TROPICAL LIFE SCIENCES RESEARCH

SHORT COMMUNICATION

Environmental Isolation of Nontuberculous Mycobacteria and Targeted PCR Detection of *Mycobacteroides abscessus*

Authors:

Zhen Yun Siew*, Hien Fuh Ng*, Nurul Asyikin Mahbud, Nazifah Fitriyah Zariman, Siew Tung Wong, Yun Fong Ngeow and Kenny Voon

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Highlights

- Nine NTM species were isolated from soil, faeces, and water samples.
- Novel primer MA16S966F enables rapid, specific detection of *M. abscessus*.
- MA16S966F primer has a detection limit of 1-10 pg of *M. abscessus* DNA.

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EARLY VIEW

SHORT COMMUNICATION

Environmental Isolation of Nontuberculous Mycobacteria and Targeted PCR Detection of *Mycobacteroides abscessus*

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Running Title: NTM Isolation and Rapid Detection of *M. abscessus*

Abstract: Nontuberculous mycobacteria (NTM) are increasingly recognised as opportunistic pathogens in humans, with *Mycobacteroides abscessus* representing one of the most clinically significant species. Rapid and reliable detection of *M. abscessus* remains challenging due to its slow growth rate and limitations in current diagnostic approaches in Malaysia. In this study, nine species of NTM were successfully isolated from soil, animal faeces, and water samples

using a Siew-modified Löwenstein–Jensen agar formulation (SMLJ), with *M. abscessus* being detected and isolated successfully. We observed that the presence of glycerol in transport or culture media interfered with DNA extraction and subsequent PCR detection, highlighting the importance of glycerol-free conditions when samples are intended for molecular identification. To improve diagnostic resolution, a novel forward primer, MA16S966F (966F), was designed to pair with the universal 1492R primer for specific amplification of the 16S rRNA gene of *M. abscessus*. The primer demonstrated robust amplification across annealing temperatures of 45–60 °C and exhibited 100% specificity against a panel of bacterial strains, including other mycobacterial species. Sensitivity testing revealed a detection limit of 10 pg of *M. abscessus* DNA, with very faint amplification still detectable at 1 pg. These findings provide a cost-effective and efficient framework for the environmental isolation and molecular detection of NTM, and establish 966F as a promising tool for rapid screening of *M. abscessus* in clinical and environmental settings.

Keywords: Detection, Environment, Mycobacteriaceae, *Mycobacteroides abscessus*, One Health

Abstrak: Mikobakterium bukan tuberkulosis (NTM) semakin diiktiraf sebagai patogen oportunistik pada manusia, di mana *Mycobacteroides abscessus* merupakan salah satu spesies yang paling signifikan dari segi klinikal. Pengecaman yang pantas dan andal bagi *M. abscessus* masih mencabar disebabkan kadar pertumbuhan bakteria yang perlahan serta kekangan dalam pendekatan diagnostik semasa di Malaysia. Dalam kajian ini, sembilan spesies NTM, termasuk *M. abscessus*, telah berjaya diasingkan daripada sampel tanah, najis haiwan dan air menggunakan formulasi agar Löwenstein–Jensen yang diubah suai oleh Siew (SMLJ). Kami mendapati bahawa gliserol dalam medium pengangkutan atau medium kultur dapat mengganggu proses pengekstrakan DNA dan seterusnya dalam pengecaman PCR. Hal ini menekankan kepentingan penggunaan medium yang bebas gliserol dalam proses pengecaman molekul. Bagi meningkatkan spesifikasi diagnostik, satu novel primer hadapan (sense), MA16S966F (966F), telah direka untuk dipadankan dengan primer universal 1492R bagi meningkatkan spesifikasi gen 16S rRNA *M. abscessus*. Primer ini menunjukkan amplifikasi gen yang teguh merentasi julat suhu sepuh lindap 45–60°C dan menunjukkan 100% kespesifikan terhadap panel strain bakteria yang diuji, termasuk spesies mikobakteria yang lain. Ujian kepekaan menunjukkan had pengesanan sebanyak 10 pg DNA *M. abscessus*, dengan jalur amplifikasi yang sangat lemah masih dapat dikesan pada 1 pg DNA. Kajian ini menyediakan satu rangka kerja yang berkos rendah dan cekap dalam pengasingan patogen dan pengesanan molekul, serta dapat menetapkan 966F sebagai primer yang berpotensi dalam saringan pantas *M. abscessus* untuk tujuan klinikal dan persekitaran.

Kata kunci: Pengesanan, Persekitaran, *Mycobacteriaceae*, *Mycobacteroides abscessus*, One Health

INTRODUCTION

Mycobacteria are a group of acid-fast bacteria often associated with lethal or opportunistic infections. Currently, more than 200 mycobacterium species have been recognised (Shafipour et al., 2021). In general, mycobacteria are classified into three groups: those that cause tuberculosis, those that cause leprosy, and those that do not cause tuberculosis. The most concerning member is *Mycobacterium tuberculosis*, which forms the *Mycobacterium tuberculosis* complex (MTC) with other related species that can cause tuberculosis in humans and animals. Meanwhile, nontuberculous mycobacteria (NTM) are emerging opportunistic pathogens that are widely distributed in the environment. They are responsible for nontuberculous mycobacterial pulmonary disease (NTM-PD), which primarily affects the respiratory tract. Thus, patients with NTM-PD often suffer from chronic nonspecific respiratory and constitutional symptoms (Nguyen et al., 2024).

