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ORIGINAL ARTICLE

Utility of RGM medium for recovery of *Mycobacterium abscessus* from presumptive pulmonary tuberculosis patients and its molecular phylogenetic characterization

Usha Kattamanchi[1,2], Rakesh Kodati[3], Neelima Angaali[4], Patil Madhusudhan[4], Vishunukanth Govindaraj[5], Gongati Kruparao Paramjyothi[3], Kumar Ebineshan[2], Noyal Mariya Joseph[1*]**

[1]Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry/Puducherry, India
[2]Department of Microbiology, LEPRA Society - Blue Peter Public Health and Research Centre (LS-BPHRC), Cherlapally, Hyderabad, India
[3]Department of Pulmonology, Nizams Institute of Medical Sciences (NIMS), Hyderabad, Telangana, India
[4]Department of Microbiology, Nizams Institute of Medical Sciences (NIMS), Hyderabad, Telangana, India
[5]Department of Pulmonary Medicine, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry/Puducherry, India

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ABSTRACT

Background and Objectives: Non-tuberculous mycobacteria (NTM), especially *Mycobacterium abscessus*, are emerging causes of pulmonary disease and are often misdiagnosed as tuberculosis in high TB burden countries. Conventional culture methods are time-consuming and may reduce viable organism recovery. Rapidly growing mycobacteria (RGM) medium enables direct inoculation of respiratory specimens and improved recovery of rapidly growing NTM. This study evaluated the utility of RGM medium for isolation of *M. abscessus* from presumptive pulmonary tuberculosis patients and characterized the isolates using molecular and phylogenetic analyses.

Materials and Methods: A total of 223 sputum samples collected from presumptive pulmonary tuberculosis patients. Untreated sputum samples were inoculated onto RGM medium with selective antibiotics and incubated at 30°C for 10 days. Suspected colonies were confirmed by Ziehl–Neelsen staining. Identification was performed by PCR and sequencing of *hsp65* and *rpoB* genes. Phylogenetic analysis was done using the Neighbour-Joining method in MEGA12. *Mycobacterium abscessus* isolates were further analysed using the GenoType NTM-DR assay.

Results: Among the 223 sputum samples processed, 7.6% (17/223) demonstrated positive growth on RGM medium. Of the culture-positive isolates, 64.7% (11/17) were identified as *Mycobacterium abscessus*, while 35.3% (6/17) were identified as other non-tuberculous mycobacterial (NTM) species. All isolates were positive for the *rpoB* and *hsp65* genes. Eleven *M. abscessus* isolates were further analysed using the GenoType NTM-DR assay, and no resistance-associated mutations were detected in the *erm(41)*, *rrl*, or *rrs* genes. Additionally, all isolates were found to be susceptible to macrolides and aminoglycosides.

*Corresponding author: Noyal Mariya Joseph, MD, Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry/Puducherry, India. Tel: +91-9843094673 Email: noyal.c@gmail.com

**Co-corresponding author: Kumar Ebineshan, Ph.D, Department of Microbiology, LEPRA Society - Blue Peter Public Health and Research Centre (LS-BPHRC), Cherlapally, Hyderabad, India. Tel: +91-8125572562 Email: ekumar@leprahealthinaction.in

Conclusion: RGM medium demonstrated good utility for recovery of clinically significant rapidly growing mycobacteria, particularly *M. abscessus*, directly from sputum samples. Molecular and phylogenetic analyses enabled accurate species identification and characterization. The absence of resistance-associated mutations suggests retained susceptibility to key therapeutic agents. These findings support the use of RGM medium for improved detection of pulmonary NTM infections in TB-endemic settings.

Keywords: Non tuberculous mycobacteria (NTM); *Mycobacterium abscessus*; Rapidly growing mycobacteria (RGM) medium; *hsp65* gene; *rpoB* gene; Phylogenetic analysis; Drug resistance; GenoType NTM-DR

INTRODUCTION

Non tuberculous mycobacteria (NTM) are increasingly recognized as important opportunistic pathogens causing pulmonary and extrapulmonary infections worldwide (1). Among the rapidly growing mycobacteria (RGM), *Mycobacterium abscessus* has emerged as one of the most clinically significant species because of its intrinsic resistance to multiple antimicrobial agents and its association with chronic pulmonary disease, particularly among patients with underlying structural lung disorders (2). The clinical presentation of pulmonary NTM disease frequently resembles pulmonary tuberculosis (TB), often leading to misdiagnosis and inappropriate anti-tubercular therapy, especially in high TB burden countries such as India (3).

