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Detection of *Mycobacterium abscessus* Complex at the Subspecies Level from Surgical Site Infection at a Tertiary Care Hospital in Bangladesh

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Abstract

Background: *Mycobacterium abscessus* complex (MABC) exhibits the highest degree of antimicrobial resistance among the rapidly growing mycobacteria (RGM). These nontuberculous mycobacteria (NTM) are capable of causing various human diseases including surgical site infections (SSI). MABC consists of 3 subspecies: *Mycobacterium abscessus* subspecies *abscessus* (*M. abscessus*), *Mycobacterium abscessus* subspecies *bolletii (M. bolletii)*, and *Mycobacterium abscessus* subspecies *massiliense (M. massiliense)*. Significant differences in antimicrobial susceptibility are observed among these MABC subspecies. Since treatment options for MABC are limited and a combination of drugs is needed for prolong period, it is very important to detect MABC at the subspecies level to ensure appropriate patient management. **Objective:** This study was conducted to detect and differentiate the three subspecies of MABC. **Methodology:** This cross-sectional study was conducted in the Department of Microbiology of BIRDEM General Hospital from August 2022 to February 2024. Pus/ wound swabs were collected from 150 patients suffering from surgical site infection (SSI). RGM was detected by microscopy and culture. DNA was extracted from the isolates and utilizing 3 sets of subspecies-specific primers, MABC were detected at the subspecies level using conventional PCR in single as well as multiplex settings. **Results:** Culture of the 150 pus/wound swab samples revealed growth of RGM in 28 cases (18.67%). Among the 28 RGM isolates, 19 (67.86%) MABC were detected by PCR of which 13 (68.42%) were *M. abscessus* and 6 (31.58%) were *M. massiliense*. **Conclusion:** This subspecies-specific PCR test can be routinely used in laboratories to detect MABC at the subspecies level, aiding the selection of appropriate treatment regimens.

Keywords: nontuberculous mycobacteria; rapidly growing mycobacteria; *M. abscessus* complex; surgical site infection

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Introduction

Nontuberculous mycobacteria (NTM) are the mycobacteria other than *Mycobacterium tuberculosis* complex (MTBC) and *M. leprae*[1]. Rapidly growing mycobacteria (RGM) are the mycobacteria that grow within 7 days of inoculation on solid culture media and fall under Group IV Runyon Classification of NTM[2]. *Mycobacterium (Mycobacteroides) abscessus* complex (MABC) is not only the most common member but also the one with the highest degree of antimicrobial resistance among all the RGM[3]. The other members include *Mycobacterium fortuitum, Mycobacterium chelonae, Mycobacterium smegmatis, Mycobacterium mucogenicum, Mycobacterium mageritense. Mycobacterium wolinskyi* and early pigmented RGM groups[4]. These ubiquitous acid-fast bacteria are

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capable of causing pulmonary as well as extrapulmonary infections in both normal and immunocompromised individuals[5-8].

A study conducted in India reported that 10.9% of surgical site infections (SSI) were caused by RGM, of which 39.4% were due to MABC[5]. Studies in Bangladesh have reported NTM as a cause of SSI[9-11] with MABC being the commonest one found in two of these studies[10-11]. This is attributed to improper sterilization of surgical equipment, especially those involved in laparoscopic surgery, as the RGM are resistant to disinfectants, including 2.0% glutaraldehyde and peracetic acid[10,12-13]. In countries with a high burden of tuberculosis (TB), infections caused by RGM are often misdiagnosed and mistreated as TB, resulting in treatment failure, since RGM are inherently resistant to the first-line anti-TB drugs[6,10,14]. It is important to identify RGM at the subspecies level not only because their treatment involves a combination of drugs for a prolonged period[8], but also because they show significant variation in susceptibility patterns within the different species of RGM groups, as well as within the subspecies of MABC[15]. The MABC are usually only susceptible to amikacin, linezolid and clarithromycin. In contrast, other RGMs are susceptible to a wider range of antimicrobials, including these 3 antimicrobials as well as ciprofloxacin, moxifloxacin, doxycycline, imipenem, and meropenem[10,16]. CLSI recommends identification of the species before performing susceptibility testing by broth microdilution[17-18].