In addition to NTM-PD, extrapulmonary NTM infections can affect the skin, soft tissue, musculoskeletal system, and other parts of the human body. These infections account for approximately a quarter of mycobacterial infection cases (Malhotra et al., 2024). In some countries, the incidence of NTM infections even exceeds that of MTC infections (Jarchow-MacDonald et al., 2022). According to the Centers for Disease Control and Prevention (CDC) Emerging Infections Program conducted in the United States (USA), *Mycobacterium avium* complex was the most common NTM, accounting for approximately 65% of all NTM infection cases, followed by *Mycobacteroides abscessus* complex, *Mycobacterium fortuitum* group, *Mycobacterium chelonae* complex, and several others. Overall, pulmonary and extrapulmonary NTM infections contributed to approximately 77% and 23% of NTM cases, respectively, across four U.S. states over six months (Grigg et al., 2023).

In Malaysia, *M. abscessus* complex and *Mycobacterium avium* complex were the most predominant NTM clinical isolates from 2018 to 2022 (Mohamad Azranyi et al., 2024). Various factors, including human-to-human transmission (rare), exposure to contaminated sources, and biological vectors, can contribute to both direct and indirect NTM infections (Siew et al., 2024). Additionally, Siew et al. (2024) and Sattar et al. (2021) provided evidence that small mammals and avians may carry NTM in their faeces. Besides, research on NTM often focuses

on their presence in soil and water sources, as they are naturally occurring environmental inhabitants (Honda et al., 2018; Falkinham J. O., 2022). However, the isolation and identification of NTM are often time-consuming due to their relatively slow growth rates compared to other bacteria, as well as the need for multiple molecular techniques (Huang et al., 2020), which can hinder the clinical diagnostic process. Therefore, the development of an affordable and convenient molecular diagnostic method is highly important.

In this study, we aimed to develop a cost-effective approach for the rapid detection, identification, and differentiation of *M. abscessus* from other mycobacterial species and common bacteria, which could be incorporated into existing molecular diagnostic approaches such as PCR-based detection. A novel inner primer was designed to accelerate the prokaryotic DNA barcoding protocol for the specific identification of *M. abscessus*. When combined with an in-house isolation protocol, this approach offers a more efficient, convenient, and cost-effective method for the isolation, detection, and identification of mycobacterial species. Besides, the effect of glycerol on bacterial DNA extraction and PCR detection was evaluated, as it is frequently incorporated into culture broth at concentrations ranging from 10% to 50% to facilitate bacterial transportation and long-term storage (Charalambous et al., 2003; Guérin-Danan C., 1999; Heep et al., 1999; Li et al., 2023).

MATERIALS AND METHODS

Animal Ethics and Sample Collection

Adopted from previous studies with modifications (Siew et al., 2024; Siew et al., 2025; Siew et al., 2026a), small mammals were trapped in a cage first before being relocated onto a plastic sheet. This project was reviewed and approved by the Department of Wildlife and National Parks (PERHILITAN), Peninsular Malaysia (Reference Nos.: JPHLTN.600-6/1/4 JLD5 (79)). Ethical clearance was also obtained from the Animal Welfare and Ethical Review Body (AWERB), UK. Briefly, small mammals from Semenyih were trapped using steel cages of various sizes, and their faeces and urine were collected before the animals were released unharmed. The details of trapping and animal study were published elsewhere (Siew et al., 2026b).

Mycobacteria Isolation

Löwenstein–Jensen (LJ) medium is traditionally dispensed into test tubes as an agar slant. In this study, we adapted the LJ medium for use on agar plates, as previously described by Siew

et al. (2024). This in-house modified formulation, referred to as Siew-modified LJ (SMLJ) agar, eliminates the need for glycerol, egg emulsion, and the Gruft Mycobacterial Supplement (FD053, HiMedia Laboratories).

Prior to sample inoculation, at least one freeze–thaw cycle (FTC) at -80 °C using a CryoCube was required before sample inoculation. If contamination by other bacteria is observed, one or two additional FTCs may be performed. Mycoplasma elimination kits offered by Beyotime (China) and Sangon Biotech (China) were used to minimise contamination.

Some previously collected samples from Tioman Island (river water) and Selangor (water, soil and animal faeces) were used in this study. Briefly, 200 µL of undiluted water or diluted soil suspension was spread onto SMLJ agar plates. For soil and faecal samples, approximately 6–12 g of sample was mixed and resuspended in sterile nutrient broth in 50 mL centrifuge tubes. The tubes were then centrifuged at 1500 rpm for 15 seconds to sediment large debris. Alternatively, tubes could be left undisturbed in a biosafety cabinet or on the bench for at least 1 minute to allow sedimentation. Subsequently, 200 µL of the supernatant was transferred onto SMLJ agar. The inoculated SMLJ plates were incubated at 37 °C for up to 21 days. To prevent the agar from drying out during long incubation periods, the following measures were employed: (1) Replace the Parafilm seal frequently and/or store plates in a sealed zip-lock bag; (2) Pour a thicker layer of SMLJ agar during plate preparation; (3) After 7 days of incubation, add a thin layer of sterile nutrient broth (approximately 200 µL to no more than 1 mL) onto the agar surface; (4) Lower the incubation temperature to no less than 30 °C after the initial 7 days of incubation; (5) Place a water reservoir inside the incubator (e.g., a container of water supplemented with $CuSO_4$ to inhibit microbial growth).

