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Prevalence and Species Distribution of Nontuberculous Mycobacteria Among Clinically Suspected Extrapulmonary Tuberculosis Cases: A Study in Bangladesh

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Abstract

Objectives: Nontuberculous mycobacteria (NTM) are a matter of concern nowadays due to the rising trend of infections worldwide. They can cause extrapulmonary infections that may be misdiagnosed as tuberculosis due to their similar presentation and course of disease, leading to treatment failure and multidrug resistance. The study aimed to observe the frequency of NTM among extrapulmonary tuberculosis (EPTB) suspects and to determine their genetic diversity and species distribution.

Methods: Microscopy, culture, and Multiplex real-time PCR were performed in 190 clinical specimens of suspected EPTB cases. Species of isolated NTM were detected by sequence analysis of the *hsp65* partial gene.

Results: NTM were detected in 24 (12.6%) cases, and *Mycobacterium tuberculosis* complex was detected in 86 (45.3%) cases. The major site of isolation of NTM was surgical site pus (50%), followed by endometrial tissue (29.2%) and lymph node (16.7%). Sequence analysis reveals *Mycobacterium abscessus* (55%) and *Mycobacterium fortuitum* (45%) as the most predominant species.

Conclusion: The increasing NTM infections among EPTB suspects in Bangladesh demand increased awareness and improved diagnostic facilities for early recovery of NTM from clinical specimens. Also, the speciation of NTM is essential as treatment management differs from species to species.

Keywords: Extrapulmonary tuberculosis; Nontuberculous mycobacteria; Genetic diversity, *hsp65* partial gene, *Mycobacterium abscessus, Mycobacterium fortuitum*

Introduction

Nontuberculous mycobacteria (NTM) are a group of mycobacteria that are typically free-living and omnipresent in the environment [1]. They are also known as environmental mycobacteria, atypical mycobacteria, or mycobacteria other than TB (MOTT). They were thought to be nonpathogenic or opportunistic mycobacteria, but nowadays they have been increasingly recognized as pathogenic microorganisms causing infection in both immunocompromised and immunocompetent patients. About 200 species and subspecies of NTM have so far been identified by DNA sequencing; among them, 140 species are potentially pathogenic to people and animals, and the remaining species are occasionally pathogenic [2,3]. The majority of NTM diseases primarily present as lung diseases, although 20%-30% of patients present as extrapulmonary diseases [4]. They infect both healthy people and people who have impaired immune systems [1].

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In a significant number of cases, NTM may cause extrapulmonary infections that may be misdiagnosed as extrapulmonary tuberculosis due to their similarity of presentation & disease sequelae. Most individuals with NTM diseases end up undergoing months or years of therapy for TB or other prevalent infections as a result of inaccurate identification and differentiation. This misdiagnosis of extrapulmonary NTM infections as extrapulmonary TB leads to treatment failure, multidrug resistance, and eventually patient suffering. Over the past ten years, reports of NTM incidence have increased globally. However, little is known about the disease's global toll. In addition, there are geographical variations in the prevalence, trends, and distribution of infections. Also, the treatment management of different species of NTM is different, which necessitates the speciation of NTM along with detection [5].

In Northern India, the prevalence of NTM was estimated to be around 8% in suspected extrapulmonary tuberculosis cases, where the most often isolated fast-growing mycobacteria were *M. fortuitum* (27.5%), followed by *M. abscessus* (14.6%) [6]. NTM was present in 8.4% of extrapulmonary mycobacterial isolates in South India [7]. Although the first report on the presence of NTM in Bangladesh was published in 2017, where NTM was found in 9.4% of the patients with suspected extrapulmonary TB [8]. The accurate prevalence, epidemiology, and genetic diversity are still greatly unrevealed in a TB-endemic country like Bangladesh due to a lack of awareness, subpar diagnostic methods, and overcrowding with other illnesses.

