Molecular methods such as real-time polymerase chain reaction (PCR) and DNA-microarray techniques are used effectively for rapid detection, identification, and differentiation between different *Mycobacterium* species [6, 7]. This is very important for the control of the disease and the prescription of appropriate and effective antimycobacterial drugs [5]. The present study was aimed at assessing the role of DNA-microarray and PCR method in the routine diagnosis of *M. tuberculosis* complex (MTBC) and NTM.

Materials and Methods

This cross-sectional study was conducted on 30 immunocompromised patients with suspected mycobacterial infection (pulmonary or extrapulmonary diseases). Different clinical specimens were obtained from these patients during the period from September 2017 to February 2018 while attending either the microbiology Lab of Medical Research Institute, Alexandria University or Alexandria Armed Forces Hospital. Patients included in the study were those suspected of having mycobacterial infection based on symptoms and signs as judged by the health-care provider or clinician at the time of sampling. These patients were clinically diagnosed as acute leukemia, lymphoma, visceral malignancies, systemic lupus erythematosus, organ transplant recipients, and hemodialysis and lymphadenitis patients. Patients who received antimycobacterial therapy during the last 6 months prior to the collection of specimen were excluded.

Preparation and Decontamination of Clinical Specimens

The clinical specimens consisted of sputum (22), lymph node aspirates (2), urine (3), and serum (3). In the case of sputum specimens, repetitive sampling was done to exclude the risk of colonization by NTM. *Sputum and lymph node aspirates* were subjected to decontamination using the NaOH method [8]. Briefly, equal volumes of the sample and 1 M NaOH were mixed in a wide-mouthed jar, shaken for 30 min at 37°C, then centrifuged for 15 min at 12,000 *g*. The supernatant was discarded, and the pellet resuspended in 1.5 mL Tris-EDTA buffer and pH was adjusted to 7 using 8% HCl. Samples were centrifuged for 15 min at 12,000 *g*, and the supernatant was discarded again. *Urine samples* (the whole urine sample for each patient on 3 consecutive days) were collected aseptically and concentrated by centrifugation at 3,000 *g* for 15 min. Serum samples (200-µL aliquots from each serum sample) were analyzed by molecular methods only. The pellets from sputum and lymph node aspirates were resuspended in a 500 µL TE buffer and each was divided into 3 aliquots to be examined using conventional and molecular methods.

The 1st aliquot was examined microscopically for acid fast bacilli (AFB) using Ziehl-Neelsen (ZN) stain, and the 2nd aliquot was cultured on Lowenstein Jensen (LJ) medium after washing several times with TE buffer. The 3rd aliquot was utilized for DNA extraction for molecular techniques.

DNA Extraction

DNA extraction was performed on decontaminated clinical specimens, including sera using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations with some modifications. 200 µL of the decontaminated sample or serum was mixed with lysis buffer (200 µL), proteinase K (20 µL), and 180 µL of lysozyme mixture (20 mg/dL lysozyme; 20 mM Tris-HCl [PH 8.0]; 2 mM EDTA; 1.2% Triton) and incubated for 30 min at 37°C and then for 30 min at 56°C. The lysate was incubated in a boiling water bath followed by rapid cooling on ice. Ethanol (200 µL) was added to the lysate and the whole mixture was transferred to the QIAamp Mini spin column. After washing the column twice, the DNA was eluted using 50 µL of elution buffer.

Polymerase Chain Reaction

All specimens were examined by real-time PCR (TaqMan assay) for mycobacterial DNA using an Artus *M. tuberculosis* RG PCR kit (Qiagen, Hilden, Germany) on Mx3000p PCR system (Stratagene-Agilent) according to the manufacturer's instructions. Briefly, the reaction consisted of 13 µL master mixture, 2 µL of $MgCl_2$ solution, and 10 µL of DNA extract. The cycling profile consisted of initial enzyme activation for 10 min at 95°C, followed by 40 cycles of 2-step PCR amplification of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. This was performed as a part of the routine work of the Microbiology Laboratory of the Medical Research Institute, Alexandria University, Egypt.

