**Original Article**

# Evaluation of Speed-oligo Mycobacteria assay for rapid differentiation and identification of *Mycobacterium tuberculosis* and nontuberculous mycobacteria in MGIT 960 system cultures from human clinical samples

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**ABSTRACT**

**Objectives:** Rapid species-specific identification is of clinical relevance since treatment varies according to the *Mycobacterium* species causing infection. The aim of this study was to evaluate the ability of Speed-oligo Mycobacteria (SpO-M) assay to correctly identify most frequently isolated *Mycobacterium* spp. cultured from clinical samples.
**Design:** Comparative study
**Setting:** Mycobacteriology Reference Laboratory, Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait
**Subjects:** *Mycobacterium* species isolates (n=82) grown from human clinical samples in mycobacteria growth indicator tube (MGIT) 960 system and previously identified by molecular methods were tested.
**Interventions:** DNA from liquid cultures was extracted, amplified by polymerase chain reaction (PCR), amplified products were detected by using the dipstick and results were interpreted according to SpO-M kit instructions. PCR-sequencing of 16S-23S internal transcribed spacer region of rDNA was used to confirm and validate SpO-M results.
**Main outcome measures:** Concordance between SpO-M and original identification
**Results:** The SpO-M correctly identified species/species complex for all *Mycobacterium tuberculosis* (n=34), and 45 of 48 nontuberculous mycobacteria. *Mycobacterium lentiflavum* was detected only as *Mycobacterium* species. One *M. kansasii* isolate was potentially misidentified as *M. tuberculosis* but a careful examination of SpO-M data resolved the discrepancy. Only one isolate, *Mycobacterium parascrofulaceum*, was misidentified as *M. tuberculosis*.
**Conclusions:** Our data show that SpO-M is a rapid and reliable oligochromatographic test for detection and identification of most frequently isolated *Mycobacterium* species from clinical specimens in MGIT 960 system cultures for proper management of mycobacterial infections.

**KEY WORDS:** *Mycobacterium tuberculosis*, nontuberculous mycobacteria, PCR-sequencing of 16S-23S rDNA, Speed-oligo Mycobacteria

## INTRODUCTION

Although the genus *Mycobacterium* comprises more than 140 species, most human infections are caused by *Mycobacterium tuberculosis* or other members of *M. tuberculosis* complex and few major nontuberculous mycobacteria (NTM) which are environmental opportunistic pathogens[1-3]. Although tuberculosis (TB) is the most common mycobacterial infection in developing countries, the incidence of NTM infections is increasing and these infections are now more common than TB in developed countries[1,3]. The increasing trend in NTM infections is mainly due to an increase in the population of susceptible hosts such as elderly subjects with one or more debilitating conditions and individuals with defects in the cellular immune system due to disease or immunosuppressive treatment[3,4].

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Similar to TB, NTM infections also mostly involve the lungs and some NTMs such as *Mycobacterium kansasii* and *Mycobacterium abscessus* can also cause pulmonary infections in immunocompetent individuals[1,5-8]. Furthermore, NTM infections often resemble TB, both clinically and radiographically, however, treatment strategies and the prognosis of NTM disease are quite different[9-13]. Thus, rapid species-specific identification of *M. tuberculosis* and NTM is crucial for appropriate treatment of mycobacterial infections.

About 95% of nearly 800 mycobacterial infections in Kuwait are caused by *M. tuberculosis*, while the remaining ~5% are caused by NTM every year[14-16]. Infections by other members of *M. tuberculosis* complex (e.g. *M. bovis*) are extremely rare. Furthermore, nearly 80% of all TB cases and nearly 90% of drug-resistant TB cases in Kuwait occur in expatriate individuals mainly originating from TB endemic countries of South/South-East Asia, while NTM infections are more common among Kuwaiti nationals[14,16-20]. Also, most NTM infections in Kuwait are caused by only seven NTM species/species complex isolates[14]. This study evaluated the ability of a novel DNA strip assay (Speed-oligo Mycobacteria, SpO-M), based on multiplex polymerase chain reaction (PCR) and double-reverse hybridization on a dipstick using probes bound to colloidal gold and to the membrane, to correctly identify mycobacterial strains isolated from clinical samples in Kuwait. A multiplex PCR assay that can differentiate *M. tuberculosis* from NTM was also used. PCR-sequencing of 16S-23S internal transcribed spacer (ITS) region of rDNA was used as gold standard to confirm the results of SpO-M.