Diagnosis of extrapulmonary NTM infections is always challenging. Conventional methods like culture and microscopy are time-consuming, laborious, and most often inconclusive for the detection of NTM. Furthermore, treatment management varies depending on the species of NTM, which necessitates the speciation of NTM along with detection [5]. Several molecular techniques, including Whole Genome Sequencing (WGS), PCR restriction-enzyme analysis, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), Line Probe Assay, sequencing of 16S ribosomal RNA, gyrB, *hsp65* gene, rpoB genes, 16S-23S internal transcribed spacer (ITS), etc., can be used to identify species of NTM. Though WGS is most accurate and confirmatory, it is very costly and laborious. Also, sequencing of 16S rRNA seems to be of higher resolution, but sequencing of *hsp65* partial gene (396bp-836bp; 441bp) is easy, cost-effective, and more discriminatory in case of identification of some species within the complex [9].

Considering the increasing trends of extrapulmonary infections caused by NTM in Bangladesh, the current study aimed to see the frequency of NTM infections in extrapulmonary sites and also to gain insight into the genetic diversity and distribution of their species by sequencing the *hsp65* partial gene for further understanding of the changing epidemiology of extrapulmonary NTM infections.

Materials and Methods

Study period and place

This is a cross-sectional observational study conducted in the Department of Microbiology & Immunology, Bangladesh Medical University (BMU), and National Tuberculosis Reference Laboratory (NTRL), Dhaka, in a period of 12 months, from August 2022 to July 2023.

Study population

A total of 190 samples, collected from clinically suspected cases of extrapulmonary tuberculosis of all age and sex groups that were sent to the Microbiology and Immunology Department of Bangladesh Medical University (BMU), and National Tuberculosis Reference Laboratory (NTRL) during the study period for laboratory investigation of *Mycobacterium tuberculosis*, fulfilling the inclusion and exclusion criteria, were enrolled in the present study.

Inclusion criteria

Samples were collected from clinically suspected EPTB patients exhibiting clinical signs and symptoms with specific diagnostic criteria suggesting tuberculosis: Tissue specimens showing granuloma (caseating or noncaseating granuloma, giant cell), chronic inflammatory cells observed in cytopathological or histopathological findings; Cerebrospinal fluid with increased total WBC count with ≥ 50% lymphocytes and/or protein level ≥ 0.45 g/L and/or glucose ≤ 2.5 mmol and/or ADA value ≥ 10 U/L [10]; Pleural/ascitic fluid/synovial fluid with increased total WBC count with ≥ 50% lymphocytes and/or protein levels > 3 g/dL and/or ADA values ≥ 40 U/L [11-13]; Urine having persistent pus cells with RBC and a negative routine bacterial culture; Pus from a clinically suspected tuberculous lesion, with negative routine bacterial culture and not responding to antibiotics and antifungals.

Data collection

Relevant data, collected from patients or their attendants or from clinical history records & investigations profile, were recorded in a predesigned data collection sheet.

Procedures in the laboratory:

Sample collection

Samples were collected in sterile, screw-capped containers and sent to the laboratory within 24 hours, maintaining the cold chain.

Sample processing, Microscopy, and Culture

Following sample collection, every step was carried out in a biosafety level-2 cabinet. After processing, the sterile samples were directly inoculated onto L-J media, while nonsterile samples were 1st decontaminated by the N-acetyl-L-cysteine-sodium hydroxide method as per the National

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guideline. About 0.2ml -0.5ml of the deposit was inoculated onto L-J media, and the rest was used for microscopy (Ziehl-Neelsen staining and fluorescence microscopy) & molecular assay.

MPT-64 Antigen test

The total of 45 culture-positive isolates was finally identified as MTBC or NTM by the MPT 64 kit (SD Bioline TB Ag MPT64 RAPID® test kit) following the manufacturer's instruction manual. Then, 20 MPT64 negative culture isolates were subjected to Z-N staining to confirm the growth of AFB.