All colonies grown on SMLJ agar were subcultured at least twice on nutrient agar. Ideally, blood agar or Middlebrook agar could be used for subculturing. However, nutrient agar served as a cost-effective alternative. Pure isolates were subsequently preserved in nutrient broth containing approximately 25–30% glycerol for long-term storage at -80°C.

Acid-Fast Staining

To confirm the species isolated using our in-house protocol, acid-fast staining was performed on all colonies using the Acid-Fast Bacillus (AFB) Stain Kit (Ziehl–Neelsen method) obtained from Solarbio, China.

PCR Amplification, Sanger Sequencing and Bioinformatic Analysis

The bacterial DNA barcoding protocol was adapted from a previous study (Siew et al., 2024). Briefly, colony PCR was performed using 2×Taq PCR MasterMix (PC1120; Solarbio, China) and a set of universal primers targeting the conserved 16S rRNA gene region. The forward and reverse primers used were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), respectively. Bacterial nucleic acids were extracted by heat lysis at 99 °C for 10 minutes. Then, PCR amplification was carried out under the following thermal cycling conditions: initial denaturation at 95°C for 2.5 minutes; followed by 35 cycles of denaturation (95°C for 40 seconds), annealing (53°C for 45 seconds), and extension (72°C for 2 minutes); with a final extension at 72°C for 7 minutes. PCR products were then analysed by gel electrophoresis using a 2% agarose gel, initially run at 300 V for 10 minutes and then at 90 V for 15 minutes. Amplicons of approximately 1400–1500 bp, as visualised on the gel, were extracted using the PrimeWay Gel Extraction/PCR Purification Kit (1st BASE, Malaysia). The purified PCR products were subsequently subjected to Sanger sequencing using universal internal primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'), together with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and an ABI 3730xl Genetic Analyzer (Thermo Fisher Scientific, USA). Finally, the sequencing chromatograms generated by the genetic analyzer were analysed and base-called using KB™ Basecaller.

The sequences obtained from bacterial DNA barcoding were subjected to BLAST analysis against the GenBank database. If the top BLAST hit corresponded to an unnamed *Mycobacterium* sp., the closest officially named *Mycobacterium* sp. was selected instead.

Effect of Glycerol and Culture Medium on Boiling Extraction

A trial was conducted to examine the effect of glycerol and culture medium on the boiling extraction method. Briefly, an adjusted bacterial suspension containing 0.5 McFarland of *M. abscessus* strain SIEW/TupaiaGlis/UNM/2023 was inoculated into four different conditions: water alone, water supplemented with 25% glycerol, nutrient broth alone, and nutrient broth supplemented with 25% glycerol, followed by PCR analysis.

Primer Design and Testing

A list of candidate internal primers targeting the conserved 16S rRNA gene region of *Mycobacteroides abscessus* was generated using the PrimerQuest Tool (https://www.idtdna.com/PrimerQuest/Home/Index), a free online tool provided by Integrated

DNA Technologies (IDT), USA. Candidate primers were analysed using BLAST against the GenBank database, and the primer with the highest sequence specificity and similarity was selected for further testing. The selected primer was used in combination with universal primers 27F and 1492R and evaluated for optimal annealing temperature, specificity, and sensitivity.

To determine the optimal annealing temperature, a gradient PCR was performed using the Eppendorf Mastercycler Nexus Gradient, with annealing temperatures ranging from 45.0 °C to 60.0 °C (45.0, 45.4, 46.3, 47.7, 49.7, 51.6, 53.4, 55.3, 57.3, 58.7, 59.6, and 60.0 °C).

Primer specificity was assessed by testing the primer set against various established bacterial isolates and American Type Culture Collection (ATCC) reference strains (refer to Supplementary Table S1).

For sensitivity testing, genomic DNA was extracted from the positive control strain, *Mycobacteroides abscessus* subsp. *massiliense* SIEW/TupaiaGlis/UNM/2023 (GenBank accession: CP163267), using the PrimeWay Genomic II DNA Extraction Kit (KIT-9022; 1st BASE, Malaysia). The concentration and purity of the extracted DNA were measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA), followed by preparation of a 10-fold serial dilution. The limit of detection (LOD) of the designed primer was subsequently determined using conventional PCR and agarose gel electrophoresis. In a total volume of 20µL PCR mixture, 8µL of extracted bacterial DNA was added with 1µL of each 20µM primer and 10µL exTEN II PCR Master Mix (1st BASE, Malaysia). Then, PCR was performed as described above.

RESULTS

SMLJ agar was used to isolate mycobacteria from previously collected, pooled environmental and animal samples. The samples were inoculated onto the agar and incubated at 37 °C (ranging from 30 - 37℃) for up to 21 days. The earliest colony formation was observed at 2 days post-inoculation. All resulting colonies were isolated, sequenced, and preserved. Acid-fast staining confirmed that all (100%) of the isolates were stained positive (pink, Fig. 1A). A total of nine different species of NTM were successfully identified from soil, water and animal samples. Soil and animal samples exhibited a greater diversity of NTM species and a higher viable bacterial density than water samples, with 80 CFU/mL and 20 CFU/mL (a four-fold difference), respectively.