There are 3 subspecies in MABC: *Mycobacterium abscessus* subspecies *abscessus (M. abscessus), Mycobacterium abscessus* subspecies *bolletii (M. bolletii)*, and *Mycobacterium abscessus* subspecies *massiliense (M. massiliense)*[15]. Though these 3 subspecies are phenotypically identical, the erythromycin ribosome methyltransferase *(erm41)* gene, which is responsible for macrolide resistance, has a large portion deleted (making it non-functional) in *M. massiliense* but not so in the other 2 subspecies[15]. Therefore, macrolides can be used as a treatment for *M. massiliense* but may lead to treatment failure in cases of *M. abscessus and M. bolletii*. It is also seen that moxifloxacin and tigecycline are more effective against *M. massiliense* than *M. abscessus*[19].

Since these 3 subspecies are phenotypically identical, conventional biochemical tests fail to differentiate them[15]. New molecular tests, such as partial gene and whole genome sequencing, line probe assay, and MALDI-TOF, are in use but these tests are expensive and require specialized equipment and trained individuals[20]. Hence, these tests are not easy to use routinely for a resource-limited country like Bangladesh. Compared to other molecular methods, conventional PCR using subspecies-specific primers can be an easy, quick, and feasible test to differentiate the subspecies of MABC[15]. In Bangladesh, NTM detection by real-time multiplex PCR for MTB/NTM[9], RGM species detection by PCR-RFLP[10] and by hsp-65 gene sequencing[11] have been reported. However, in these studies, subspecies of MABC were not detected. In this present study, MABC were detected at the subspecies level using conventional PCR from SSI in a tertiary care hospital in Bangladesh.

Methodology

Study Settings and Population: This cross-sectional study was conducted in the Department of Microbiology of BIRDEM General Hospital from August 2022 to February 2024. Pus or wound swab was collected from 150 surgical site infection cases with the following inclusion criteria: cases with chronic discharge from surgical site, onset of discharge after 2 weeks following operation, clinically suspected tuberculosis, not responding to common antimicrobials and/or first line anti- tubercular drugs, together with the absence of bacterial growth on conventional culture for 48 hours irrespective of age, sex or comorbidities. To exclude tuberculosis, all samples were sent for GeneXpert. Written informed consent was taken and information of patients including their age, sex, history, clinical and laboratory findings were documented in a data collection form.

Laboratory Procedure: Pus or wound swab samples were subjected to Ziehl-Neelsen (ZN) staining and culture on sheep blood agar and MacConkey agar. The plates were incubated at 37°C for up to 7 days according to standard procedure[21]. The presence of growth of bacterial colony was checked each day, and the culture growth was subjected to ZN staining to detect AFB. A total of 28 RGM were isolated. As reference strains, *Mycobacterium abscessus* (Accession number: SAMN44467547), *Mycobacterium massiliense* (Accession number: SAMN44467509), *Mycobacterium fortuitum* (Accession number: SAMN44416141), and *Mycobacterium conceptionense* (Accession number: SAMN44467510) were included. These reference strains were also isolated from SSI, characterized earlier by whole genome sequencing, and were archived at -20°C in trypticase soy broth.

They were thawed and revived on blood agar media, followed by incubation at 37°C for 5-7days[22]. As negative controls, *Mycobacterium tuberculosis* (MTB) H37Rv strain, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 25922 were used. All the control strains were collected from the Institute of Epidemiology, Disease Control and Research (IEDCR), Bangladesh. The MTB H37Rv strain was inoculated onto Löwenstein-Jensen media and incubated at 37°C for 21 days. The rest of the control strains were inoculated onto sheep blood agar and incubated at 37°C overnight.

PCR Amplification

DNA extraction, Primers, and parameters: DNA was extracted from all the clinical RGM isolates (including those used as references) and the control strains by the boiling method[23]. Purity of all DNA was checked by observing 260/280 in Ultraviolet (UV) photometer (Eppendorf BioPhotometer® D30, Germany), which was between 1.6-1.7. Extracted DNA was stored at - 20°C until PCR amplification. Primers listed in Table 1 were obtained from Macrogen Incorporation, Korea, and were used in both single and multiplex PCR settings for differentiating the 3 subspecies of *M. abscessus*[15].

For the single PCR assay, a total volume of 25 µl mixture was prepared by mixing 12.5µl of EmeraldAmp® GT PCR Master Mix (Takara Bio USA, Inc.) (composed of PCR buffer, $MgCl_2$, deoxyneucleotide triphosphate/ dNTP and taq polymerase enzyme), 1 µl forward primer (10µM working solution), 1 µl reverse primer (10µM working solution), 1µl of DNA template and 9.5µl of nuclease free water. For the multiplex PCR assay, 12.5 µl Master Mix, 1 µl of extracted DNA, 0.5 µl of each of the forward and reverse primers, 1 µl of dimethyl sulfoxide (DMSO), and 7.5 µl nuclease-free water were used (total volume = 25 µl).