DNA-Microarray Analysis

Amplification and hybridization of extracted DNA for detection of mycobacterial infection were performed using a DNA/DNA hybridization MYCO^{Direct} 1.7 kit (low cost/density-array, manufactured by Chipron GmbH [Berline, Germany]) [9]. It is based on a PCR amplification of an internal transcribed spacer region (225–265 bp fragment depending on species) of rRNA gene (Primer Mix A), and a 126-bp fragment from the Insertion Sequence (IS*6110*) element (Primer Mix B). The previous two DNA targets of PCR products hybridize with species-specific immobilized DNA probes that were designed depending on available database entries of mycobacterial species. Each microarray-chip contains eight identical microarrays in rectangular reaction chambers that can be addressed individually. The formed array is an 8 × 8 pattern where the capture probes are immobilized as duplicates (vertical), and the functional controls are in three angles (Fig. 1).

DNA amplification was performed on a Viriti thermal cycler (Applied Biosystems, Waltham, MA, USA). Thermal cycler conditions were set initially at 1 cycle at 96°C for 3 min for reactivation step followed by 45 cycles of amplification consisting of denaturation step at 94°C for 20 s, primer annealing step at 58°C for 30 s, and extension step at 72°C for 30 s. Finally, 1 cycle of final extension at 72°C for 3 min. PCR amplification and microarray hybrid-

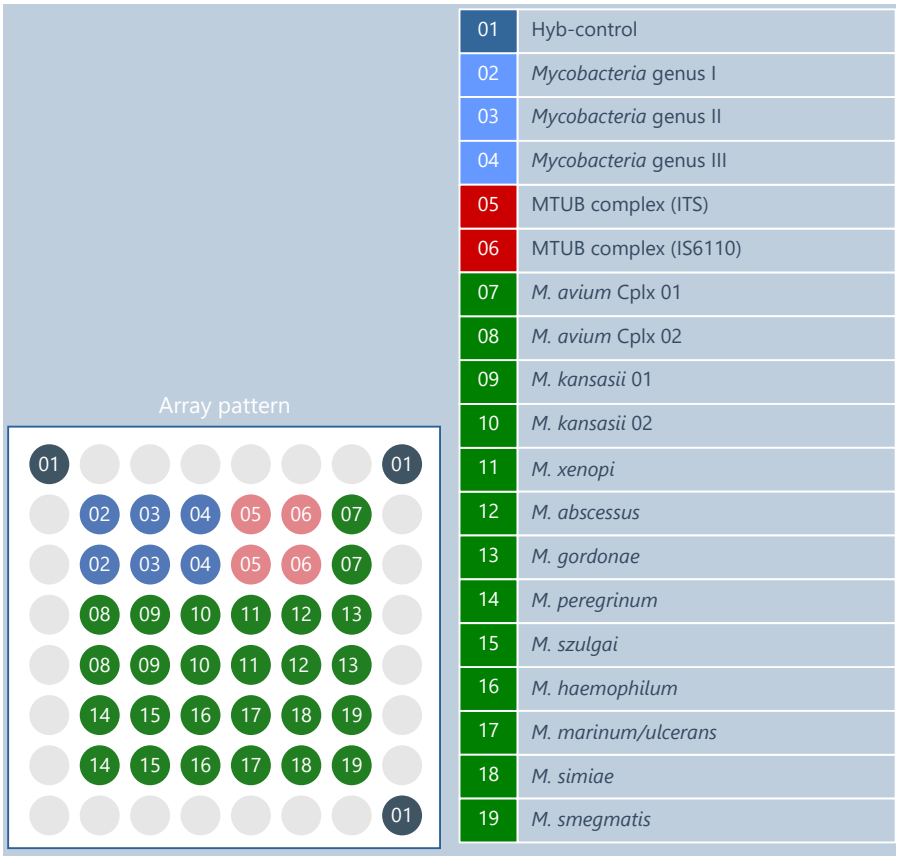

Fig. 1. MYCO^{Direct} 1.7 kit microarray pattern for results interpretation.

ization were performed according to the provided protocol of the microarray kit (Myco^{Direct} 1.7). The microarray-chip was scanned using the Chip Scanner PF 2700, and the data were analyzed by SlideReader V7.00.01 software provided by Chipron GmbH.

Results

Out of the 30 immunocompromised patients included in this study, 19 were males and 11 were females. The age of patients ranged from 1 to 75 years, and a majority of patients (56.7%) fell in 35- to 65-year age group. Twelve patients were suffering from leukemia, 5 patients presented with renal failure, 6 patients were diagnosed with cancer, 2 patients had undergone renal transplantation, and 2 infants (<2 years) presented with cervical lymphadenitis. The remaining 3 patients each suffered from pancytopenia, systemic lupus erythematosus, and bone marrow failure.