Traditional culture-based diagnostic methods for NTM isolation are time-consuming and require harsh decontamination procedures that may reduce the recovery of viable organisms (4). Liquid culture systems such as the BACTEC MGIT 960 System are widely used for mycobacterial detection; however, contamination, prolonged incubation time, and limited selectivity for rapidly growing mycobacteria remain important challenges (5). Recently, rapidly growing mycobacteria (RGM) medium has been introduced as a selective agar-based culture medium that allows direct inoculation of untreated respiratory specimens and facilitates improved recovery of clinically important rapidly growing NTM, particularly the *M. abscessus* complex (4).

Accurate species and subspecies identification of *M. abscessus* is clinically important because treatment outcomes and antimicrobial susceptibility profiles differ significantly among subspecies (6). Molecular methods targeting conserved genes such as *hsp65* and *rpoB* have demonstrated high discriminatory power for the identification of NTM species (7, 8) In addition, phylogenetic analysis of clinical isolates provides valuable insights into genetic diversity, strain relatedness, and evolutionary patterns (9). Detection of mutations in *erm* (41), *rrl*, and *rrs* genes is particularly relevant because these mutations are associated with resistance to macrolides and aminoglycosides, the cornerstone drugs for treatment of *M. abscessus* infections (10).

Despite the increasing recognition of pulmonary NTM disease, information regarding the utility of RGM medium for recovery of *M. abscessus* from presumptive pulmonary TB patients in India remains limited. Furthermore, molecular phylogenetic characterization and resistance profiling data on clinical *M. abscessus* isolates from this region are scarce. Therefore, the present study aimed to evaluate the utility of RGM medium for the isolation of *M. abscessus* from sputum specimens of presumptive pulmonary TB patients and to characterize the recovered isolates using molecular identification, phylogenetic analysis, and drug resistance-associated mutation profiling.

MATERIALS AND METHODS

Clinical samples. A cross-sectional study was conducted on 223 sputum samples collected from presumptive pulmonary tuberculosis patients attending Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry and Nizam's Institute of Medical Sciences, Hyderabad during the period from September 2023 to December 2025. Approximately 2 mL of expectorated sputum was collected from each patient in a sterile 50 mL centrifuge tube. Each sample was divided into two aliquots for parallel processing by RGM and MGIT culture methods.

Inoculation on RGM medium. Rapidly growing mycobacteria (RGM) medium was prepared as previously described by Preece CL et al. (4). The culture medium was prepared using Middlebrook 7H9 broth base (4.70 g/L) and agar (19.70 g/L) dissolved

in 850 mL distilled water prior to sterilization. After autoclaving and cooling, OADC supplement (100 mL; two 50 mL vials) was aseptically added. An antibiotic cocktail (total 50 mL) was then incorporated, prepared in sterile water (40 mL) containing colistin (32 mg), fosfomycin (400 mg), glucose-6-phosphate (25 mg), and C-390 (32 mg). Amphotericin B stock solution (10 mL) was prepared separately by dissolving 5 mg amphotericin B in 200 µL N-methyl-2-pyrrolidinone and adjusting the volume to 10 mL with sterile water, and was included within the antibiotic supplementation.

The prepared medium was poured into sterile 90 mm Petri dishes and stored at 4°C for a maximum of 12 weeks before use. A 100 µL aliquot of untreated sputum was directly inoculated onto RGM medium and incubated aerobically at 30°C for 10 days. Plates were examined for colony growth after 4, 7, and 10 days of incubation. Cream-colored, non-pigmented, dry, smooth or rough, crumbly colonies suggestive of rapidly growing non-tuberculous mycobacteria (NTM) were subjected to Ziehl–Neelsen (ZN) staining for confirmation of acid-fast bacilli (AFB). AFB-positive isolates were subcultured onto Middlebrook 7H11 agar until visible growth appeared. A loopful of colonies was suspended in 0.8 mL of Middlebrook 7H10 broth supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC). The suspension was mixed with 0.8 mL of 25% glycerol in cryovials and stored at -80°C until DNA extraction and molecular analysis.