Biochemical tests for the identification of species of NTM

A Niacin test was done with all 45 culture-positive isolates to differentiate NTM from MTBC phenotypically, and then, the Citrate utilization test and the Urea Hydrolysis tests were performed with the 20 NTM culture-positive isolates to identify species of NTM biochemically.

Multiplex real-time PCR for detection of MTBC and NTM:

Molecular diagnosis of MTBC and NTM in extrapulmonary clinical specimens (n=190) was carried out by the MTB / NTM Detection Kit for Real-Time PCR (GENMARK, TURKEY) that can detect both MTBC and NTM, according to the manufacturer's instructions, respectively. DNA extraction was performed by the Extraction Solution that was available in the MTB Real-Time Detection kit**.** The real-time PCR instrument was a CFX Opus 96 Touch™ real-time detection system (Bio-Rad). PCR was considered positive when for a single sample, the CT value was ≤40 for MTBC detection and ≤42 for NTM detection.

Conventional PCR for the detection of the *hsp65* partial gene

Conventional PCR was carried out in 20 NTM culture isolates for amplification of the *hsp65* partial (positions 396bp-836bp;441bp) gene. DNA of isolated NTM was extracted by a kit-based method (GENMARK, TURKEY). Purity and load of all NTM DNA were checked by observing 260/280 in an ultraviolet (UV) photometer, and stored at -20°C until PCR amplification. A single set of oligonucleotide primer(Tb 11-5'-ACC AAC GAT GGT GTG TCC AT-3' and Tb 12- 5'-CTT GTC GAA CCG CAT ACC CT-3') (Takara Bio INC, Japan) designed to generate a 441 base pair (bp) PCR product of *hsp65* gene (positions 396bp to 836bp) were used and then amplification of the 441 bp product of the *hsp*65 gene was detected by 1.5% agarose gel electrophoresis, as previously described [14].

Gene Sequencing

Sequence was determined from final PCR products of the 20 NTM culture isolates by the Sanger dideoxy method using the PCR forward primer (Tb 11-5'-ACC AAC GAT GGT GTG TCC AT-3') in a 3500 Genetic Analyzer [15].

Analysis of sequencing data

The 20 sequences of data of the isolated NTM were edited and converted to FASTA format by Chromas software. Species identification was performed by determining sequence homology using the NCBI BLAST (National Center for Biotechnology Information- The Basic Local Alignment Search Tool) program. Multiple sequence alignment was conducted using the MEGA ver. 11 program. The sequences of isolated NTM species in this study were submitted to GenBank under accession numbers **PP103230-PP103234**, **PP130621-PP130628**, and **PP481157-481163**.

Phylogenetic tree construction

By using the BLAST (Basic Local Alignment Search Tool), available at GenBank (http:/www.ncbi.nlm.nih.gov.blast), the 441bp sequence of *hsp65* (positions 396bp-836bp) of NTM species was compared among each other and to the available reference sequences of their identical strains. Phylogenetic dendrogram of the 20 isolated NTM species was constructed by maximum- likelihood method in MEGA 11 software using sequencing data of *hsp65* partial gene (441bp) of isolated NTM species and those of reference strains obtained from Genbank **(**https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers (supplementary Data). The reference strains were designated as 'Name of the strain/Genbank accession number/country of isolation/year of collection or isolation' (Table 1). Trees were statistically supported by bootstrapping with 1000 replicates.

Results

Demographics

In this study, 190 extrapulmonary samples from suspected extrapulmonary tuberculosis patients were analyzed, where most of them were Lymph node 57, 30%), followed by surgical site pus 30, 15.5%) & pleural fluid 26, 13.7%) (Table 2).

Frequency of MTBC and NTM among the extrapulmonary samples

Out of 190 extrapulmonary samples, a total of 24 (12.6%) were detected as NTM, and 86 (45.3%) were detected as MTBC. Multiplex RT-PCR detected MTBC in 86 (45.3%) cases and NTM in 23 (12.1%) cases. Among 45 culture-positive samples, 25 (55.6%) had positive culture growth for MTBC, and 20 (44.4%) had positive culture growth for NTM, as shown in Table 3. Of the positive microscopy cases, 6 (20.7%) Z-N staining positive and 7(21.9%) FS positive cases were detected as NTM by Multiplex rtPCR (Supplementary Table A. 1).

