Original Article

Detection of Non-Tuberculous Mycobacteria by PCR from Non-healing Surgical site Infections

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ABSTRACT

Surgical Site Infection (SSI) is an infection that occurs after any surgical procedure with adverse effects on a patient's prognosis. SSIs are caused by a variety of bacteria including Non-Tuberculous Mycobacteria (NTM). Nowadays, NTM is reported as an important pathogen in delayed healing of surgical site infections in post-operative cases in many countries, however, there is no report yet in the country due to lacking any sensitive detection method. By multiplex PCR we previously reported the existence of NTM in a variety of clinical specimens and here we report NTM in SSI. Out of 98 patients with SSI, the total NTM positive cases were 20(20.4%) and Mycobacterium Tuberculosis (MTB) was 6(6.1%). NTM positivity rate was higher in female 13(65%) than male 7(35%). The majority (13) of the patients were treated with a combination of Clarithromycin and Ciprofloxacin with or without the first line of Anti-Tubercular Therapy (ATT) and cured. In the case of patients with delayed recovery of SSI, there should be a high level of clinical suspicion for the NTM as a causative agent and Multiplex PCR can be utilized to diagnose NTM and to discriminate from MTB.

Key words: *Surgical site infection, mycobacteria, NTM, PCR*

INTRODUCTION

Surgical site infection (SSI) is one of the common conditions of post-operative complications which represent a substantial clinical and economic burden on patients and the healthcare system[1]. SSI creates adverse conditions for patients by increasing the duration of wound healing, prolonged use of antibiotics, increased length of hospital stays and increased mortality[2]. In the last two decades, the surveillance data shows that there has been a change in the profile of microorganisms causing SSI, with available reports of SSI which are caused by mycobacteria.[3] In the case of mycobacteria, the most global attract concern is Mycobacterium tuberculosis (MTB) infection, however, infections caused by non-tuberculous mycobacteria (NTM) exist and may be unnoticed due to limitations of conventional diagnostics techniques. Several reports on NTM in SSI were found in many countries including our neighboring country, which were detected by using advanced diagnostics tools[3-4].

NTM are mycobacteria other than MTB (the cause of tuberculosis) and M. leprae (the cause of leprosy). NTM are also referred to as atypical mycobacteria, mycobacteria other than tuberculosis (MOTT), or environmental mycobacteria. There are more than 180 recognized species of nontuberculous mycobacteria (NTM) and some of these cause disease in humans. NTM can be found in soil, dust, and water including natural water sources[5]. NTM existed in nature and reported as causative agents for different types of infections including post-operative infections in cutaneous and soft tissue[6-8]. It is assumed that contaminated water or surgical instruments might be possible sources of post-operative infection[9].

The traditional method for tuberculosis detection is AFB staining which cannot differentiate the MTB from NTM because both take AFB staining. Anatomical pathology reports can find the granulomatous lesion but there is no specific point that the lesion is caused by MTB or NTM.[10]. Improper or missed diagnosis may lead to inappropriate treatment and produce adverse emergence of antimicrobial resistance. Therefore, advanced, and specific laboratory diagnosis is required[11-12]. Culture is

one of the standard techniques for laboratory confirmation of NTM but is very time-consuming (takes at least 1–2 months) and laborious, resulting in a delay of treatment[13]. We are using multiplex real-time PCR for routine diagnosis of infections including mycobacteria in our Evercare Hospital Dhaka (EHD) and previously reported the existence of NTM in a variety of clinical specimens including samples from surgical site infections[10].

Here we report the accumulated data of mycobacterial infections detected from 98 non-healing surgical site infections from 2020 to 2022.

MATERIAL AND METHODS

Method of data collection:
The data of the patients were collected from Hospital Information System (HIS) of Evercare Hospital Dhaka (Ex. Apollo Hospitals Dhaka), Bangladesh. All patients with surgical site infections visited our hospital over a period of three years (January 2020 to December 2022). Age, sex, types of specimens, types of surgery and clinical history were used for data analysis. To protect patients' private information except for age & sex all samples were de-identified and patient consent was not needed as data was extracted from the routine test result available in the hospital information system.