DNA extraction. Total genomic DNA was extracted from mycobacterial colonies recovered from RGM medium. Briefly, a loopful of colonies grown on RGM medium was emulsified in 1000 µL of sterile deionized water in a screw-cap microcentrifuge tube and centrifuged at 13,000 rpm for 10 minutes. The supernatant was carefully discarded. The resultant pellet obtained from solid culture was resuspended in Tris–EDTA (TE) buffer and subjected to heat lysis at 95°C for 20 minutes in a dry bath. The lysate was immediately cooled on ice and centrifuged at 10,000 rpm for 10 minutes as previously described by Awua AK et al. (11). The supernatant containing genomic DNA was carefully transferred into sterile 0.5 mL microcentrifuge tubes and stored at -20°C until further analysis. The concentration and purity of extracted DNA were assessed using a NanoDrop spectrophotometer.

PCR-based amplification of *hsp65* and *rpoB* genes. Approximately 2 µL of purified genomic DNA was used as the template for PCR amplification using standard working concentrations of forward and reverse primers (Table 1) along with Emerald Amp Max PCR Master Mix (Takara, Japan). PCR amplification was performed with an initial denaturation step at 95°C for 1 minute, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds, and extension at 72°C for 90 seconds. A reaction mixture without DNA template was included in each run as a negative control. The amplified PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination. PCR amplicons were purified using the QIA quick PCR Purification Kit and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit on the 3730XL DNA Analyzer. The obtained nucleotide sequences were aligned and analysed using the BLAST (Basic Local Alignment Search Tool) database for species identification.

Genotyping and drug resistance pattern analysis. The isolates identified as *Mycobacterium abscessus* by PCR were further subjected to the Geno Type NTM-DR Line Probe Assay Version 1.0 for subspecies identification and detection of antimicrobial resistance-associated mutations. The assay was used to identify *M. abscessus* subspecies and to detect mutations associated with inducible macrolide resistance at position 28 of the *erm(41)* gene, chromosomal macrolide resistance at positions 2058/2059 of the *rrl* gene, and aminoglycoside resistance at position 1408 of the *rrs* gene.

PCR amplification and hybridization procedures were performed according to the manufacturer's instructions provided by Hain Lifescience. Hybridization banding patterns were interpreted visually using the evaluation sheet supplied with the assay kit.

Phylogenetic analysis. The evolutionary history was inferred using the Neighbour-Joining method (12). The optimal phylogenetic tree with a total branch length of 2.932 was generated. Bootstrap analysis was performed with 1000 replicates, and the percentage of replicate trees in which the associated taxa clustered together is shown next to the branches (13). Evolutionary distances were computed using the Maximum Composite Likelihood method (14) and expressed as

Table 1. Primers used for PCR amplification and sequencing

Target Gene	Primer Name	Primer Sequence (5′–3′)	Amplicon Size	Reference
hsp65	HSPF3	ATC GCC AAG GAG ATC GAG CT	644 bp	Kim et al., 2005
	HSPR4	AAG GTG CCG CGG ATC TTG TT		
rpoB	MycoF	GGC AAG GTC ACC CCG AAG GG	764 bp	Adekambi et al., 2003
	MycoR	AGC GGC TGC TGG GTG ATC ATC		
rpoB Sequencing	MycoseqF	GAA GGG TGA GAC CGA GCT GAC	—	Adekambi et al., 2003
	MycoseqR	GCT GGG TGA TCA TCG AGT ACG G	—	

the number of base substitutions per site. The analysis included 51 nucleotide sequences comprising first, second, third codon, and non-coding positions. Ambiguous positions were removed for each sequence pair using the pairwise deletion option, resulting in a final dataset of 758 positions. Evolutionary analyses were performed using MEGA12 (15).

Institutional ethical committee approvals. Ethical approval was obtained from Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India (Approval No. JIP/IEC-OS/222/2023), LEPRA Society – Blue Peter Public Health and Research Center, Hyderabad, India (Approval No. 10/LEPRA/IEC/2024-2025), and Nizam's Institute of Medical Sciences, Hyderabad, India (Approval No. EC/NIMS/3717/2025).