Distribution of NTM among the extrapulmonary specimens

The surgical site pus sample showed maximum test

Table 1: Reference strains with their details

Reference Strains	Gene	GenBank accession number	Country of origin	Year
M.fortuitum strain TNTM10	*hsp*65 partial gene (386bp-816bp)	MK713929.1	North Tunisia	2019
M.fortuitum strain MYC 28	*hsp*65 partial gene (386bp-816bp)	MW731489.1	Italy	2021
M.fortuitum strain 37	*hsp*65 partial gene (386bp-816bp)	OP598241.1	Iran	2022
M.fortuitum strain InDRE Guerrero 538DT	*hsp*65 partial gene (386bp-816bp)	JX154098.1	Mexico	2012
M.fortuitum ATCC 6841	*hsp*65 partial gene (386bp-816bp)	JF491295.1	USA	2011
M.fortuitum strain wg86	*hsp*65 partial gene (386bp-816bp)	MK341527.1	Japan	2018
M.fortuitum strain CIP 104534	*hsp*65 partial gene (386bp-816bp)	AY458072.1	France	2003
M.fortuitum strain ATCC 13756	*hsp*65 partial gene (386bp-816bp)	DQ866789.1	USA	2006
M.abscessus subsp.bolletii strain CIP 108541	*hsp*65 partial gene (386bp-816bp)	FJ607778.1	Korea	2009
M.abscessus subsp.massiliense strain 107	*hsp*65 partial gene (386bp-816bp)	OL704626.1	China	2021
M.abscessus subsp. massiliense strain CIP 108297	*hsp*65 partial gene (386bp-816bp)	EU191919.1	France	2007
M.abscessus subsp.massiliense strain 05I284	*hsp*65 partial gene (386bp-816bp)	KT185514.1	Spain	2015
M.abscessus subsp.abscessus strain 11I110	*hsp*65 partial gene (386bp-816bp)	KT185524.1	Spain	2015
M.abscessus subsp.abscessus ATCC 19977	*hsp*65 partial gene (386bp-816bp)	EF486338.1	korea	2007
M.abscessus subsp.abscessus strain MIS005	*hsp*65 partial gene (386bp-816bp)	OP422735.1	Taiwan	2022
M.abscessus subsp.massiliense strain	*hsp*65 partial gene (386bp-816bp)	MT428414.1	India	2020
M.lehmannii strain CECT 8763	*hsp*65 partial gene (386bp-816bp)	KY933786.1	UK	2017
M.lehmannii strain DWMJ-1145B1	*hsp*65 partial gene (386bp-816bp)	MW451702.1	USA	2021

Table 2: Demographic characteristics (n=190)

Characteristics	Category	Frequency, n	Percentage (%)
Sex	Male	88	46.3
	Female	102	53.7
	Total	190	100
Age	≤20	37	19.5
	21-30	58	30.5
	31-40	46	24.2
	41-50	13	6.8
	51-60	15	7.9
	≥60	21	11.1
	Total	190	100
Sample types	Lymph node	57	30
	Pleural fluid	26	13.7
	Ascitic fluid	16	8.4
	Pus	30	16.3
	Endometrial tissue	24	12.1
	Synovial tissue	8	4.2
	Bone tissue	5	2.6
	CSF	10	5.3
	Urine	9	4.7
	Skin tissue	5	2.5
	Total	190	100

*Data were presented as n (%).