Among the soil and animal (*Paradoxurus* sp.) isolates (Fig. 1B), *Mycolicibacterium mucogenicum* was the most frequently isolated mycobacterium (n = 11, 47.8%), followed by *M. abscessus* (n = 7, 30.4%), *Mycolicibacterium porcinum* (n = 2, 8.7%), *Mycolicibacterium phocaicum* (n = 1, 4.3%), *Mycolicibacterium houstonense* (n = 1, 4.3%) and *Mycolicibacterium alvei* (n = 1, 4.3%). In the water samples (Fig. 1C), *Mycolicibacterium conceptionense* was the most frequently detected species (n = 2, 50.0%), followed by *Mycobacterium cosmeticum* (n = 1, 25.0%) and *Mycolicibacterium senegalense* (n = 1, 25.0%). The details of each bacterial isolate will be published elsewhere.

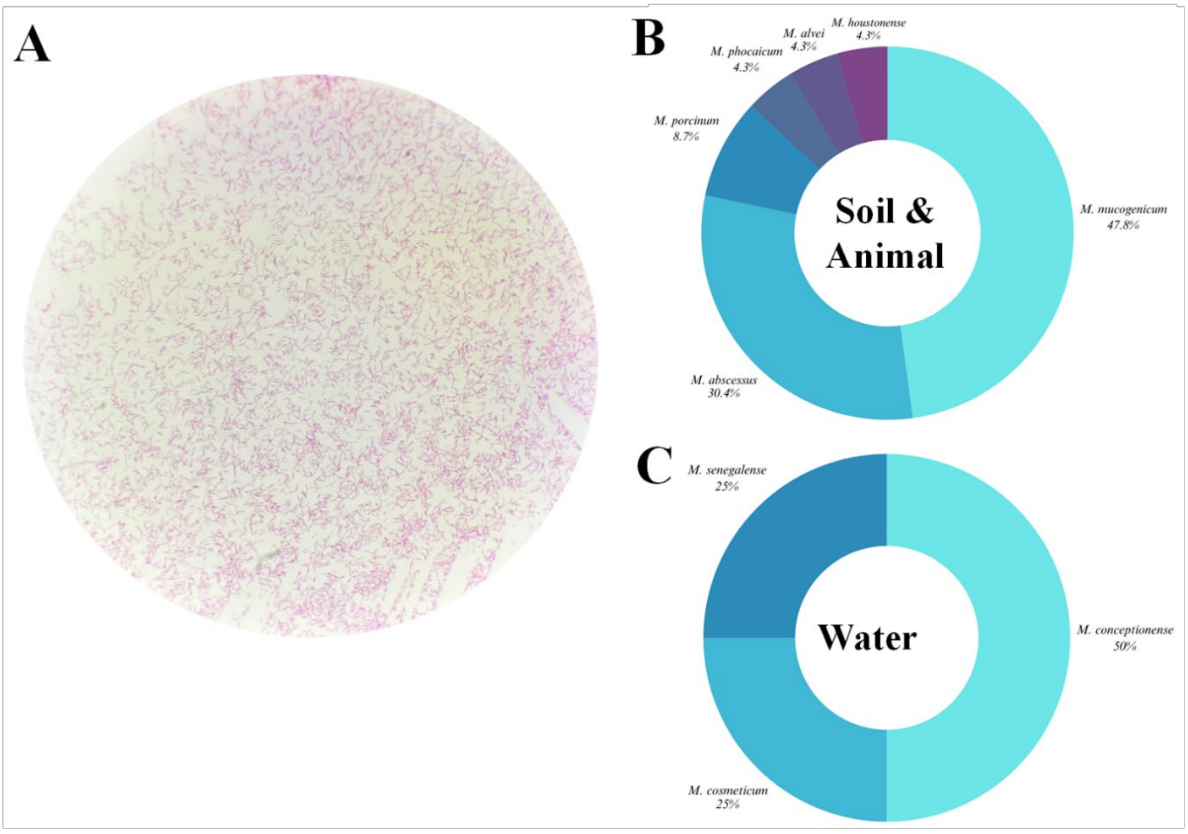


Figure 1. Identification of mycobacteria isolated from different samples. (A) Representative figure of acid-fast positive bacilli. *M. abscessus* ATCC 19977 was used as the positive control. All isolates obtained in this study were stained positive (100%). For the staining protocol, alternative acid-fast staining can be carried out using the modified Kinyoun method or the Auramine O method, both of which are available from Solarbio (China) and other suppliers. Nevertheless, the Ziehl–Neelsen method is recommended, as it generally provides clearer staining results and can be observed with a standard compound microscope. (B) Mycobacteria isolated from soil and animal samples. (C) Mycobacteria isolated from water samples.

In the present study, direct boiling of bacterial suspensions in glycerol-supplemented broth or water did not permit successful extraction. Conversely, boiling extraction performed in broth or water devoid of glycerol resulted in positive PCR detection (Fig. 2).