## MATERIALS AND METHODS

### *Mycobacterium* species isolates

A total of 82 clinical mycobacterial isolates belonging to *M. tuberculosis* and 10 different NTM species most frequently encountered in clinical specimens[3-9] were obtained from Kuwait National TB Reference Laboratory. The isolates were grown from 66 pulmonary (sputum, n=58; bronchoalveolar lavage, n=6 and endotracheal aspirate, n=2) and 16 extra-pulmonary (fine needle aspirate/pus, n=12; urine, n=2; lymph node, n=1 and tissue biopsy, n=1) specimens collected from 82 patients as part of routine patient care and diagnostic work-up and data are reported in this paper anonymously. Non-sterile clinical specimens were processed by the standard *N*-acetyl-L-cysteine and sodium hydroxide method while sterile samples were processed directly for culture in automated mycobacteria growth indicator tube (MGIT) 960 system (Beckton-Dickinson, Sparks, MD, USA) according to the manufacturer's instructions and as described previously[16,21]. The isolates were identified to the species-level previously by a commercial line probe assay and/or by PCR-sequencing of 16S-23S ITS region of rDNA, as described earlier[14]. The study was approved by the Ethics Committee of the Faculty of Medicine, Health Sciences Center, Kuwait University, Kuwait (Approval no. VDR/EC/2 dated 9-2-2015).

### Template DNA preparation and multiplex PCR assay

Genomic DNA was prepared from MGIT 960 system cultures of *Mycobacterium* species isolates by incorporating the removal of PCR inhibitors with Chelex-100 (Sigma-Aldrich Co. St. Louis, MO, USA) as described previously[22]. The isolates were also tested by a multiplex PCR assay designed to differentiate *M. tuberculosis* from NTM species[23]. The multiplex PCR assay with six primers (IGRF, 5′-AGCGTCTGGTCGCGTAGGCAGTG-3′; IGRR, 5′-GGTGAAGTAGTCGCCGGGCTGCT-3′; MTCF, 5′-TACGGTCGGCGAGCTGATCCAAA-3′; MTCR, 5′-ACAGTCGGCGCTTGTGGGTCAAC-3′; NTMF, 5′-GGAGCGGATGACCACCCAGGACGTC-3′ and NTMR, 5′-CAGCGGGTTGTTCTGGTCCATGAAC-3′) was performed as described previously[23]. This assay yields two amplicons of 473 bp and 235 bp from *M. tuberculosis* isolates but only a single amplicon of 136 bp from NTM members[23].

### Speed-Oligo Mycobacteria assay

The SpO-M assay kit (Vircell, Granada, Spain) identifies the bacterial isolates belonging to *Mycobacterium* genus. The test also simultaneously detects *M. tuberculosis* complex (*M. tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti* and *Mycobacterium caprae*) members and several different NTM species/ species complex comprising *Mycobacterium abscessus/M. chelonae* complex, *Mycobacterium gordonae, Mycobacterium kansasii*, *Mycobacterium avium/M. intracellulare/M. scrofulaceum* complex and *Mycobacterium fortuitum*. The SpO-M kit was used according to the kit instructions. Briefly, the DNA (5 µl) from each isolate was amplified by using 15 µl of ready-to-use reconstituted PCR mix, the PCR products were denatured by heating at 95 °C for one min and then cooled on ice immediately. A 10 µl portion of the denatured PCR product was then added to 35 µl of hybridization solution (provided with the kit) preheated to 55 °C in a 1.5 ml microcentrifuge tube, the test strip (provided with the kit) was inserted into the tube and the results were interpreted after ten minutes of incubation at 55 °C according to kit instructions. The test strip has seven (TL1 to TL7) probes in addition to product control and PCR control. Probe TL1 reacts with *M. abscessus/M. chelonae* complex, TL2 reacts with *M. gordonae*, TL3 reacts with *M. kansasii*, TL4 (in addition to weak reaction with TL3) reacts with *M. tuberculosis* (and other *M. tuberculosis* complex)