Amplification was carried out in an automated DNA thermal cycler (ASTEC thermal cycler, Gene Atlas, Japan) using these PCR cycling conditions: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 1 min; annealing at 60°C for 1 min; and extension at 72°C for 1.5 min; with final extension at 72°C for 10 min. The amplified products were separated by 3.5% agarose gel electrophoresis at 135V for 45 minutes and were visualized by using a UV transilluminator.

Statistical Analysis: Statistical analysis was performed by Windows-based software named Statistical Package for Social Science (SPSS), version 26.0 (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). Categorical data were summarized in terms of frequency counts and percentages.

Ethical Consideration: The study was ethically approved by the Institutional Review Board (IRB) of the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) Academy. All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional Research Ethics. Participants in the study were informed about the procedure and purpose of the study, and confidentiality of information was maintained. All participants willingly consented to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Table 1: Primer Sequences, Amplicon Sizes, Melting Temperature, and GC Ratio for *Mycobacterium abscessus* subspecies-specific PCRs

Target *M. abscessus* subspecies	Primer Sequence (5′→3′)	Tm (ºC)	%GC ratio	Amplicon size (bp)	Reference
M. abscessus subspecies *abscessus*	5’-TCC AAC CGA GAT GAC CAG AG	56.2	55	161	Akwani et al[12]
	5’-CCG ATA TAG AAT TCG GCC AGC AAG T	58.8	48		
M. abscessus subspecies *bolletii*	5’-GGC TTC ACG TTC AAT CAG TTT CTA	55.4	41.7	176	
	5’-CGA TTC ACT GCT CCG CAT TC	56.5	55		
M. abscessus subspecies *massiliense*	5’-AGG GTA TTT CAC TTG ATG ACC TAT G	54.4	40	134	
	5’-GAT CGC CGT CAG CGA ATA AT	55.3	50		

Note: Tm=melting temperature; °C= degree centigrade; %= percentage

Results

The presence of RGM (growth within 7 days and AFB on culture smear) were detected in 28 (18.67%) pus/wound swab samples (Figures II, III) of which 21 (75%) isolates also had AFB in their corresponding direct smear of pus/wound swab samples (Figure I). The age range was 20-80 years; female to male ratio was 14:9; 22 (78.57%) patients underwent laparoscopic surgery, 6 (21.43%) patients had open surgery; 3 patients had diabetes, 1 patient was hypertensive, and 3 patients were smokers. Only 1 sample tested positive for MTB without Rifampicin resistance in GeneXpert but a total of 8 patients had previously completed first-line anti-TB therapy (Table 2).

Table 2: Demographic, Clinical, and Laboratory Parameters of RGM Causing SSI (n=28)

Parameters	Frequency	Percent
Female	18	64.3
Male	10	35.7
Type of surgery:		
• Laparoscopic cholecystectomy	11	39.3
• Laparoscopic hernioplasty with mesh repair	4	14.3
• Other laparoscopic surgery	7	25.0
• Open surgery	6	21.4
Comorbidities		
• Diabetes	3	10.7
• Hypertension	1	3.6
GeneXpert: +ve for MTB	1	3.6
Anti-TB therapy	8	28.6
AFB on ZN on Pus/ Wound swab	21	75.0

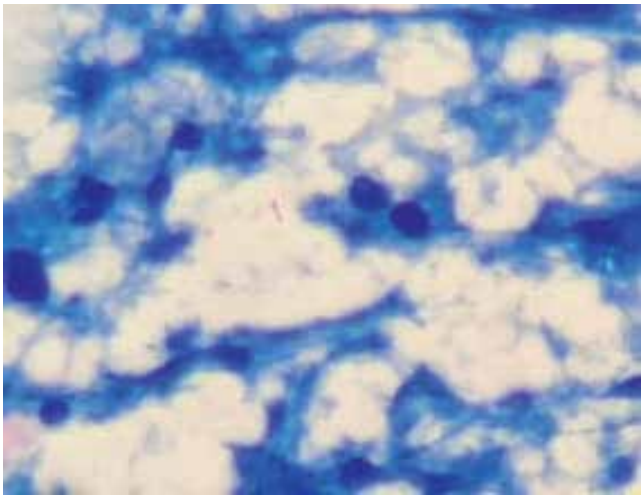

Figure I: AFB in Ziehl-Neelsen (ZN) stain of direct smear microscopy of pus sample from a surgical site infection