Table 3: Results of Multiplex real-time PCR and Culture in clinically suspected extrapulmonary tuberculosis (EPTB) cases (n=190)

Test Methods	Positive cases Number(n)	Percentage (%)	*P* value*
Multiplex rtPCR MTBC	86	45.3	<0.001
Multiplex rtPCR NTM	23	12.1	<0.001
Culture MTBC Positive	25	13.2	-
Culture NTM Positive	20	10.5	-

*MTBC: *Mycobacterium tuberculosis* complex; NTM: Nontuberculous mycobacteria; Multiplex rtPCR: Multiplex real-time Polymerase Chain Reaction;

*Data were presented as n (%) and *P*-value was calculated by the chi-square test and was significant when compared with culture (<0.001)

positivity for NTM, followed by endometrial tissue and lymph node. Out of 30 pus samples, 12(40%) samples tested positive for NTM in total. NTM was found in 7(29.1%) cases of the 24 endometrial tissue samples, followed by 4 (7.01%) in lymph nodes by Multiplex rtPCR, but only 2 (3.5%) of them yielded growth in culture. One(3.8%) of the 26 pleural fluid samples tested positive for NTM by Multiplex rtPCR but didn't grow in culture. None of the other samples yielded NTM in any test method (Table 4). Among the total 24 NTM detected, 20(83.3%) yielded growth in culture. The remaining 4(16.7%) couldn't be isolated from culture. Species of the 20 isolated NTM were identified by NCBI (National Center for Biotechnology Information) nucleotide blast using sequencing data of the *hsp65* partial gene (positions

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396bp-836bp;441bp). Out of 20 isolated NTM, 11 (55%) were isolated from surgical site pus; among them, 6 (54.5%) were *M. abscessus* species; 4 (36.4%) were *M. fortuitum* species; 1 (9.1%) was *M. lehmannii* species. Out of 7 (35%) isolated NTM species in endometrial tissue, 4 (57.1%) were *M. fortuitum* species; 3 (42.9%) were *M. abscessus* species. *M. abscessus* species accounted for 100% of the two (10%) isolated NTM species found in the lymph node (Table 4).

Clinical relevance of extrapulmonary NTM infections

Among the total 24 extrapulmonary NTM infections detected, 12 (54.2%) had a history of surgery, either open or laparoscopic, followed by discharge from the surgical site & not responding to conventional antibiotics. All four (100%) lymph node tissues and two (28.5%) of the seven endometrial tissues had features of granulomatous inflammation, and five endometrial tissues (71.4%) had chronic inflammatory cells in tissue histopathology. The ADA value was high in only one pleural fluid specimen. Clinical signs and symptoms like a history of fever, weight loss, infertility, or subfertility were found in variable percentages, which were consistent with true extrapulmonary infection by NTM (Table 5).

Phylogenetic analysis of isolated NTM species

The species of 20 NTM isolates were identified by phylogenetic analysis of the *hsp65* as *M. abscessus* (n = 11), *M. fortuitum* (n = 10), and *M. lehmannii*, with BLAST searches revealing 97–100% identity to those of specific species. In line with phenotypical identification, they were all citrate negative, urease positive, indicating rapidly growing mycobacteria. The dendrogram tree showed all three different species of RGM (*M. fortuitum*, *M. abscessus,* and *M. lehmanni*) formed well-supported distinct clusters with bootstrap value 100% for *M. fortuitum* species, 88% for *M. abscessus,* and 100% for *M. lehmanni* species (Figure 1).

Table 4: NTM positivity by culture and Multiplex real-time PCR (rtPCR) in different clinical specimens of suspected extrapulmonary tuberculosis cases with their species distribution.