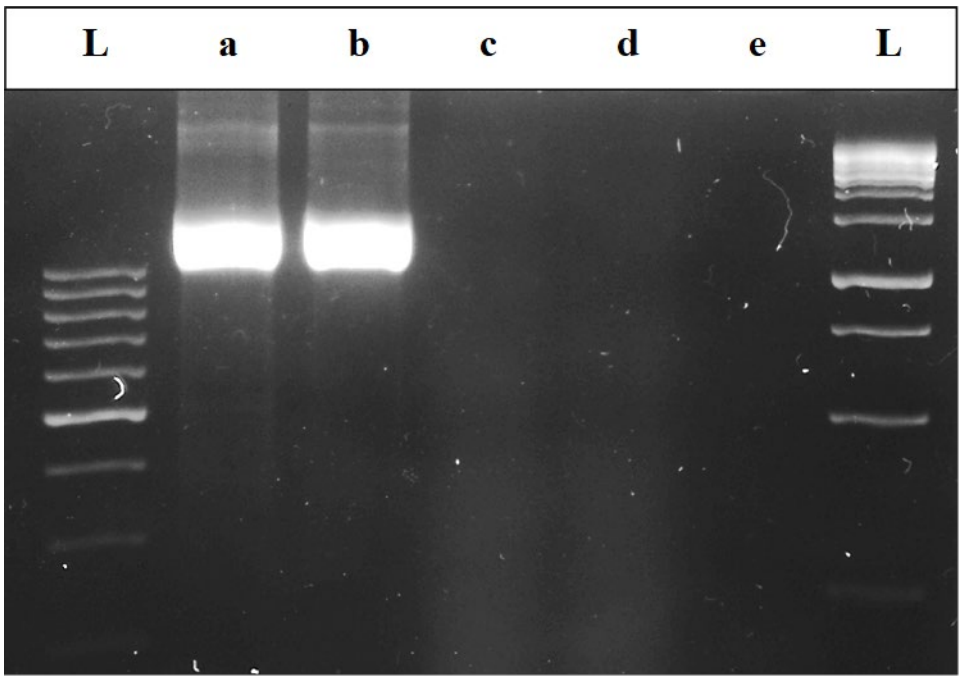


Figure 2. Effect of glycerol and culture medium on boiling extraction. An approximately 1400 bp band was amplified from DNA extracted from bacterial suspensions in nutrient broth and water without glycerol. L, DNA ladder; a, water; b, nutrient broth; c, water with 25% glycerol; d, nutrient broth with 25% glycerol; e, no-template control (NTC).

A new inner primer, MA16S966F (abbreviated as 966F), was designed and tested extensively in this study. The primer was designed to bind to position 966 of the *M. abscessus* 16S rRNA (Supplementary Fig. S1). The sequence of 966F is 5'-CACAGGACGTACCTAGAGATAG-3'. However, primer BLAST also suggested that the primer might bind to certain strains of *Mycobacteroides chelonae* and *Mycobacteroides saopaulense*. To determine the optimal annealing temperature of 966F, it was tested together with the 1492R primer within a range of temperatures. This inner primer set can produce a positive band with a size of around 526 bp at annealing temperatures between 45 °C and 60 °C (Fig. 3A).