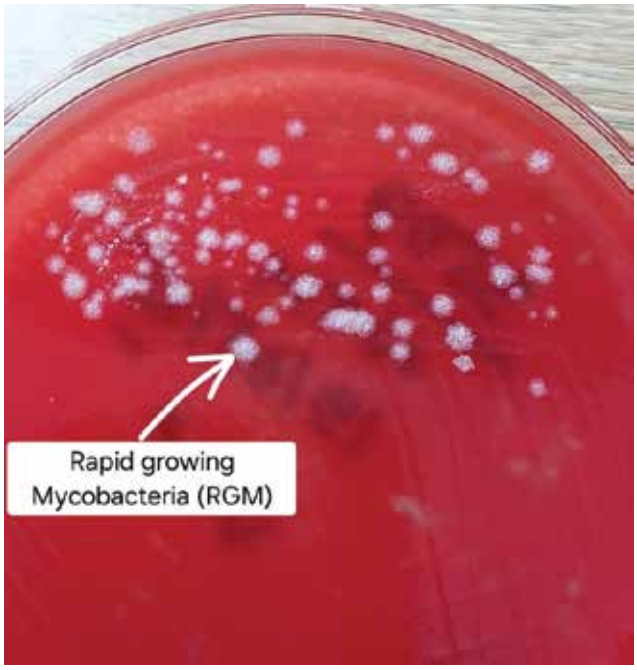

Figure II. RGM colony in blood agar media on Day 5 of inoculation

Figure III. AFB in Ziehl-Neelsen (ZN) stain of culture smear from RGM colony on blood agar media

Using PCR it was seen that out of 28 RGM species, 19 (67.86%) were MABC, of which 13 (68.42%) were *M. abscessus* and 6 (31.58%) were *M. massiliense* (Figures IV-VI).