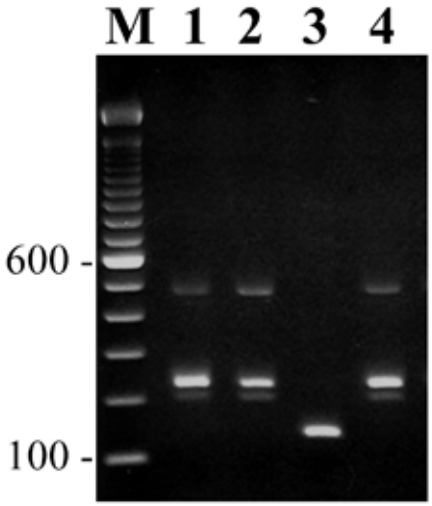


**Fig 1:** Representative agarose gel of multiplex PCR products using genomic DNA extracted from mycobacterial growth indicator tube (MGIT) 960 system cultures of four selected *Mycobacterium* species isolates and showing MTB-specific pattern with 473 bp and 235 bp products (lanes 1, 2 and 4) or NTM-specific pattern with 136 bp product (lane 3). Lane M is 100 bp DNA ladder and the position of migration of 100 bp and 600 bp fragments are marked.

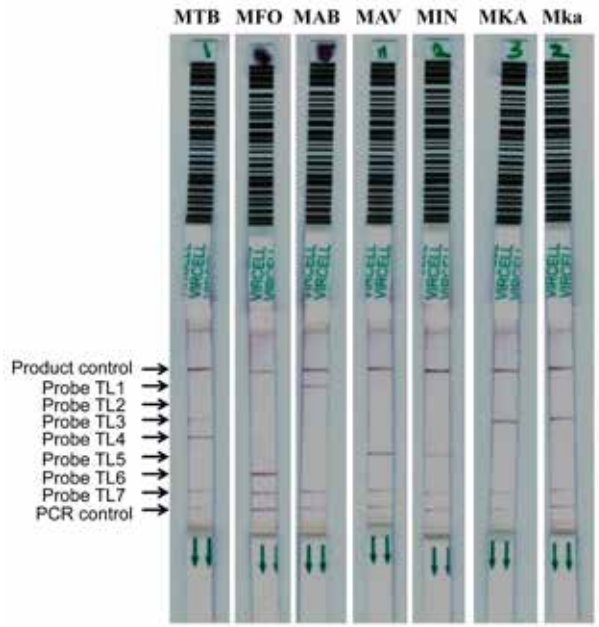


**Fig 2:** Representative hybridization results of Speed-Oligo Mycobacteria (SpO-M) test strips with DNA from *M. tuberculosis* (a member of *M. tuberculosis* complex) (lane MTB), *M. fortuitum* (lane MFO), *M. abscessus* (a member of *M. abscessus/M. chelonae* complex) (lane MAB), *M. avium* (a member of *M. avium/M. intracellulare/M. scrofulaceum* complex) (lane MAV), *M. intracellulare* (a member of *M. avium/M. intracellulare/M. scrofulaceum* complex) (lane MIN), *M. kansasii* (lane MKA) and *M. kansasii* isolate showing additional weak reactivity with TL4 (in addition to TL3 and TL7) that is potentially misidentified as *M. tuberculosis* (lane Mka). The arrows mark the positions of species/complex-specific probes TL1 to TL7. The band positions for product control and PCR control are also marked.

isolates, TL5 reacts with *M. avium/M. intracellulare/M. scrofulaceum* complex, TL6 reacts with *M. fortuitum* and TL7 reacts with all *Mycobacterium* species isolates. The visual interpretation of test results was based on the presence or absence of different bands appearing on the test strip according to the reference band patterns provided with the kit instructions. The *M. tuberculosis* $H_{37}Rv$ DNA was used as positive control while water instead of DNA was used as negative control.

**Resolution of discrepant results**

Discrepant results between SpO-M assay and the original identification were resolved by PCR-sequencing of 16S-23S ITS region of rDNA.

**PCR-sequencing of 16S-23S ITS region of rDNA**

The results of SpO-M assay for selected isolates were confirmed by PCR-sequencing of 16S-23S ITS region of rDNA. The ITS region of rDNA was amplified by using forward (SAKW135; 5′-GATTGGGACGAAGTCGTAACAAG-3′) and reverse (SAKW136, 5′-AGCCTCCCACGTCCTTCATCGGCT-3′) primers and the touchdown PCR amplification and cycling conditions described previously[22]. The amplicons were purified by using PCR product purification kit (Qiagen, Hilden, Germany) which was used according to kit instructions. Both strands of purified amplicons were sequenced by using the same amplification primers as well as two internal (ITSIF, 5′-TGGATAGTGGTTGCGAGCAT-3′ and ITSIR, 5′-GATGCTCGCAACCACTATCCA-3′) primers as sequencing primers and ABI BigDye terminator (version 3.1) cycle sequencing kit (Life Technologies Corp., Austin, TX, USA). Sequencing reactions were performed and processed as described previously[24]. Basic local alignment search tool searches (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) of DNA sequence data were performed with data from GenBank and sequence identity of >99% with reference strains or well-characterized clinical isolates of various *Mycobacterium* species was used for species identification.