Conventional Methods

A total of 27 specimens were positive either by the ZN stain or culture or both, as shown in Figure 2. The remaining three serum specimens were excluded from culture and ZN staining. Out of 27 specimens, 23 were AFB-positive and 24 were culture-positive, and 20 specimens were both AFB- and culture-positive. Time of positivity for culture ranged from two to 4 weeks in the case of *M. tuberculosis*. The colonies were buff, dry, and rough non-chromogenic, and no photoreactivity was observed. The reddish brown pigmented colonies of *M. gordonae* were isolated from one sputum sample from patient number 5. *M. kansasii* colonies were buff in color with a slightly rough colony surface, while *M. avium* complex (MAC) colonies were buff with a smooth surface.

Molecular Methods

The results of the molecular methods showed that all specimens were positive for *M. tuberculosis* DNA using real-time PCR. Also, DNA-microarray results confirmed that all 30 clinical specimens (100%) were positive for typical MTBC. Additionally, coinfection with 4 different atypical species belonging to NTM was identified in 7 out of 30 (23.3%) sputum specimens (*M. kansasii* [n = 3, 10%], MAC [n = 2, 6.6%], *M. gordanae* [n = 1, 3.3%], and

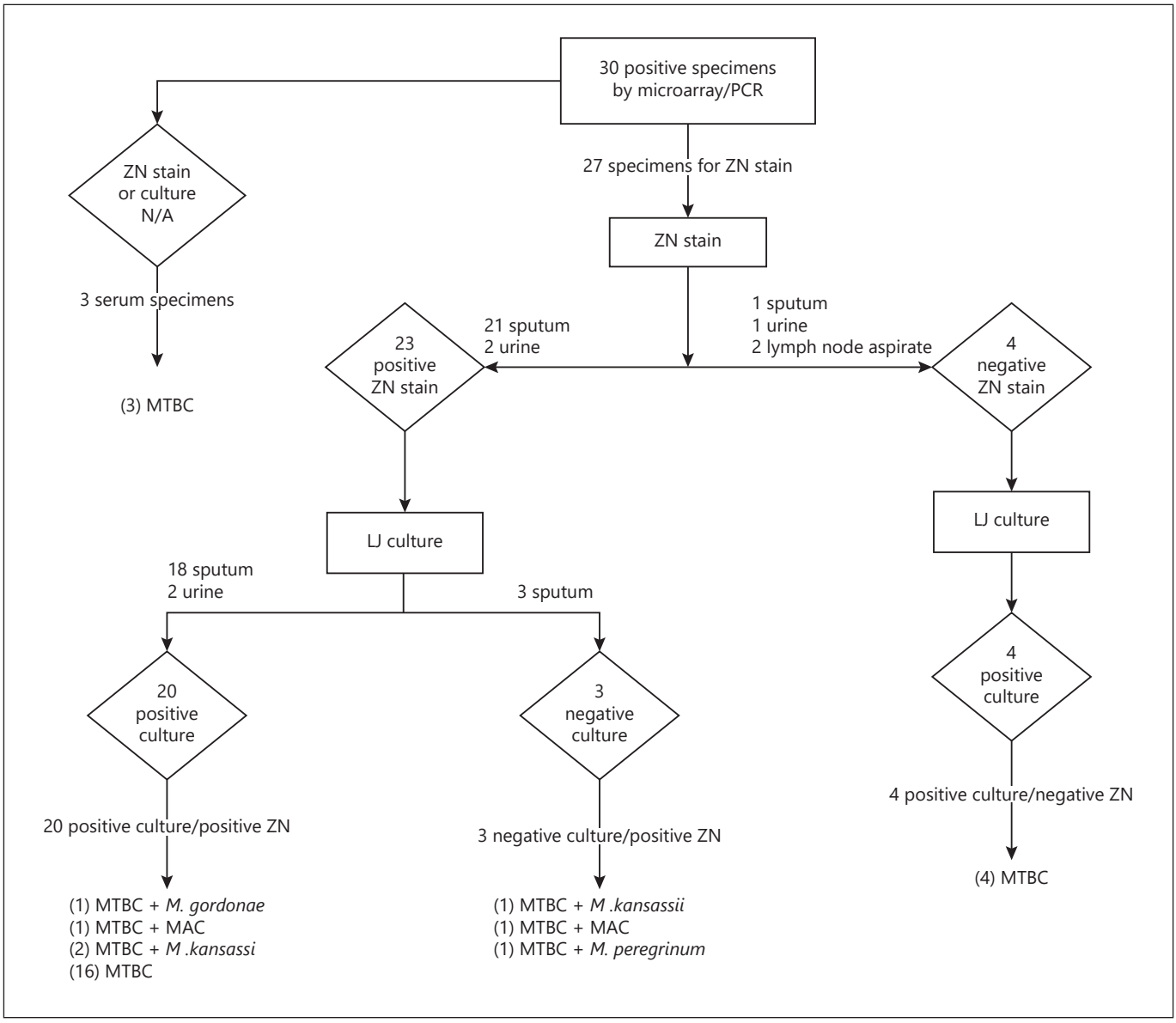

Fig. 2. Flow diagram of grouping of clinical specimens on the basis of molecular techniques, ZN staining, and LJ culturing results. Three serum specimens were neither subjected to ZN nor LJ culturing. The remaining 27 specimens demonstrated 3 different patterns of results as follows: culture positive/ZN positive (74%), culture negative/ZN positive (11%), and culture positive/ZN negative (15%). MTBC was detected in all specimens with 100% sensitivity by molecular techniques while NTM were co-detected in 7 (23.3%) sputum specimens by Microarray.

M. peregrinum [n = 1, 3.3%]). According to the DNA-microarray technique, 4 different patterns of four species of NTM and 2 different patterns of typical MTBC were detected (as shown in Fig. 3).

Discussion

Immunosuppression is one major contributor to the development of mycobacterial infection. Organ transplantation, malignancies, chemotherapy, and long-term use of glucocorticoids are the main factors to increase immunosuppression level within population [10].