Clinical Samples:
All samples were collected from surgical site infections under aseptic precautions by the Surgery & Gynecological departments of Evercare Hospital Dhaka. A total of 98 samples were collected from the age group of 3-81 years old. Pus, wound swabs, wound tissue/fluid were collected from patients. In the case of pus and swab samples, 1-2 ml of sterile phosphate buffer saline (PBS) was added to the specimen tube and mixed it with vortex mixture. For tissue samples, a tiny, suspected part of tissue was collected in a sterile container with normal saline by the clinician. Then the tissue samples are homogenized by tissue ruptor using disposable probe. All samples were stored at 2-8°C for no longer than 24 hours until DNA extraction and then DNA extracted and was stored at -80°C. All the laboratory work was performed in Molecular Laboratory of Evercare Hospital Dhaka.

DNA extraction & Multiplex Real time PCR:
The DNA was isolated by using QIAamp DNA Mini (Qiagen, Germany) spin column-based extraction kit according to the manufacturer's instructions. 200µl of sample was used for DNA extraction. The elution volume was 50 µl. We used CE-IVD approved VIASURE MTB/NTM commercial multiplex real time PCR kit from CerTest Biotech, S.L. Spain for the detection of M. Tuberculosis complex and Nontuberculous mycobacteria. The total PCR volume was 20 µl where 15 µl rehydration buffer dispenses in lyophilized master mix containing 0.2 ml PCR strip tube for each sample, Negative control (NC) and Positive control (PC). Then 5 µl of extracted DNA, negative control and positive control were added, respectively. PCR amplification was done by QuantStudio-5Dx (Applied Biosystems) thermocycler according to kit manufacturer's instruction which was programmed as follows: Hold-95˚C for 2 minutes, then 45 cycles of 95˚C for 10 seconds, 60˚C for 50. Signal was acquired at 60˚C and analysis was performed on the linear scale. Thresholds were set manually in each run. The fluorescence was detected in FAM channel & CY5 channel for M. Tuberculosis complex, ROX channel for Nontuberculous mycobacteria, and HEX/VIC channel for amplification of internal control. The recommendations of the manufacturer were strictly followed for DNA extraction and Real Time PCR.

Data analysis:
The PCR result of each batch was analyzed by two technical persons. Finally, accumulated data were analyzed by finding the percentage of total samples that were either detected positive or negative.

RESULTS
A total of 98 patients with SSI attended our hospital over the three-year period. Among the attendees, male was 41(41.84%) and female was 57(58.16%). Out of 98 processed samples of surgical site infected patients PCR showed positive in 26 samples (26.5%). From these 26 positive samples, 20 samples (20.4%) were positive for NTM and 6 (6.1%) for MTB. Among 20 NTM positive patients' female were 13(65%) and male were 7(35%). The age range of patients was from 3-81 years. In our study, majority of patients 22 (22.5%) were from 31-40 years old and the positivity rate was higher 5(25%) in the age group of 21-30. (Table: 1 and Figure: 1)

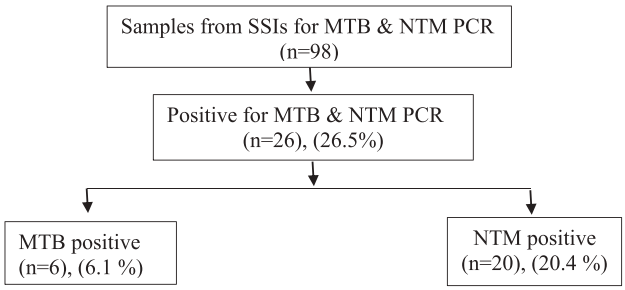

Figure 1: Flowchart of molecular laboratory results of SSI samples

Table 1: PCR tested SSIs patient's age distribution

Age range (years)	Male (%)	Female (%)	No. of patients (%)	No. of NTM +ve patients (%)
≤20	11	2	13(13.3)	2(10)
21-30	3	13	16(16.3)	5(25)
31-40	10	12	22(22.5)	4(20)
41-50	4	9	13(13.3)	4(20)
51-60	5	13	18(18.3)	2(10)
>60	8	8	16(16.3)	3(15)
Total	41 (41.84)	57 (58.16)	98 (100)	20(100) M- 7(35) & F- 13(65)