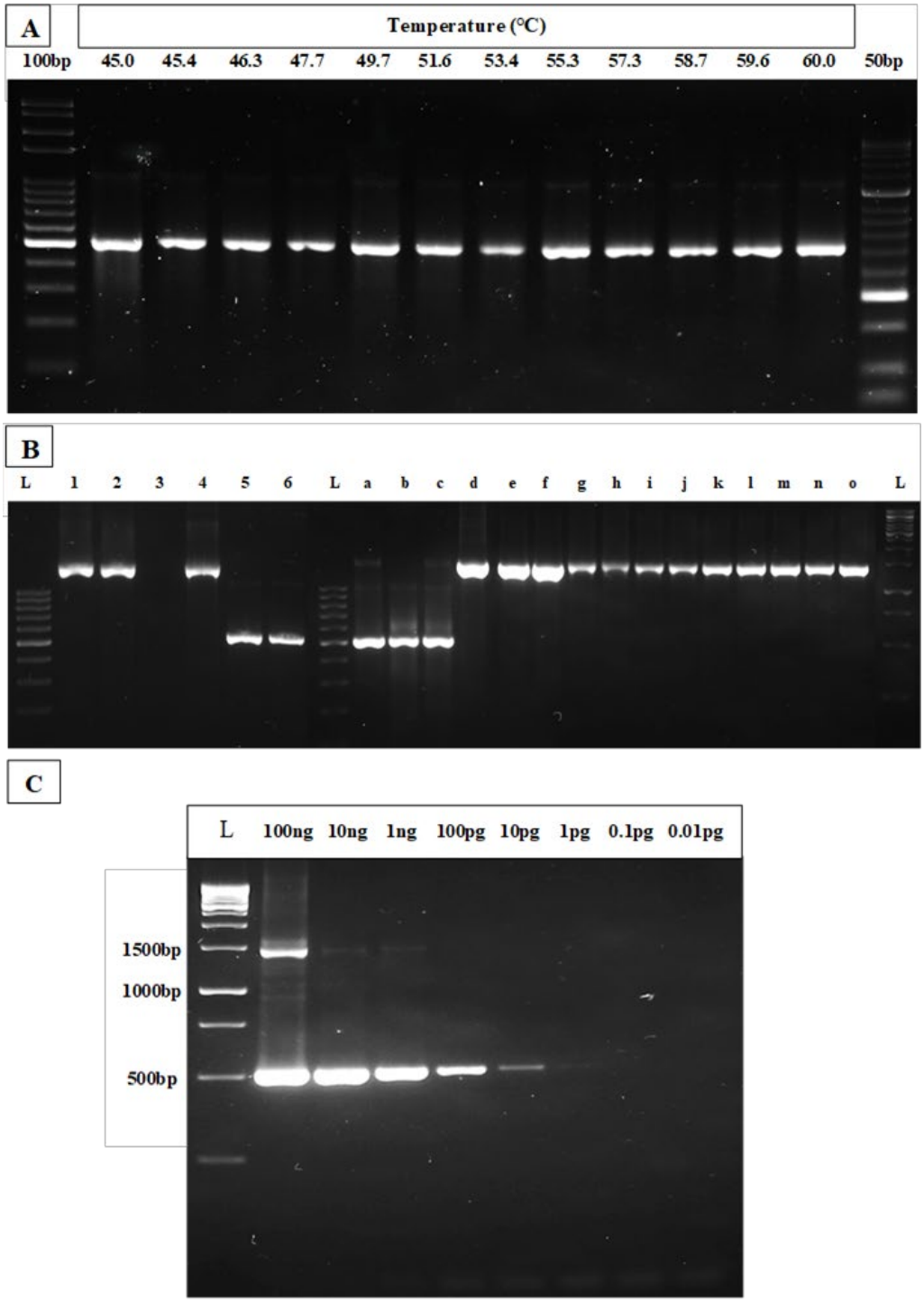


Figure 3. Primer testing under different conditions and primer combinations. (A) The inner primer pair 966F and 1492R produced a positive band at annealing temperatures between 45 °C and 60 °C. (B) The inner primer pair functioned both independently and in combination with the outer universal primer pair. Lanes: 1, *L. paracasei* strain Shirota with outer primers only; 2, *L. paracasei* strain Shirota with outer + 966F; 3, *L. paracasei* strain Shirota with inner primers only; 4, *M. abscessus* ATCC19977 with outer primers only; 5, *M. abscessus* ATCC19977 with outer + 966F; 6, *M. abscessus* ATCC19977 with inner primers only. Lanes

a–o: outer primers + 966F tested on different mycobacteria. a, *M. abscessus* ATCC19977; b, *M. abscessus* subsp. *massiliense* DSM 45103; c, *M. abscessus* subsp. *massiliense* strain SIEW/TupaiaGlis/UNM/2023; d–o, *Mycobacterium* isolates obtained in this study. Complete results of all tested bacteria are presented in Supplementary Table S1. Lanes: L, 100bp and 50bp are all DNA ladders. (C) Two thick bands were observed with 100 ng of *M. abscessus* DNA, while the ~1500 bp band appeared faintly with 10 ng and 1 ng inputs. The limit of detection using the inner primer pair was 10 pg with 35 amplification cycles, although an extremely faint ~526 bp band was visible at 1 pg.

The 966F primer can also be used alongside the outer universal primer set (27F and 1492R). In such cases, amplification of the shorter amplicon (inner region) is generally favoured over the longer amplicon (outer region) (Fig. 3B). For the primer specificity test, a panel of bacterial strains was evaluated, and the results demonstrated 100% specificity of 966F across all tested bacteria (Fig. 3B, Supplementary Table S1).

When 100 ng of *M. abscessus* DNA was used as the template, two prominent amplification products of approximately 1500 bp (amplicon of 27F and 1492R) and 526 bp (amplicon of 966F and 1492R) were generated. The larger ~1500 bp fragment remained detectable, though faint, at lower DNA inputs of 10 ng and 1 ng. In contrast, at template concentrations ranging from 1-100 pg, only the ~526 bp fragment was consistently observed, with the band at 1 pg appearing extremely faint (Fig. 3C).

DISCUSSION

Apart from *M. tuberculosis*, several emerging NTMs have been associated with infections and even mortality (Siew et al., 2024). Among these, *M. abscessus* poses a substantial threat to public health. In this study, nine mycobacterial species were isolated and identified from environmental reservoirs, including soil, animal faeces, and water samples.

These reservoirs may serve as potential sources of opportunistic mycobacterial exposure to both humans and animals. Such NTM are commonly found in environmental settings worldwide, including Malaysia. Notably, several of these species have previously been associated with human infections and mortality in Malaysia (Faridah et al., 2021; Mohamad Azranyi et al., 2024). This highlights the interconnected nature of environmental and clinical settings in the transmission and persistence of NTM. By recognising these reservoirs, the study underscores the broader public health significance of environmental surveillance. In turn, this strengthens the rationale for implementing rapid and cost-effective molecular diagnostic

approaches, which are essential not only for clinical diagnosis but also for monitoring environmental sources that may contribute to infection risk.

Regarding the *Mycobacteria* isolation protocol, at least one FTC at -80 °C using a CryoCube was required before sample inoculation. This step is critical, as mycobacteria are generally more tolerant of freezing in the absence of cryoprotectants (e.g., glycerol, DMSO) than most other bacterial species. If contamination by other bacteria is observed, one or two additional FTCs may be performed. However, a total of four or more FTCs is not recommended, as Portaels et al. (1988) reported that slow-growing mycobacteria, such as *M. leprae*, exhibit reduced tolerance to repeated FTCs compared to other species. In support of this, Shu et al. (2012) demonstrated that mycobacteria can survive FTCs even in liquid nitrogen (LN_2) without glycerol, although the cooling rate was shown to be a critical factor for survival and recovery. Similarly, Siew et al. (2024; 2025) observed that *M. abscessus* remained viable after multiple FTCs in viral transport medium (VTM), which contains high concentrations (2X working concentration) of penicillin–streptomycin–amphotericin B, also known as Antibiotic-Antimycotic (Nacalai, Japan), despite the absence of glycerol.