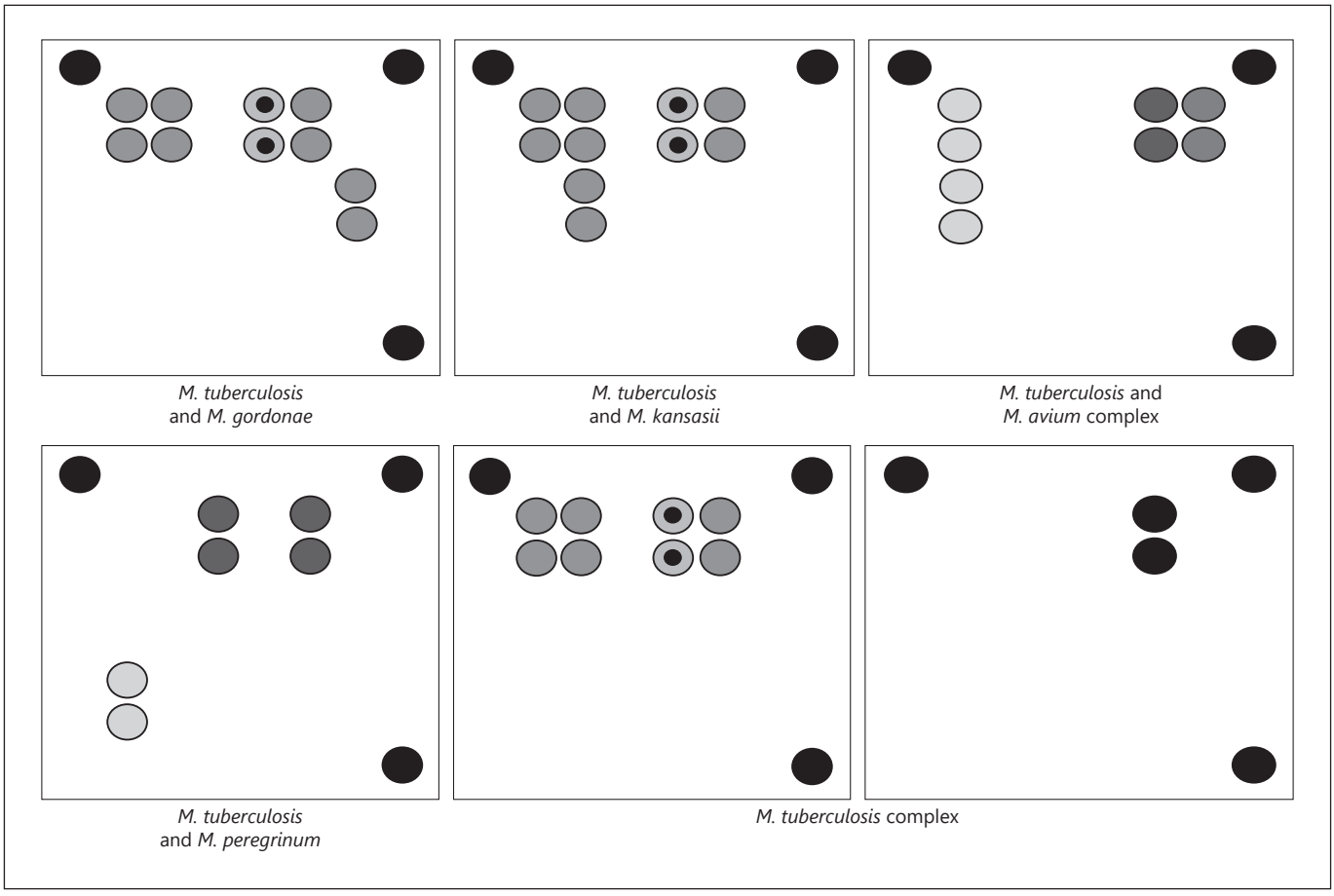

Fig. 3. DNA-microarray of 4 different patterns of four species of atypical *Mycobacteria* and 2 different patterns of typical *M. tuberculosis* species.

In this study, we examined 30 different clinical specimens from immunocompromised patients suspected of having mycobacterial infection. These patients either attended the Hospital of Medical Research Institute University of Alexandria or Alexandria Armed Forces Hospital, and they were suffering mostly from renal or cancer diseases. 27 out of the 30 clinical samples studied were examined microscopically by ZN stain and were cultured on LJ medium. The results revealed that 23 (85.2%) were AFB-positive and 24 (88.8%) were slow-growing culture positive. However, all 27 specimens were positive by ZN staining and/or culture confirming that all the patients were suffering from mycobacterial infections. The culture results of these 24 positive specimens showed nonpigmented buffy creamy dry colonies. In addition, reddish brown pigmented colonies were isolated from one sputum specimen (from patient number 5) suggesting NTM infection. The remaining 3 specimens were serum and were not examined by these conventional methods.

All the 30 specimens were examined using molecular (real-time PCR and DNA-microarray) methods. Results of all molecular techniques showed 100% sensitivity. Overall, a total of 20 specimens were both smear- and culture-positive. Three serum specimens were excluded from ZN staining or LJ culturing.

DNA-microarray was developed for rapid, easy, and reliable identification of *Mycobacteria* belonging to the MTBC and clinically relevant NTM. *M. tuberculosis* has been found in various biological samples such as sputum, urine, pus, and lymph node aspirates. Although culturing is the standard method for the specific diagnosis of TB, it is time-consuming (up to 8 weeks) [11]. Thus, nucleic acid amplification methods are increasingly in use to rapidly detect and accurately diagnose *M. tuberculosis*. The present study aimed to assess the role of the PCR method and DNA-microarray in the routine diagnosis of MTBC and NTM.