According to year wise distribution, PCR tests were performed 28(26.6%) in 2020, 38(38.8%) in 2021 and 32(32.6%) in 2022 from SSI patients' samples and among them, NTM positive patients found 16(57.1%), 3(7.9%), 1(3.1%), respectively. There were no co-infections of MTB and NTM found during the whole study period. (Figure-2)

Various types of specimens were tested in this study period. Samples were pus (52/53.1%), wound swab (41/41.8%), fluid and tissue from wound (5/5.1%). Among all specimens, NTM was identified 50%, 45%, 5%, respectively (Table: 2). These results show that NTM was mainly detected from pus and wound swabs. Fluid/tissue were the least requested specimens by the clinicians and NTM detection rate was also least in fluid/tissue.

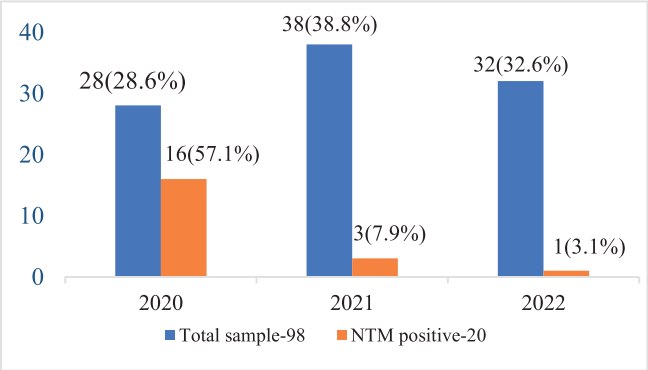

Figure 2: Yearly distribution of NTM positive Samples

Table 2: PCR tested SSIs specimen types and distribution

Type of specimen	Number	%	NTM +ve	%
Pus	52	53.1	10	50
Wound swab	41	41.8	9	45
Fluid/ tissue from wound	5	5.1	1	5
Total	98	100	20	100

In our study, all NTM positive patients had undergone surgery in different surgical settings. Surgeries were mainly carried out in various hospitals across different cities in Bangladesh. This information was gathered from the history of patients. The positivity rate was higher 8(40%) in laparoscopic surgery (Laparoscopic Cholecystectomy, Oophorectomy and Hernioplasty) and followed by Caesarean Section-6(30%) and Excision Biopsy-6(30%). (Table: 3)

Table 3: Types of surgery (Only NTM positive patients)

Name of Surgery	Number	%
Laparoscopic Cholecystectomy	4	20
Laparoscopic Oophorectomy	2	10
Laparoscopic Hernioplasty	2	10
Caesarean Section	6	30
Excision Biopsy	6	30
Total	**20**	**100**

Out of 20 NTM positive patients, we found a complete treatment history of 13 patients. All patients were treated with a combination of Clarithromycin and Ciprofloxacin in doses of 500mg twice daily for at least six months. Among these 13 patients, 10(76.9%) patients were treated by ATT with Clarithromycin and Ciprofloxacin and the rest of 3(23.1%) patients were treated only by Clarithromycin and Ciprofloxacin. We found one patient who was treated by ATT (Rifampicin, Isoniazid) with Clarithromycin and Ciprofloxacin for the first six months. As there was no significant improvement, this patient was then treated by

Levofloxacin instead of Ciprofloxacin for another 3 months and the patient was cured. Besides these oral antibiotics local antibiotic ointment dressing was used over the ulcers and abscesses until they heal. All patients showed good response, but treatment duration varied (6 months to 2 years). We classified our patients into two groups (6 Months and >6 Months) according to treatment. Among these two groups 5(38.5%) were treated for 6 months duration and 8(61.5%) were treated for >6 months. It is noticeable that one patient was treated for continuous two years. The condition of this patient was improved but not cured completely. This patient had surgical mesh infection which was placed during laparoscopic hernioplasty. The first 9 months patient was treated only by ATT and then treated by Clarithromycin and Ciprofloxacin for 15 months. (Table: 4)

Table 4: NTM positive patients therapy given, duration and response:

Therapy given	Duration		Response	Patients' number (%)
	6 Months	> 6 Months		
1st line ATT* with Ciprofloxacin & Clarithromycin	4	6	9-Cured 1-Improved	10 (76.9)
Ciprofloxacin & Clarithromycin	1	2	Cured	3 (23.1)
Total (%)	5(38.5)	8 (61.5%)		13(100)