Besides, mycoplasma represents a significant source of contamination, particularly in animal samples from farms or animal husbandry. Similar to mycobacteria, mycoplasma can withstand FTCs in culture medium lacking cryoprotectants, although reduced cell recovery and viability are typically observed (Gille et al., 2018; Al-Farha et al., 2018). They are also capable of proliferating in standard culture media and agar, albeit at slower growth rates under suboptimal conditions (Volokhov et al., 2008). To minimise contamination, *Mycoplasma* removal agents such as Normocin, Plasmocin, and Primocin (InvivoGen) are available, while more cost-effective alternatives of elimination kits are offered by Beyotime (China) and Sangon Biotech (China). In addition, PCR-based *Mycoplasma* detection kits (Solarbio, China) were utilised to monitor potential mycoplasma contamination in the culture medium.

Molecular detection approaches were further evaluated, revealing that the presence of glycerol could interfere with colony PCR or boiling extraction. Therefore, glycerol should not be added to transport media if samples are intended for the isolation and PCR-based detection of mycobacteria. Previous studies have reported that glycerol concentrations of approximately 15–20% may significantly improve PCR amplification and yield, whereas concentrations that are too low or too high (≤10% or ≥25%) may negatively affect PCR amplification (Jurišić et al., 2016). However, in the present study involving transport and storage media, glycerol appeared to interfere primarily with the boiling extraction process rather than the PCR amplification itself. The heat-protective effect of glycerol on proteins has been demonstrated previously (Müller et

al., 2007), which may explain its negative impact on the efficiency of the boiling extraction protocol, possibly by reducing effective cellular lysis and subsequent DNA release.

A novel forward primer, MA16S966F (966F), was designed for the rapid detection of *M. abscessus*. This primer can be used in combination with the universal primers 27F and 1492R for PCR screening in clinical settings, providing a cheaper, faster, and more convenient alternative to next-generation sequencing (NGS). In general, PCR detection followed by Sanger sequencing requires a turnaround time of 1–2 days, whereas NGS workflows typically require a longer turnaround time, often exceeding 5 days. Despite the higher sensitivity and specificity of NGS, it is not routinely used in hospitals due to its higher cost and greater resource requirements compared to PCR and Sanger sequencing. Therefore, the incorporation of 966F for the detection of *M. abscessus* could provide substantial benefits for public health and clinical diagnostics.

To further discuss the diagnostic approaches, phenotypic methods such as acid-fast staining and biochemical assays are insufficient for definitive identification of mycobacterial species. Therefore, genotypic methods, including line probe assays (LPA), oligochromatographic assays, PCR, NGS, and MALDI-TOF, are more commonly employed for species-level confirmation of mycobacteria (Reis et al., 2026; Jia Khor et al., 2021). Among these approaches, PCR remains one of the most widely used diagnostic methods due to its sensitivity, relatively short turnaround time, affordability, and broad accessibility, particularly in Southeast Asia and other low- and middle-income countries.

Notably, 966F exhibited robust amplification across a wide range of annealing temperatures (45–60 °C), consistently generating a detectable band. The primer also demonstrated 100% specificity against the bacterial panel tested, effectively distinguishing *M. abscessus* from other mycobacterial species. The LOD for 966F was 10 pg of *M. abscessus* DNA, with only a very faint band observable at 1 pg. Perhaps increasing the amplification to 40 cycles may enhance band intensity at this lowest concentration.

This study has several limitations: (1) More than 200 *Mycobacterium* sp. are currently recognised (Brown-Elliott et al., 2025), but not all were tested here. In particular, *Mycobacteroides chelonae* and *Mycobacteroides saopaulense* were not examined, and primer-BLAST analysis indicated that the 966F primer pair may produce non-specific amplifications in these species. Besides, the closely genetically related *M. abscessus* subsp. *bolletii* was not tested in this study. Remarkably, all *M. abscessus* subspecies share an identical sequence of the 16S rRNA gene. (2) Large-scale validation

using retrospective patient samples with confirmed *M. abscessus* infection was not performed and should be considered in future work.

CONCLUSION

In conclusion, the primers developed in this study represent a promising tool for the detection of *Mycobacteroides abscessus*. Although further validation across a wider range of mycobacterial species and retrospective clinical samples is necessary, these findings provide a foundation for the development of rapid and reliable molecular assays to enhance the diagnosis and management of *M. abscessus* infections. Moreover, this study highlights that the addition of glycerol to the transport and storage medium may impede the direct PCR detection of mycobacteria, except when intended for long-term cryopreservation.