RESULTS

Culture. Out of the 223 sputum samples obtained from presumptive tuberculosis patients and processed, 7.6% (17/223) demonstrated positive culture growth on RGM medium. Among the culture-positive isolates, 64.7% (11/17) were identified as *Mycobacterium abscessus*, while the remaining 35.3% (6/17) were identified as other non-tuberculous mycobacterial (NTM) species. The rough and smooth colony morphologies of *M. abscessus* observed on RGM medium are presented in Fig. 1. All isolates were positive for both *rpoB* and *hsp65* gene amplification. Representative PCR amplicon images of the *hsp65* gene from clinical isolates demonstrated successful amplification on 2% agarose gel electrophoresis (Fig. 2).

Drug resistance. All 11 isolates demonstrated the characteristic banding pattern of *Mycobacterium abscessus* subsp. *abscessus* on the GenoType NTM-DR Line Probe Assay Version 1.0. The *erm(41)* T28 band

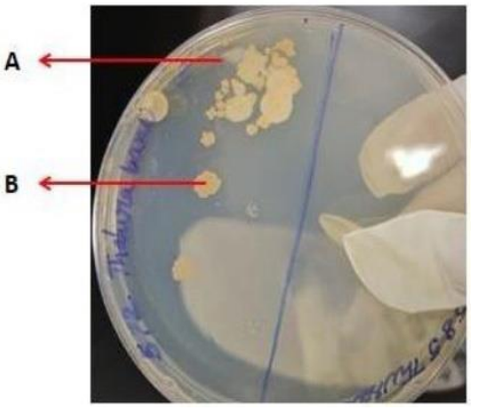

Fig. 1. Growth of *Mycobacterium abscessus* on RGM showing characteristic colony morphology.
A: Smooth colony
B: Rough colony

was present in all isolates, while the *erm(41)* C28 band was absent. Ten isolates exhibited the wild-type banding pattern at the *rrl* and *rrs* loci, indicating susceptibility to macrolides and aminoglycosides. However, one isolate demonstrated an *erm(41)*-associated resistance pattern suggestive of inducible macrolide resistance. Overall, 10 of the 11 *M. abscessus* isolates were susceptible to both macrolides and aminoglycosides, whereas one isolate showed resistance-associated findings related to the *erm(41)* gene (Fig. 3).

Phylogenetic analysis. All M. abscessus isolates clustered closely with reference *Mycobacterium abscessus* strains in the phylogenetic tree, confirming their genetic relatedness and species identity (Fings modified as Figs. 4 and 5).

DISCUSSION

The present study evaluated the utility of rapidly growing mycobacteria (RGM) medium for the isolation of *Mycobacterium abscessus* from sputum

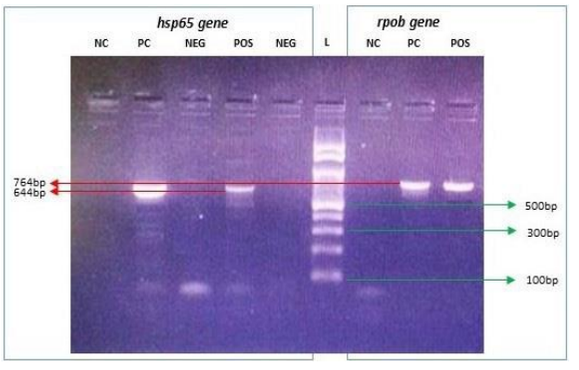

Fig. 2. Agarose gel electrophoresis (2%) showing PCR amplification of the *hsp65* and *rpoB* gene from clinical isolates.
NC: Negative control; PC: Positive control
NEG: Negative; POS: Positive; L: DNA ladder

Fig. 3. Drug resistance pattern of *Mycobacterium abscessus* isolates as determined by GenoType NTM-DR Line Probe Assay (LPA), showing banding patterns for resistance-associated genes.

specimens of presumptive pulmonary tuberculosis patients and characterized the isolates using molecular and drug resistance profiling methods. The findings highlight the growing importance of non-tuberculous mycobacteria (NTM) in TB-endemic regions and support the use of selective RGM medium for improved recovery of clinically significant rapidly growing mycobacteria. In this study, 7.6% of sputum samples demonstrated positive growth on RGM medium, indicating the presence of rapidly growing NTM among presumptive TB patients. Similar observations have been reported in previous studies, where NTM isolation rates among patients suspected of pulmonary TB ranged from 5-15% depending on geographic region and diagnostic methodology (1, 3). The increasing prevalence of NTM pulmonary disease in countries with a high TB burden presents a major diagnostic challenge because clinical and radiological findings frequently overlap with tuberculosis, leading to delayed or inappropriate therapy (2).