Sample Types	Only Culture NTM Positive n (%)	Only Multiplex RT-PCR NTM Positive n (%)	Both Culture and Multiplex rtPCR positive n (%)	Total test positivity for NTM n (%)	Identified species of isolated NTM obtained by NCBI Blast using the sequencing data of the *hsp65* partial gene (positions 396bp-836bp;441bp) n (%)
Lymph node (n=57)	-	2 (3.4)	2 (3.4)	4 (7.0)	*M. abscessus*-2 (100)
Pleural fluid (n=26)	-	1 (3.8)	-	1 (3.8)	-
Ascitic fluid(n=16)	-	-	-	-	-
Pus(n=30)	1(3.3)	1(3.3)	10(33.3)	12(40)	*M. abscessus*-6 (54.5) *M. fortuitum*-4 (36.4) *M. lehmannii*-1 (9.1)
Endometrial tissue(n=24)	-	-	7 (29.1)	7 (29.1)	*M. abscessus*-3 (42.9) *M. fortuitum*-4 (57.1)
Synovial fluid(n=08)	-	-	-	-	-
Bone tissue(n=05)	-	-	-	-	-
CSF(n=10)	-	-	-	-	-
Urine(n=09)	-	-	-	-	-
Skin tissue(n=05)	-	-	-	-	-
Total(n=190)	1 (0.5)	4 (2.1)	19 (10)	24 (12.6)	20 (10.5)

*The data were presented as n (%); One pus sample was culture positive for NTM but negative by Multiplex rtPCR, and another pus sample was Multiplex rtPCR positive for NTM but negative by culture.

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Table 5: Baseline characteristics of patients with extrapulmonary NTM (n=24) to evaluate clinical relevance

Characteristics	Frequency(n%)
Mean age (years)±SD	31.75±14.33
Gender	
Male	5(20.8)
Female	19 (79.2)
Site of isolation	
A. Surgical site	**12 (50)**
Features:	
H/O Laparoscopic oophorectomy	1 (7.7)
H/O Laparoscopic cholecystectomy	1 (7.7)
H/O Laparoscopic hernioplasty	1(7.7)
H/O Open hernioplasty	2(15.4)
H/O Caesarean section	2(15.4)
H/O Open nephrolithotomy	1(7.7)
H/O Open appendicectomy	2(15.4)
H/O Dental surgery	1(7.7)
H/O Open total abdominal hysterectomy	1(7.7)
H/O Surgical site discharge	12(100)
No growth of other organisms in routine culture/sensitivity	12(100)
B. Endometrium	**7 (29.2)**
Features:	
H/O Infertility or subfertility	7(100)
H/O Tubal block in laparoscopic evaluation	7(100)
Granulomatous inflammation	2(28.6)
Chronic inflammatory cells	5(71.4)
C. Lymph node	**4 (16.6)**
Features:	
Fever, Weight loss	2(50)
Enlarged lymph node	4(100)
Granulomatous inflammation	4(100)
D. Pleura	**1 (4.2)**
Features:	
Weight loss, fever, cough, dyspnea	1(100)
Raised ADA	1 (100)

*The data were presented as n (%)

Discussion

Bangladesh is 8th among the high TB burden countries globally[16]. Extrapulmonary Tuberculosis (EPTB) comprises a significant portion (15-20%) of total TB cases in this country. In a significant number of cases, extrapulmonary nontuberculous mycobacterial infections are misdiagnosed as EPTB due to similar presentation and lack of differentiating properties of conventional diagnostic methods, which puts clinicians in a dilemma and leads to the maltreatment of the patients. Moreover, the incidence of EP-NTM in the post-COVID era has increased alarmingly.