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DECLARATION OF COMPETING INTEREST

The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTIONS

Zhen Yun Siew, Hien Fuh Ng, Yun Fong Ngeow: Conceptualisation, research funding, study design, execution and data collection, research supervision, sampling and field support, data analysis, manuscript writing and editing.
Nurul Asyikin Mahbud, Nazifah Fitriyah Zariman, Siew Tung Wong, Kenny Voon: Study design, execution and data collection, sampling and field support, data analysis, manuscript writing and editing.
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SUPPLEMENTARY MATERIALS

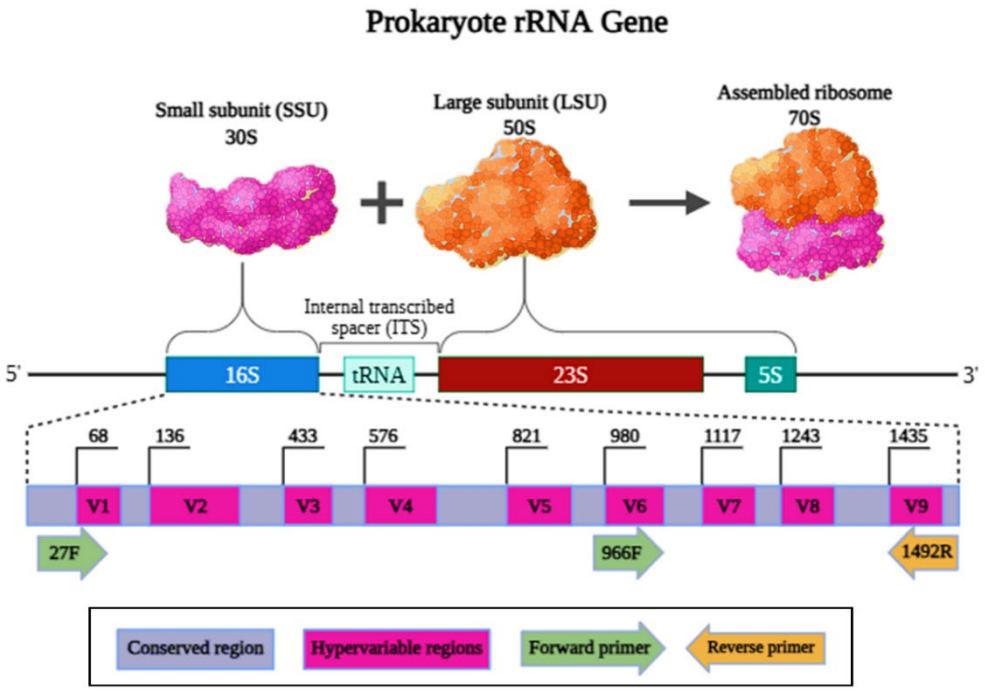


Figure S1. Graphical illustration of primers targeting the 16S rRNA gene. The universal primers 27F and 1492R, commonly used in bacterial DNA barcoding, together with the newly designed MA16S966F (abbreviated as 966F), were employed to amplify the 16S rRNA gene, which encodes the 30S small ribosomal subunit. The outer primer pair (27F and 1492R) spans the hypervariable regions V1 to V9, whereas the inner primer pair (966F and 1492R) targets only regions V6 to V9. The expected amplicon size generated by the inner primer set is approximately 526 bp.

Table S1. The results of primer testing using 966F and 1492R primers.

	Result
Mycobacteroides abscessus strain L948 ATCC 19977	Positive
Mycobacteroides abscessus subsp. *massiliense* DSM 45103	
Mycobacteroides abscessus subsp. *massiliense* strain SIEW/TupaiaGlis/UNM/2023	
M. abscessus isolates obtained in this study[A]	
Acinetobacter baumannii strain 2208 [81, DSM 6974] ATCC 19606	Negative
Bacillus cereus FDA strain PCI 213 [ATCC 9634, NCTC 10320, S.A. Waksman strain O] ATCC 11778	
Escherichia coli strain CDC AR-0346	
Escherichia coli strain Crooks ATCC 8739	
Klebsiella pneumoniae strain AIS 2007023 [6179] ATCC BAA-1706	
Klebsiella pneumoniae strain ART 2008133 [D-05, 1338] ATCC BAA-1705	
Lacticaseibacillus paracasei strain Shirota[B]	
Mycobacterium tuberculosis strain H37Ra	
Mycolicibacterium alvei[C]	
Mycolicibacterium conceptionense[D]	
Mycobacterium cosmeticum[D]	
Mycobacterium fortuitum subsp. *fortuitum* strain [TMC 1529] ATCC 6841	
Mycolicibacterium houstonense[C]	
Mycobacterium porcinum[A]	
Mycolicibacterium senegalense[D]	
Mycobacterium smegmatis strain mc(2)155 ATCC 700084	
Mycolicibacterium mucogenicum[A]	
Mycolicibacterium phocaicum[A]	
Pseudomonas aeruginosa (Schroeter) Migula strain [CCEB 481, MDB strain BU 277, NCIB 8295, NCPPB 1965, NCTC 10332, NRRL B-771, R. Hugh 815] ATCC 10145	
Salmonella enterica subsp. *enterica* serovar Newport strain NCTC 129 ATCC 6962	
Staphylococcus aureus subsp. aureus Rosenbach strain S13 ATCC 11632	
Staphylococcus aureus subsp. *aureus* strain Mu50 [NRS1] ATCC 700699	
Staphylococcus epidermidis FDA strain PCI 1200 ATCC 12228	

A positive result indicates the presence of a band of approximately 526 bp generated using the universal primer pair plus 966F, whereas a negative result indicates the absence of a band at this size. Bacteria strain sources: A, isolated from soil samples; B, obtained from Yakult; C, isolated from the faeces of *Paradoxurus* sp.; D, isolated from water samples.