Microarray-based species identification for *Mycobacterium* is a widely used technology. The DNA-microarray

used in this study was based on two mixes of multi-primers, which amplify 225–265 bp of internal transcribed spacer of 16S rRNA depending on *Mycobacterium* species and a 126-bp fragment from the IS*6110* element. The targets amplified by PCR primer mixes were highly conserved sequences, and the probes spotted on the array had been thoroughly evaluated for their species, group, or genus specificity. The probe design is based on available database entries.

Our results showed 100% of sensitivity and specificity for MTBC and NTM when DNA-microarray was applied. The provided primer mix was designed for PCR amplification of all species. However, as the primer-binding sites were located in highly conserved regions, DNA from other *Mycobacteria* species would also be amplified; three genus-specific capture probes detecting a broad range of mycobacterial species are included.

Over the last decade, several studies were conducted regarding the burden of *M. tuberculosis*, its genetic diversity, and drug resistance to antimycobacterial drugs in Egypt [12–15]. However, very few studies have reported the clinical existence of NTM in Egypt [16, 17]. The resemblance of the clinical presentation of NTM to the well-established TB presents a tough challenge in diagnosis and treatment. NTM can be easily misdiagnosed as TB or multidrug-resistant TB, leading to poor management and serious morbidity and mortality [18].

A few studies have investigated the incidence of NTM and NTM/MTBC coinfection. The fact that all NTM in our study were co-isolated with MTBC (23.3%) is quite interesting. A recent study in India reported coinfection between NTM and MTBC in 21 cases (60%, 21/35) [19]. The same scenario was reported in one case (0.2%, 1/444) in a previous study from Nigeria [20]. On the other hand, another study in Ethiopia reported no coinfection with MTBC among their detected NTM [21].