**RESULTS**

A total of 82 mycobacterial isolates cultured in MGIT 960 system from 66 pulmonary and 16 extra-pulmonary clinical specimens obtained from 82 patients were tested by SpO-M assay. The isolates were previously identified by molecular methods and included 34 *M. tuberculosis* and 48 NTM. When multiplex PCR assay that differentiates *M. tuberculosis*

**Table 1:** Differentiation of MGIT 960 system cultures as MTB or NTM by multiplex (m)PCR and species-specific identification of isolates by Speed-Oligo Mycobacteria (SpO-M) assay and PCR-sequencing of 16S-23S ITS region of rDNA

| No. of isolates | *Mycobacterium* species identified by* | | |
|---|---|---|---|
| | mPCR | Probe pattern in SpO-M assay and interpretation | DNA Sequencing of rDNA |
| 10 | MTB | TL3, TL4, TL7: *M. tuberculosis* | *M. tuberculosis* |
| 24 | MTB | TL3, TL4, TL7: *M. tuberculosis* | Not done |
| 6 | NTM | TL6, TL7: *M. fortuitum* | *M. fortuitum* |
| 5 | NTM | TL6, TL7: *M. fortuitum* | Not done |
| 8 | NTM | TL5, TL7: *M. avium/M. intracellulare/M. scrofulaceum complex* | *M. avium complex* |
| 7 | NTM | TL5, TL7: *M. avium/M. intracellulare/M. scrofulaceum complex* | *M. intracellulare complex* |
| 1 | NTM | **TL3, TL4, TL7: *M. tuberculosis*** | ***M. parascrofulaceum*** |
| 8 | NTM | TL3, TL7: *M. kansasii* | *M. kansasii* |
| 1 | NTM | TL3, TL4 (weak), TL7: *M. kansasii* | *M. kansasii* |
| 7 | NTM | TL1, TL7: *M. abscessus/M. chelonae complex* | *M. abscessus* |
| 2 | NTM | TL1, TL7: *M. abscessus/M. chelonae complex* | *M. chelonae* |
| 1 | NTM | TL1, TL7: *M. abscessus/M. chelonae complex* | *M. immunogenum* |
| 1 | NTM | TL2, TL7: *M. gordonae* | *M. gordonae* |
| 1 | NTM | TL7: *Mycobacterium* species | *M. lentiflavum* |

*Discordant results by Speed-Oligo Mycobacteria and PCR-sequencing of rDNA are indicated in bold

from NTM was performed (data from four selected isolates are shown in Fig. 1), 34 isolates yielded *M. tuberculosis*-specific pattern of two amplicons of 473 bp and 235 bp while 48 isolates yielded a single amplicon of 136 bp which is characteristic of NTM species.

The SpO-M assay yielded interpretable results (data from seven selected isolates are shown in Fig. 2) for all 82 isolates. The SpO-M assay correctly identified all 34 *M. tuberculosis* isolates (Table 1) as they showed strong reaction with probe TL7 and TL4 in addition to weak reaction with probe TL3 (Fig. 2, lane MTB). The SpO-M assay also correctly identified all 11 *M. fortuitum* isolates as they showed reaction with probes TL7 and TL6 (Fig. 2, lane MFO) and one *M. gordonae* isolate as it reacted with probes TL7 and TL2 (Table 1). Seven isolates of *M. abscessus* (Fig. 2, lane MAB), two isolates of *M. chelonae* and one isolate of *M. immunogenum* were also correctly identified as *M. abscessus/M. chelonae* complex isolates (Table 1) as they reacted with probes TL7 and TL1. Similarly, eight isolates of *M. avium* (Fig. 2, lane MAV) and seven isolates of *M. intracellulare* (Fig. 2, lane MIN) were identified as *M. avium/M. intracellulare/M. scrofulaceum* complex isolates as they reacted with probes TL7 and TL5. However, one *M. parascrofulaceum* isolate was misidentified as *M. tuberculosis* (Table 1) as it reacted with probes TL7 and TL4 in addition to weak reaction with TL3. Of nine *M. kansasii* isolates, eight isolates were correctly identified as they reacted with probes TL7 and TL3 only (Fig. 2, lane MKA); however, one isolate was potentially misidentified as *M. tuberculosis* (Table 1) since probe TL4 also reacted, albeit weakly, in addition to probes TL7 and TL3 (Fig. 2, lane Mka). One *M. lentiflavum* isolate was identified to the genus level as it reacted with probe TL7 only. The *M. kansasii* isolate showing additional weak reaction with probe TL4 was confirmed as *M. kansasii* by PCR-sequencing of ITS region of rDNA. The *M. parascrofulaceum* identified as *M. tuberculosis* by SpO-M assay was confirmed as *M. parascrofulaceum* by PCR-sequencing of ITS region of rDNA (Table 1).