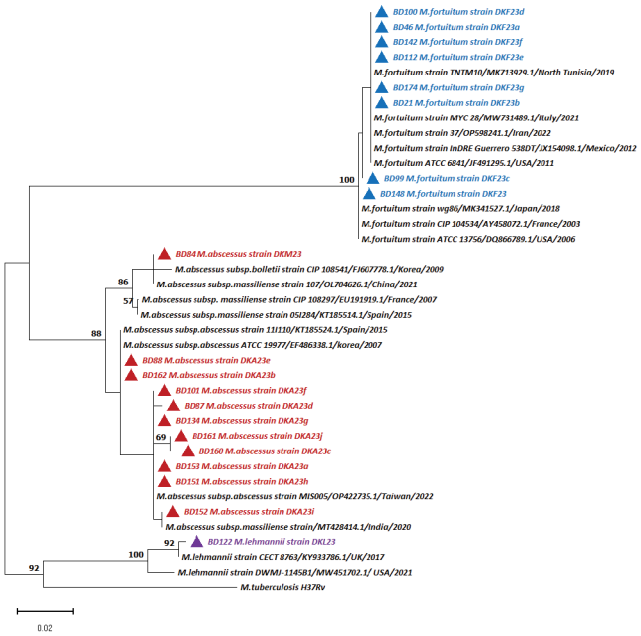

Figure 1: Phylogenetic analysis of the isolated NTM species (n=20), based on *hsp65* partial gene (positions 396bp-836bp; 441bp) sequencing, by Maximum–likelihood method using Tamura-Nei model in MEGA11 software. The reference strain sequences were obtained from GenBank under accession numbers. The tree was statistically supported by 1000 times bootstrapping and rooted with *M. tuberculosis* H37Rv (Genbank accession number nub-JF491311.1). Percent bootstrap was indicated by the value at each node (values <50 were omitted). The variation scale was described at the bottom. Closed red, blue, and purple triangles indicated *M. abscessus*, *M. fortuitum,* and *M. lehmanni* species isolated in the present study.

The frequency of NTM (12.6%) in extrapulmonary sites in the present study is comparable to another study of India, where they reported that the prevalence of NTM among extrapulmonary TB suspects was 12.4% [17]. It is more than the prevalence mentioned in a study of Bangladesh, where it was 9.4% in extrapulmonary samples [8]. Majority of NTM positivity was found in surgical site pus 12(50%), followed by in endometrial tissue 7(29.2%), lymph node 4(16.7%) and pleural fluid 1(4.2%). Several other studies showed skin and soft tissue as the most common extrapulmonary site of isolation of NTM, which is consistent with the present study [18,19]. This preponderance of NTM infection in the surgical site suggests that the preoperative sterilization and disinfection protocols were insufficient. Among the 12 (50%) NTM-positive surgical site pus samples, one NTM that grew in culture media was missed by Multiplex rtPCR, which can be explained by the inappropriate extraction of mycobacterial DNA from the sample. A total of 4 samples; two from lymph node, one from a pus sample, and one from pleural

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fluid, failed to yield growth in culture but were detected as NTM by Multiplex rtPCR. This can be explained by the low bacilli load and loss of viability of mycobacteria during the decontamination procedure.

Biochemical test results indicated that they are rapid growers (RGM) and belong to either *M. abscessus* or *M. fortuitum* species, but none of them could differentiate whether the species was *M. abscessus* or *M. fortuitum,* as both types of species show the same result [20]. Sequencing was carried out in the isolated 20 (83.3%) of 24 NTM cases. Three (3) different rapidly growing NTM (RGM) species were identified here. A similar study in Iran identified five (5) different NTM species from suspected pulmonary and extrapulmonary TB specimens by *hsp65* partial gene sequencing [21].

The predominance of *M. abscessus* 11(55%) and *M. fortuitum* 8(40%) species in extrapulmonary samples found here is consistent with a number of other studies [6,22]. Among the two, *M. abscessus* species are most prevalent in East Asia, and A similar study conducted in Thailand showed *M. abscessus* as the most common NTM causing extrapulmonary infections, comprising 25.4% of all clinical NTM isolates [22]. Moreover, the high prevalence of *M. abscessus* species in surgical site pus is comparable to several other studies where it is recognized as one of the most common NTM isolates causing surgical site infections [23].

M. fortuitum species are another rapidly growing mycobacterium frequently recognized as the causative agent of localized skin and soft tissue infections. A study conducted in India showed *M. fortuitum* as the most common species isolated from surgical site pus [24]. The preponderance of these two RGM in surgical site pus indicates that disinfectant failure, contamination of medical devices & hospital water supply may be the common source of these infections. *M. lehmannii* species was detected in one (5%) pus sample, which is a novel, rapidly growing mycobacterium under the genus Mycobacterium, discovered in 2017, UK. They produced yellow-orange colonies in L-J media and were citrate negative, urease positive [25]. However, their existence in surgical site pus in the present study, with complete clinical concordance, calls for more understanding of the role of this unique species in surgical site infections.