NTM are environmentally emerging pathogens that represent more than 190 species and subspecies (http://www.bacterio.net/mycobacterium.html); some of them induce disease in immunocompetent or immunocompromised of all ages and can affect both pulmonary and extrapulmonary sites. Generally, the genus *Mycobacterium* is divided into two main groups according to their culture growth rates, slowly growing mycobacteria (SGM) (more than 7 days) and rapidly growing mycobacteria (RGM) (less than 7 days) [22]. Among detected NTM, *M. kansasii, M. gordonae*, and *M. avium* complex are SGM, while the *M. peregrinum* is RGM. Recently, an increasing trend in NTM isolation from the Middle East countries was observed [23].

In the present study, 7 out of 30 clinical specimens were positive for NTM (*M. kansasii* [*n* = 3, 10%], MAC [*n* = 2, 6.7%], *M. gordanae* [*n* = 1, 3.3%], and *M. peregrinum* [*n* = 1, 3.3%]). *M. kansasii* is the most common NTM detected among 3 immunocompromised patients (10%) and among the NTM (3/7 = 42.9%) in this study. Similarly, a previous study from Egypt reported *M. kansasii* (37.5%) as the most prevalent NTM [17]. *M. kansasii* was also previously reported in other countries such as Iran and Turkey as the third most common SGM in clinical isolates in the Middle East region [23]. On the other hand, another Egyptian study reported that *M. marinum* is the most prevalent atypical mycobacteria clinically detected from extrapulmonary infections [16]. However, this mycobacterial species was not detected among our isolates.

Globally, MAC is the most prevalent cause of NTM pulmonary disease [24, 25]. In this study, MAC was detected sputum specimens (6.6%) from a 5-year-old boy and a young adult. Because treatment outcome differs according to the NTM species, the accurate identification of NTM species is very important [26]. For example, in the case of *M. avium* complex, pulmonary disease macrolides and amikacin are preferred, while in the case of *M. kansasii* pulmonary disease rifampicin is preferred according to updated clinical practice guidelines published in 2020 [27].

M. gordonae is a rare species of SGM that is commonly found in water and soil. In this study, this species was detected in an immunocompromised patient with bone marrow failure. Also, it was previously reported in Egypt [17]. Although it is rare and less virulent than other NTM, a recent study describes *M. gordonae* in a series of 7 immunocompetent and immunocompromised patients [28].

M. peregrinum is a rare subspecies of *Mycobacterium fortuitum* group that belongs to RGM that is environmentally abundant [29]. *M. peregrinum* has been associated with soft tissue, skin infections, and surgical sites/central catheters [30]. However, it is less commonly associated with pulmonary infections. According to treatment guidelines published in 2007, it is usually susceptible to multiple oral antimicrobial agents, including newer macrolides and quinolones, doxycycline, minocycline, and sulfonamides [5]. In this study, *M. peregrinum* was detected once from a sputum specimen in an 8-year-old male with leukemia. To the best of our knowledge, this is the first clinical identification of this subspecies in Egypt.

Conclusions

This study documents the presence of certain species of atypical mycobacteria among immunocompromised patients in Egypt. One species in particular, *M. peregrinum*, was detected in an 8-year-old boy with leukemia. The DNA-microarray is a valuable tool for specific detection of mycobacteria directly from different clinical specimens. The ability of the microarray method to identify mixed mycobacterial infections (MTBC and NTM) is of advantage because this dual infection cannot be detected easily by conventional methods. This would help clinicians to prescribe suitable antimycobacterial drugs and reduce excessive antibiotic exposure to avoid or minimize drug resistance in the future.

Statement of Ethics

Approval of this study was obtained from the Ethics Committee at Medical Research Institute, Alexandria University. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines website, have been adhered to and the appropriate Ethical Review Committee approval has been received in accordance with the Declaration of Helsinki principles of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Ahmed Gaballah: conceptualization, methodology, validation, formal analysis, investigation, supervision, writing, and editing of the original draft. Abeer Ghazal: conceptualization, methodology, validation, writing and editing, and supervision. Reda Almiry: methodology, validation, formal analysis, and investigation. Rasha Emad: formal analysis, writing, reviewing, and editing. Nadia Sadek: study supervision. Mohamed Abdel Rahman: study supervision. Eglal El-Sherbini: conceptualization, methodology, formal analysis, validation, reviewing and editing, visualization, and supervision.

Data Availability Statement

All original data are available and will be provided upon request.

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