## DISCUSSION

Although TB, particularly pulmonary TB, is the major mycobacterial disease in most of the developing countries, the incidence of NTM infections has surpassed TB in resource-rich developed countries[1-3]. The NTM infections are usually acquired through contact with contaminated environment[2,25]. Lung disease is the most common manifestation of NTM infection, while extra-pulmonary infections usually include lymphadenitis and cutaneous or disseminated disease but may also involve lymph nodes, soft tissue, bone and joints[2,4-8,26,27]. Patients with NTM infections often mimic disease presentation indistinguishable from TB both clinically and radiographically and are older with predisposing pulmonary abnormalities[4-7,10,11,26]. A definitive diagnosis of NTM lung disease also requires differentiation from colonization or contamination due to their abundance in the environment[25]. Successful treatment of TB is dependent on rapid diagnosis but also requires accurate drug susceptibility of *M. tuberculosis* isolates to anti-TB drugs due to increasing reports of multidrug-resistant TB (infection with *M. tuberculosis* strains resistant at least to rifampicin and isoniazid, the two most active anti-TB drugs) in many countries[28]. The virulence of NTM varies considerably and the susceptibility of NTM to anti-TB drugs and other antibiotics also varies markedly among members belonging to different species/species complex, which necessitates different treatment strategies for different NTM infections[5-9,12,27]. Furthermore, *in*

*vitro* susceptibility does not always correlate with effective *in vivo* response to antibiotics[4,8,9,27]. Thus, rapid differentiation of MTB from NTM and accurate identification of NTM species is crucial for proper treatment and appropriate patient management.

Kuwait is a low (23 cases per 100,000 population) TB incidence country[15,16,29]. Nearly 800 mycobacterial infections are diagnosed every year, with 95% of these infections occurring due to *M. tuberculosis* while 5% of infections are caused by NTM[14-16,26]. Furthermore, 80% of TB cases and ~90% of drug-resistant TB cases occur in expatriate patients, while NTM infections are more common in Kuwaiti subjects[14,16-18,26,29]. Most NTM infections in Kuwait are caused by only few species which mainly include *M. fortuitum, M. kansasii, M. abscessus* complex, *M. avium* complex and *M. intracellulare* complex members[14]. In this study, we evaluated SpO-M assay that has been developed for rapid differentiation of mycobacterial strains in MGIT 960 system cultures. This dipstick test directly detects denatured PCR amplicons from *M. tuberculosis* and most common NTM species in only two pipetting steps. The procedure requires a total time of nearly three hours and a hands-on time of only 30 minutes[30]. Our data on 82 mycobacterial strains showed that all *M. tuberculosis* (n=34), all *M. fortuitum* (n=11), all *M. abscessus*/*M. chelonae* complex members isolates (n=9) (including seven *M. abscessus* and two *M. chelonae* isolates), all *M. avium* (n=8), all *M. intracellulare* (n=7) and one *M. gordonae* isolate were correctly identified. The probe for *M. abscessus*/*M. chelonae* complex also reacted with *M. immunogenum*, a species closely related to *M. abscessus* and *M. chelonae*[31]. One *M. lentiflavum* isolate was identified to the genus level as it reacted with probe TL7 only since no specific probe is included in the SpO-M kit for this rare *Mycobacterium* species. Although eight *M. kansasii* isolates were correctly identified, one isolate was potentially misidentified as *M. tuberculosis* and one *M. parascrofulaceum* isolate was misidentified as *M. tuberculosis*.