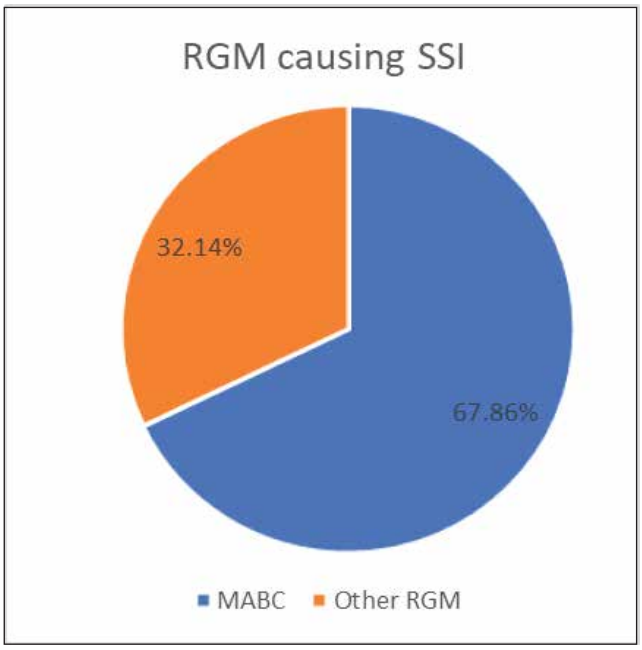

Figure IV. Percentage of RGM causing SSI

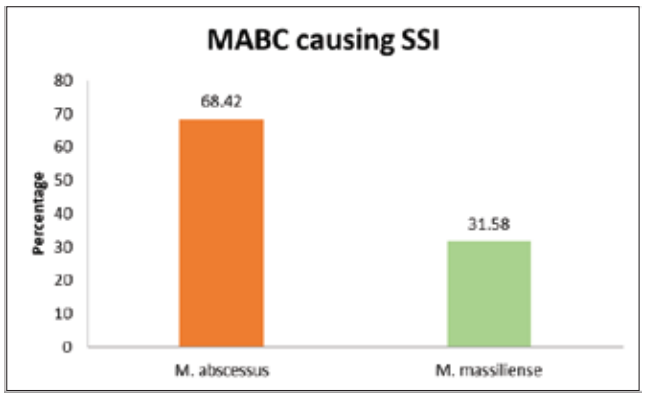

Figure V: Frequency of MABC subspecies detected by PCR

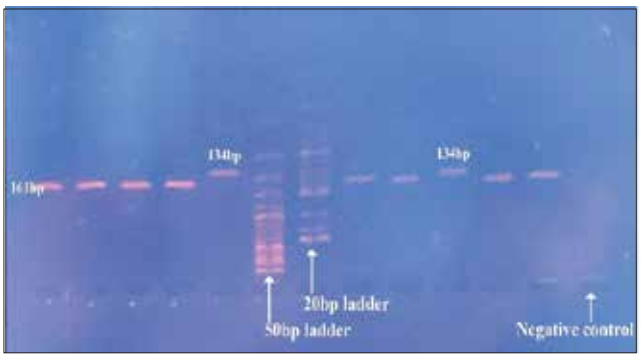

Figure VI: Snapshot of PCR gel electrophoresis showing 134bp PCR amplicon of *Mycobacterium abscessus* subspecies *massiliense* and 161bp PCR amplicon of *Mycobacterium abscessus* subspecies *abscessus* using subspecies-specific primers

Discussion

An increase in awareness, together with the practice of prolonged culture (up to 7 days) and improvement in molecular diagnostic tools, has led to many cases of SSI caused by rapidly growing mycobacteria (RGM) being reported recently[10-11,24]. Being ubiquitously distributed in air, water, and soil, these highly resistant acid-fast bacilli can contaminate disinfectants, surgical instruments, and wounds[10]. The prevalence of rapidly growing mycobacteria SSI varies worldwide[16]. In Bangladesh, 7.7% to 20.4% of SSI have been reported to be caused by NTM with MABC being the commonest NTM found in these studies[9-11]. Whereas, in India 10.9% of SSI is reported to be due to RGM of which 39.4% were MABC[5].

In this study, 18.7% of SSI were caused by RGM, among which 67.9% were due to MABC. It is important to differentiate MABC from other RGM, because MABC is susceptible to only a few antimicrobials, while other RGM are susceptible to wider range of antimicrobials[3,16]. Although the studies conducted in Bangladesh and India give an idea about the prevalence, they lack information regarding the subspecies of MABC involved. However, it is crucial to differentiate the subspecies of MABC because treatment with clarithromycin may lead to failure in *Mycobacterium abscessus* subspecies *abscessus* and *bolletii*. This PCR assay can be employed routinely as a feasible tool for differentiating the subspecies of MABC. In this study, 13(68.4%) *Mycobacterium abscessus* subspecies *abscessus* and 6 (31.6%) *Mycobacterium abscessus* subspecies massiliense were identified by the subspecies-specific PCR assay. This was comparable to a study conducted in China in which with 47.4% *Mycobacterium abscessus* subspecies *abscessus* and 73.7% *Mycobacterium abscessus* subspecies *massiliense* were found[25].

In this study, 78.6% of the rapidly growing mycobacteria SSI occurred following laparoscopic surgery with 64.3% of the patients being female. Similar results were found in other studies from Bangladesh[10-11]. Only 10.71% of the patients had diabetes showing rapidly growing mycobacteria can infect both healthy and immunocompromised individuals. In this study, AFB were found in 75.0% of the samples by ZN staining compared to 41.9% in another study[10]. Although GeneXpert was negative, 25.0% of the cases of this study and 14.5% cases of another study[10] were still misdiagnosed and wrongly treated with first-line anti-tubercular drugs.

The sample size was small due to lack of availability

of RGM isolates within the study period.

Conclusion

Rapidly growing mycobacteria infection should be suspected in cases of chronic discharging SSI, with the absence of bacterial growth on conventional culture for 48 hours, and not responding to common antimicrobials. RGM infection can be routinely diagnosed in the laboratory by prolong culture for 7 days, but additional detection of *Mycobacterium abscessus* complex to the subspecies level is needed to aid in the proper treatment regimen. This PCR assay can be used routinely for the detection of subspecies of *Mycobacterium* (*Mycobacteroides*) *abscessus* complex for culture positive isolates. Its use on direct samples can be explored in the future.

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Conflict of Interest
The authors declare that there is no conflict of interest

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Authors' contributions
Marium Sukanya: conceptualized and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Lovely Barai, Mili Rani Saha, and Tanjila Rahman contributed to the data analysis, interpretation of the results, and critical review of the manuscript. Minhaj Rashidur Rahman and Md. Rokibul Hasan contributed to the analysis of the data. All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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