Again, 4(57.1%) *M. fortuitum* species and 3(42.9%) *M. abscessus* species found in endometrial tissue samples in this study draw attention to the rising infection by RGM in reproductive women in Bangladesh. A similar recent study in India showed *M. chelonae*/*M. abscessus* and *M. fortuitum* species are the most commonly isolated NTM species in endometrial tissue and correlate with clinical findings, coming to the point that NTM infections (23.7%) in endometrium might be the hidden cause of endometrial damage leading to subfertility and infertility in reproductive women, which is consistent with the present study [26]. Among the 2 NTM isolated from the lymph node, both of them were *M. abscessus* species. Lymphadenitis caused by *M. abscessus* species was also mentioned in a study in Japan [27].

Four (4) NTM that were detected by Multiplex rtPCR but didn't yield growth in culture media, couldn't be identified as sequencing was carried out only in the culture isolates of NTM. They were positive by Multiplex rtPCR because as few as 10 DNA copies/ml of sample can be detected by this highly sensitive molecular test [28]. The phylogenetic analysis using sequence data of the *hsp65* partial gene (441bp) of isolated NTM and that of reference sequences obtained from Genbank under accession number represented that all three different RGM (*M.fortuitum*, *M.abscessus* and *M.lehmanni)* species identified here formed well-supported and separated clusters with the reference strains with a bootstrap value >80%. A small sample size is a limitation. A population-based investigation utilizing a generalized technique could not be carried out due to time and financial constraints. Again, speciation could not be performed in 4 Multiplex rtPCR NTM positive samples due to non-retrieval from culture, and Whole Genome Sequencing (WGS), the confirmatory technique of species identification, was not possible to carry out due to budget limitations and lack of laboratory resources.

The major outcome of the study is to draw attention to the increasing trend of NTM infections in extrapulmonary sites, which are usually misdiagnosed and maltreated as EPTB, leading to multidrug resistance. In a TB-endemic country like Bangladesh, this study will enhance the knowledge about the evolving epidemiology of NTM infections and will help clinicians rethink the diagnosis of NTM infections and initiate appropriate treatment, which will eventually improve patient care and disease control initiatives.

Conclusion

The overall frequency of NTM in extrapulmonary areas is 12.6%, and the most common site of isolation is surgical site pus according to this study, which indicates that inappropriate sterilization and disinfection procedures before any surgery may be an important cause of the disease burden. Among the isolated species in this study, *M. abscessus*, followed by *M. fortuitum,* appears to be the most common species causing extrapulmonary infections in Bangladesh. As the selection of appropriate treatment management varies from species to species in case of NTM infections, identification of the species of NTM alongside its differentiation from MTBC is crucial and of utmost importance for the prevention of developing antimicrobial resistance. A greater understanding of the changing epidemiology of NTM, better diagnostic facilities, and more extensive research are essential, considering the ambiguity of NTM with MTBC in the case of diagnosis and the rising number of illnesses caused by NTM species in Bangladesh.

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Conflict of interest

The authors have nothing to declare.

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Credit authorship contribution statement

Fahmida Hoque: Writing – original draft, Validation, Software, Methodology, Investigation, Data curation.

Md Zaber: Writing – original draft, Software, Investigation, Data curation.

S.M. Nakibul Mazed: Writing – original draft, Software, Investigation, Data curation.

Shirin Tarafder: Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization.

Author agreement

The authors have given their approval to the submitted work. It is the writer's original work that has not been published before and hasn't been submitted for publication anywhere else.

Ethical approval

The research protocol was approved by the Institutional Review Board (IRB) of BMU (Former-BSMMU), Dhaka (No BSMMU/2022/12620) on 18.12.2022. Informed written consent was taken from the patient in a predesigned consent form.

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