Thus, application of SpO-M on MGIT 960 system cultures of mycobacterial strains in Kuwait showed concordant results for 79 of 82 (96%) isolates. Other investigators have also reported similar results. Quezel-Guerraz *et al*[32] reported a concordance of 97.2% for 182 mycobacterial isolates collected from various Spanish mycobacteriology laboratories with only 13 of 157 NTM isolates identified at genus level while the remaining 144 NTM isolates were identified to the correct species/species complex level. Significant discordance was noted only for two *Mycobacterium marinum* isolates which were identified as *M. kansasii*[32]. Similar results were also reported for another isolate from Czech Republic[33]. O'Donnell *et al*[34] also reported a concordance of 98% between SpO-M and AccuProbe assay for the identification of mycobacterial strains. On the contrary, Ramis *et al*[35] using an updated version reported a concordance of only 93.5% with one *M. chelonae* isolate failing to react with any probe, one *M. kansasii* isolate misidentified as *M. tuberculosis* and one *M. peregrinum* isolate misidentified as *M. abscessus*. These misidentifications are significant due to differences in antimicrobial susceptibility profiles of these NTM, which could influence treatment decisions and outcome[2,5,7-9]. Similar to the findings of Ramis *et al*[35], one *M. kansasii* isolate in our study was also potentially misidentified as *M. tuberculosis*. However, a closer look at our data showed that this isolate exhibited stronger reaction with probe TL3 and much weaker reaction with probe TL4, while *M. tuberculosis* isolates exhibit stronger reaction with probe TL4 and weaker reaction with probe TL3. Thus, careful examination of SpO-M data identified the isolate correctly as *M. kansasii*. Similar to our study, *M. lentiflavum* was also not specifically identified in other studies using SpO-M assay as there is no specific probe for its identification[32,33]. One *M. parascrofulaceum* isolate in our study was misidentified as *M. tuberculosis*. It is not known at present if *M. parascrofulaceum* isolates are routinely misidentified as *M. tuberculosis* by this test since other studies using SpO-M assay have not used *M. parascrofulaceum* isolates[30,32-35].

Other tests such as matrix assisted laser desorption ionization-time of flight mass spectrometry has also been used for rapid detection of *Mycobacterium* species. However, the method requires expensive equipment and also needs cultures on solid media, which is time consuming, while the performance in rapid liquid cultures is only around 90%[36-38].

A limitation of our study is that many mycobacterial isolates (such as those belonging to *M. abscessus* and *M. chelonae* or *M. avium* and *M. intracellulare*) were only identified to the species complex level rather than specifically to the individual species due to inclusion of only few probes on test strips. Although an updated version of SpO-M assay has been developed recently that can specifically identify *M. tuberculosis* complex members (*M. tuberculosis, M. bovis* etc.) and 13 NTM species (including *M. abscessus, M. avium, M. chelonae, M. fortuitum, M. gordonae, M. interjectum, M. intracellulare, M. kansasii, M. malmoense, M. marinum*/*M. ulcerans, M. peregrinum, M. scrofulaceum* and *M. xenopi*), many other NTM species such as *M. lentiflavum, M. mucogenicum, M. porcinum* and several other rarely occurring NTM species (e.g. *M. canariasense*) still cannot be specifically identified[39].

## CONCLUSION

Our data on 82 mycobacterial isolates showed that all 34 *M. tuberculosis* isolates were accurately identified

and 45 of 48 NTM isolates were accurately identified to the species/species-complex level. Careful examination of SpO-M data also correctly identified one *M. kansasii* isolate that showed additional weak reaction with probe TL4 while one isolate (*M. lentiflavum*) was identified only at the genus level. Only one isolate (*M. parascrofulaceum*) yielded inaccurate identification. Our data show that Speed-Oligo Mycobacteria is a reliable test for routine diagnostics of *Mycobacterium* species isolates in MGIT 960 system cultures.

## ACKNOWLEDGMENT

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### Competing Interests

The authors declare that they have no competing interests.

### Author Contributions

Suhail Ahmad and Eiman Mokaddas designed the study. Noura M Al-Mutairi performed the experiments and analyzed the data. Suhail Ahmad and Eiman Mokaddas wrote the manuscript. All authors read and approved the final version of the manuscript.

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