A total of 11 *M. abscessus* isolates were recovered on RGM medium, whereas two of these isolates were not detected by MGIT culture. This discrepancy may be attributable to the decontamination procedure required for MGIT processing. Specimens inoculated into the MGIT system undergo N-acetyl-L-cysteine–NaOH (NALC–NaOH) decontamination, a process that may reduce the viability of rapidly growing mycobacteria, particularly when the bacterial burden is low. As a result, viable *M. abscessus* organisms may be lost during specimen processing, leading to reduced culture recovery. In contrast, RGM medium permits direct inoculation of untreated sputum specimens and contains selective antimicrobial agents that suppress contaminating flora while supporting the growth of rapidly growing NTM. The recovery of two *M. abscessus* isolates on RGM medium that were not detected by MGIT culture may therefore reflect improved preservation of viable organisms due to the absence of a decontamination step. These observations are consistent with previous studies demonstrating enhanced recovery of the M. abscessus complex using RGM medium compared with conventional culture methods (4). Furthermore, the agar-based format of RGM medium enabled visualization of both rough and smooth colony morphotypes, facilitating preliminary phenotypic characterization of *M. abscessus* isolates. Colony morphology has clinical significance, as rough morphotypes have been associated with increased virulence, enhanced biofilm formation, and invasive disease (16). Collectively, these findings support the use of RGM medium as a valuable complementary culture method for the recovery of rapidly growing non-tuberculous mycobacteria from respiratory specimens and suggest that its implementation may improve the detection of *M. abscessus* in routine clinical microbiology laboratories.

Molecular identification using amplification and sequencing of *hsp65* and *rpoB* genes successfully confirmed all isolates as mycobacterial species. The *hsp65* and *rpoB* genes are well-established molec-