**ATT: Anti-tubercular therapy (Rifampicin, Isoniazid)*

DISCUSSION

In surgical site infection, NTM is not a lesser concern than MTB.[14] Last couple of decades NTM gained much clinical significance in immune-compromised as well as immune-competent patients.[15] To the best of our knowledge this is the first report in Bangladesh to show the existence and prevalence of NTM in surgical site infections by using real time PCR techniques. The study of NTM from any types of clinical samples in our country is still limited. By doing this study we have tried to make awareness about NTM in post-operative surgical site infections and to avoid being mistaken for MTB as both can take AFB stain. A multiplex PCR is the best method for the identification and differentiation of NTM from MTB rapidly and accurately.[16] By using the multiplex real time PCR, 20.4% of NTM were detected from our study population and positivity rate was higher in female than male population. Different studies from other countries show the existence of NTM in surgical site infection and majority were in female population.[17-19]

In year-wise distribution, we found a significant difference in positivity rate. The positivity rate is higher (16/57.1%) in 2020 followed by (3/7.9%) in 2021 and (1/3.1%) in 2022 among all study population. Positivity rate was decreased gradually, most probably due to increased awareness about NTM infection among healthcare personnel and use of proper sterilization techniques for all surgical instruments. NTM infection can be prevented by implementation of strict aseptic protocol and routine use of different appropriate sterilization methods.[20]

In our study, among all positive cases the highest number (8/40%) of NTM positive in laparoscopic surgery. Our data matched with another study in Nepal where the highest number of NTM was found in laparoscopic surgery.[17] Due to high positivity rate in laparoscopic port site infection, it is mentionable here that most laparoscopic instruments are not sterilized by autoclave because they are not able to withstand the heat. As a result, chemical sterilization method that is glutaraldehyde commonly used. There is no evidence found to increase NTM infection due to use of glutaraldehyde disinfection,[21-22] but increased resistance to glutaraldehyde has been documented in different species of NTM (M. chelonae, M. massiliense and M.smegmatis),[14, 23-25] which may increase the risk of NTM infection in surgical site. To prevent these types of infection, the use of disposable laparoscopic instruments is the gold standard method. 20 Another way is to use ethylene oxide gas and hydrogen peroxide gas plasma sterilization method for heat sensitive instruments and replace the glutaraldehyde solution with Ortho-phthalaldehyde (OPA).[26]

The proper line of NTM treatment has much controversy. NTM shows a limited response to the first line of ATT. The standard treatment for NTM consists of a combination of second line anti-tubercular drug including macrolides such as Clarithromycin, quinolones such as Ciprofloxacin or

Levofloxacin and aminoglycosides such as Amikacin[27,28]. To prevent recurrence and for complete healing of wound, it is recommended that the antibiotics can be given for at least three months or for a period of 3-6weeks after the wound heals completely[29,30].

In our study population we found complete treatment history of 13 patients. All patients were treated with combination treatment of Clarithromycin and Ciprofloxacin with or without first line of anti-tuberculous drugs (Rifampicin, Isoniazid) for a period of minimum six months. Among them 12 patients are cured, and 1 patient was improved. Similarly, NTM infection in chronic breast lesions were also previously treated with Clarithromycin and Ciprofloxacin and mostly cured[31].

The limitation of the current study is the small sample size. Further study is needed with large volumes of samples covering more surgical settings in the country.

CONCLUSION

Clinicians should be aware of the appropriate diagnosis of non-tuberculous mycobacteria. NTM should be differentiated from MTB. Multiplex PCR can differentiate NTM from MTB within a short time, thus can help clinicians for early diagnosis and specific treatment. As it is a single center study in Bangladesh, this study may not show the original scenario of our country. Multicenter based study is required for knowing the exact prevalence. Also, detection of specific NTM species by sequencing or other advanced molecular techniques is required for more specific diagnosis and treatment. Generally, multidrug therapy with prolonged duration becomes successful if NTM is diagnosed properly. Maintaining all aseptic precautions before any surgical procedure and following strict sterilization protocols is necessary to prevent these NTM infections.

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