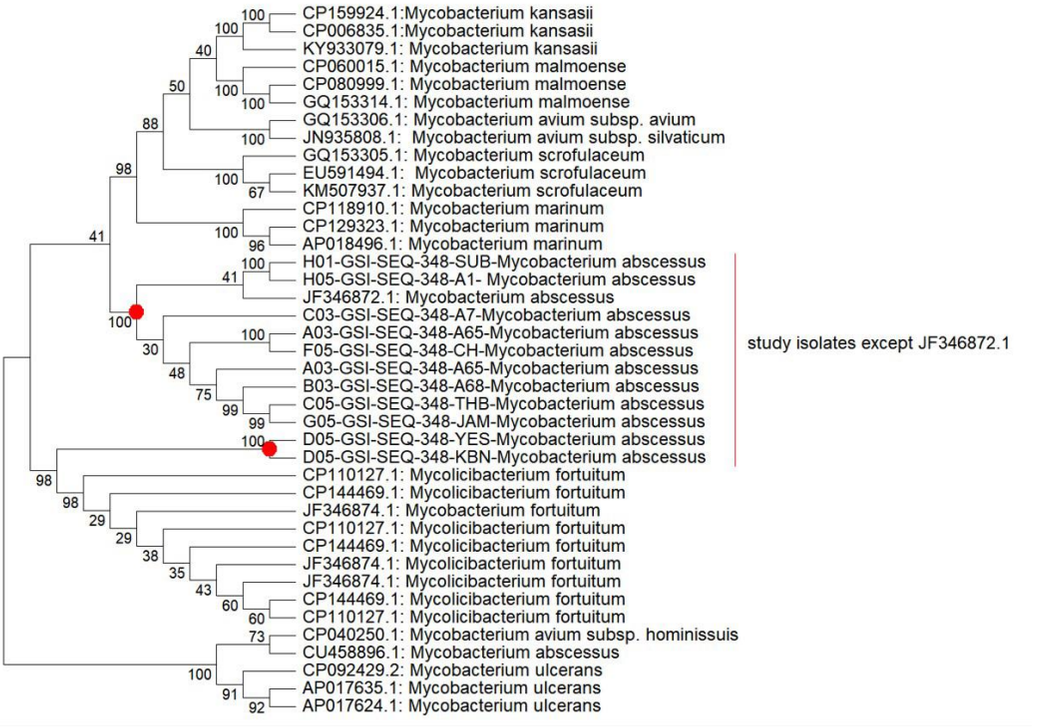

Fig. 4. Phylogenetic analysis based on *rpoB* gene

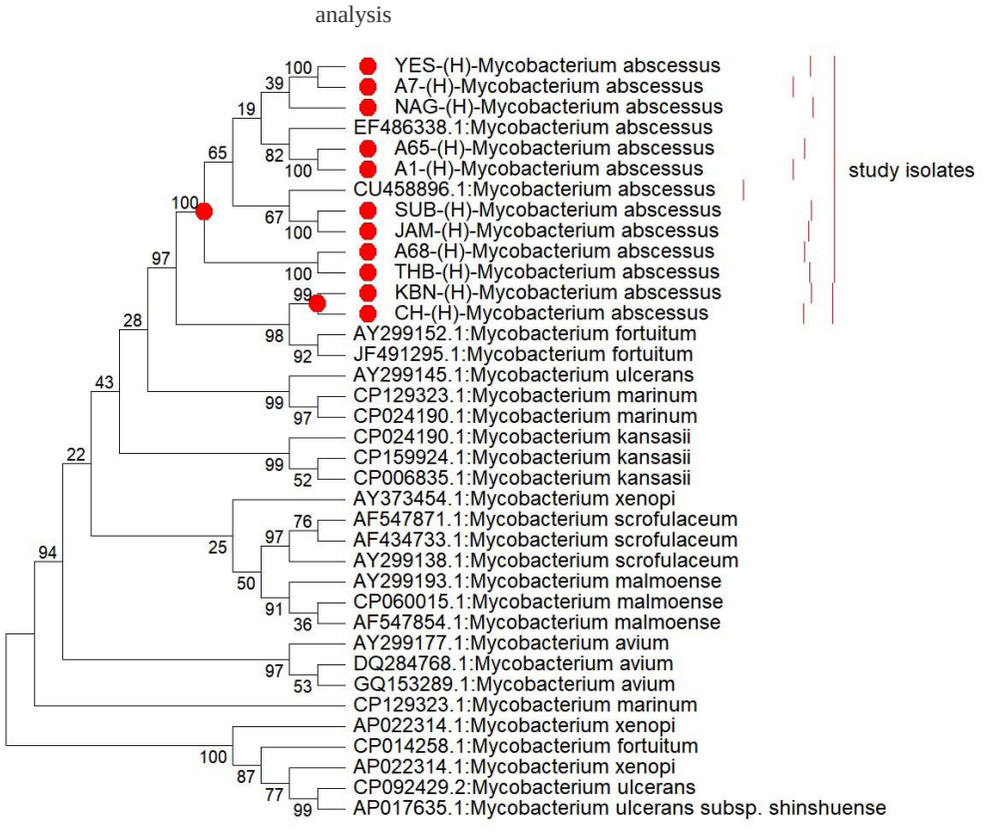

Fig. 5. Phylogenetic analysis based on *hsp65* gene

ular targets for accurate identification of NTM because they possess sufficient interspecies sequence variability while remaining highly conserved within species (7, 8). Previous studies have shown that *rpoB* sequencing provides superior discriminatory power for differentiating closely related rapidly growing mycobacteria compared with conventional biochemical methods (7). In the present study, the combined use of *hsp65* and *rpoB* sequencing enabled reliable species-level identification and minimized the possibility of misclassification. Phylogenetic analysis performed using the Neighbour-Joining method demonstrated close genetic relatedness among the clinical isolates and reference strains. Molecular phylogeny has become an important tool for understanding the epidemiology, transmission dynamics, and evolutionary relationships of NTM species (8). The clustering pattern observed in the present study supports the taxonomic placement of the isolates within the *M. abscessus* complex. Similar phylogenetic clustering patterns have been reported in studies from East Asia and Europe, where clinical isolates of *M. abscessus* showed high genomic similarity despite geographic variation (6).

Drug resistance profiling using the GenoType NTM-DR assay revealed that all isolates belonged to *Mycobacterium abscessus* subsp. *abscessus.* Only one isolate harbored a resistance-associated mutation in the *erm(41)* gene, whereas the remaining isolates showed no resistance-associated mutations in the *erm(41)*, *rrl*, or *rrs* genes. The absence of mutations at positions 2058/2059 of the *rrl* gene suggests susceptibility to macrolides, while the absence of mutations at position 1408 of the *rrs* gene indicates preserved susceptibility to aminoglycosides. These findings are clinically significant because macrolides and aminoglycosides remain the cornerstone drugs for treatment of *M. abscessus* pulmonary disease (10).

Interestingly, all isolates in the present study exhibited the *erm(41)* T28 sequevar pattern. Previous studies have demonstrated that the T28 polymorphism is commonly associated with inducible macrolide resistance in *M. abscessus* subsp. (17). However, no phenotypic resistance-associated mutations were detected in the present isolates. Similar findings have been reported by Mougari et al. (18) who observed that the presence of T28 polymorphism alone may not always correlate with high-level resistance, particularly in isolates lacking additional *rrl* mutations. These findings emphasize the importance of combining molecular assays with phenotypic susceptibility testing for accurate antimicrobial resistance interpretation. The absence of aminoglycoside resistance-associated mutations in the *rrs* gene is encouraging because amikacin remains one of the most effective agents against *M. abscessus* infections (10). Reports from several countries have demonstrated increasing rates of acquired macrolide and aminoglycoside resistance among *M. abscessus* isolates due to prolonged antimicrobial exposure (2, 6). In contrast, the isolates in the present study retained susceptibility to both drug classes, suggesting limited circulation of resistant strains in the study population.

In addition to culture-based methods, several phenotypic and molecular approaches are available for identification of non-tuberculous mycobacteria (NTM). Conventional solid media such as Lowenstein–Jensen and Middlebrook agar are widely used but limited by slow growth and reduced sensitivity for rapidly growing mycobacteria. The MGIT liquid culture system is a standard method due to higher sensitivity and faster detection; however, prior decontamination may reduce viability of rapidly growing organisms such as *Mycobacterium abscessus.* In contrast, RGM medium allows direct inoculation of untreated sputum and improves recovery of rapidly growing NTM, with complete concordance with MGIT observed in the present study. Molecular methods further improve diagnostic accuracy. While 16S rRNA sequencing provides genus-level identification, it has limited resolution within the *M. abscessus* complex. In this study, *hsp65* and *rpoB* sequencing enabled accurate species identification, with *rpoB* offering higher discriminatory power. Line probe assays (GenoType NTM-DR) rapidly detect subspecies and resistance-associated mutations in *erm(41)*, *rrl*, and *rrs* genes, supporting treatment decisions, although they do not detect all resistance mechanisms. Whole genome sequencing (WGS) provides the most comprehensive insight into strain diversity and resistance but remains limited by cost and infrastructure. Overall, combining RGM culture with targeted molecular methods (*hsp65*, *rpoB*, and line probe assays) offers an effective approach for accurate identification of M. abscessus in TB-endemic, resource-limited settings. Future studies incorporating whole genome sequencing may further enhance understanding of strain diversity, transmission patterns, and resistance mechanisms of *Mycobacterium abscessus* in TB-endemic settings.

Study limitation. This study was not designed as a population-based epidemiological survey; therefore, the sample size was not calculated for prevalence estimation. The findings primarily reflect the utility of RGM medium in a clinical diagnostic setting. In addition, the study has certain limitations, including a relatively small sample size and its conduct at a single center, which may limit the generalizability of the findings. Whole genome sequencing was not performed, restricting detailed analysis of strain diversity and resistance mechanisms. Despite these limitations, the study provides important preliminary data on the utility of RGM medium and the molecular characteristics of *Mycobacterium abscessus* isolates in a tuberculosis-endemic setting and may serve as a foundation for future larger-scale studies.

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