ORIGINAL ARTICLE

Detection of Nontuberculous Mycobacterium by Real Time PCR from Variety of Clinical Specimens

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Abstract

Aim: Nontuberculous mycobacterium (NTM) causes many types of infections including respiratory and non-respiratory infections such as skin and soft tissue infections, lymphadenitis, meningitis, gastro-intestinal infections, disseminated infections and even intravenous catheter-related infections. Increasing incidence of NTM is reported worldwide in last decade. However, incidence of NTM in Bangladesh is not known as detection of NTM is not undergoing in Bangladesh which is necessary to know as these NTM species are resistant to first-line anti-TB drugs and, when mistaken for M. tuberculosis, give rise to erroneous identification of multidrug-resistant TB (MDR-TB). We wanted to know the existence of NTM from various clinical specimens including tissues from tuberculosis suspected patients visited in Apollo Hospitals Dhaka in 2013 to 2015. **Material and Method:** Sample processing, DNA extraction and real time PCR (polymerase chain reaction) were done according to the commercial LyteStar TB/NTM PCR kit developed by Altona Diagnostics, Germany. The target DNA sequences are amplified with IS6110-specific primers for MTB complex and ITS-specific primers for NTM. Probes specific for MTB complex and NTM DNA are labeled with fluorophore dye FAM and HEX, respectively. We have analyzed 579 clinical specimens from tuberculosis suspected patient. **Result:** Among 579 specimen different types of tissues were 201 and histopathology data were available for 166 cases. In tissues NTM was detected by PCR in 31(19%) cases, 8 of which were compatible with histopathology findings and rest 23 cases showed no evidence of granulomatous lesion. We analyzed 378 different varieties of clinical specimens such as sputum, bronchial lavages, body fluids, pus and swabs. Among 378 samples 215 samples were requested for AFB staining. NTM was detected by PCR in 19(8.8%) samples and out of 19 NTM positive specimens only one was AFB positive. **Conclusion:** This is the first report in the country about detection of NTM in variety of clinical specimens and warrants further elaborate investigation. Our results showed that PCR is an effective tool for the rapid identification of NTM from tissues and AFB negative clinical specimens having suspicion for mycobacterial infection.

Keywords

Nontuberculous mycobacteria; mycobacterium tuberculosis; Acid Fast Bacilli; Polymerase chain reaction

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Introduction

The membership list of genus mycobacterium is ever expanding. While leprosy and tuberculosis are specific diseases caused by mycobacteria, other members are usually saprophytes but can be opportunistic and at times deadly pathogens. These other mycobacteria are referred to as atypical mycobacteria, non-tuberculous mycobacteria (NTM) or mycobacteria other than tubercle bacilli (MOTT).[1] Nontuberculous mycobacteria (>150 species such as Mycobacterium avium, Mycobacterium kansasii, Mycobacterium chelonae and Mycobacterium abscessus) are opportunistic pathogens causing lung and extra respiratory infections, beside M. ulcerans and M. marinum that are pathogens causing specific skin and soft tissue infections. Disseminated infections occur only in severe immunosuppressed conditions such as AIDS.[2] Based on reports from many countries, the prevalence and numbers of diseases caused by NTMs are on the rise.[3-12] The rising number of NTM species is also of concern as these are both difficult to diagnose and treat. Clinical presentation of Mycobacterium tuberculosis complex (MTBC) and NTM may or may not be the same, but the treatment regimen is always different for both the infection.[13] Unfortunately, most of the NTM species are inherently resistant to the anti-tuberculosis (TB) agents, which make the treatment of these infections more difficult. This concern is even more serious in economically challenged countries - the prevalence of diseases caused by NTM is expected to rise due to inadequate laboratory facilities. In many cases identification of mycobacterium to the species level is not done and NTM diseases are frequently misdiagnosed as TB.[14]

Method of detection of tuberculosis by AFB staining cannot differentiate the mycobacterium tuberculosis from other NTM because both take AFB stain. Histopathology can find the chronic granulomatous lesion compatible of TB but there is no clear differentiating point between MTB and NTM. Moreover, in addition to mycobacterial infections, granuloma and/or necrosis can be found in some other diseases, including fungal infections, bacterial infections, sarcoidosis, and rheumatologic diseases.[15]

In countries like Bangladesh, where TB is still a major public health problem, the prevalence or incidence of NTM diseases among TB suspects has not yet been reported whereas in our neighbor country India, Thailand a lot of reports exist about NTM and its rising trends are also published and a comprehensive analysis from different parts of country has been performed.[16-17] Here, we aimed to establish a concern regarding the presence of NTM in TB suspected cases along with the implementation of most effective molecular method of detection.

Material and method

Specimen processing

Total 378 different varieties of clinical specimens such as sputum (35), bronchial lavages (55), CSF (92), pleural fluid (71), urine (42), synovial fluid (27), tracheal aspirate (4), other body fluids (52) and total 199 tissue specimens were received at the Molecular Diagnostic Laboratory of Apollo Hospitals Dhaka for the detection of Mycobacterium tuberculosis complex and NTM.

Sample processing was done by pretreatment solution 1 as per instruction of LyteStar TB/NTM PCR kit. Cell pellet was stored at -80°C until the cell-disruption was done by the addition of pretreatment solution.[2]

DNA extraction

DNA was extracted by the addition of Extraction buffer to the pellet according to manufacturer's instruction. For small pellet 50 ul and for large pellet 100 ul of Extraction buffer was used. The mixture was heated at 100°C for 20 minute and then centrifuged. 5 ul of supernatant was used in PCR reaction.

TB/NTM PCR

The LyteStar TB/NTM PCR kit 1.0 is based on real-time PCR technology, utilizing polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. Target DNA sequences are amplified with IS6110-specific primers for MTBC, and ITS-specific primers for NTM. The probes are labeled with fluorescent reporter and quencher dyes. Probes specific for MTBC and NTM DNA are labeled with the fluorophore FAM and HEX, respectively. The probe specific for the internal control (IC) is labeled with the fluorophore Cy.[5] Using probes linked to distinguishable dyes enables the parallel and differential detection of MTBC and NTM-specific DNA and the parallel detection of the Internal Control in the corresponding detector channels of the real-time PCR instrument.

The test consists of two processes in a single tube assay:

-PCR amplification of target DNA and Internal Control

-Simultaneous detection of PCR amplicons by fluorescent dye labeled probes.

5 ul Primer-Probe Mix and 10 ul 2X PCR Mix were used per sample. 15 ul master mix and finally 5 ul extracted DNA was used into each PCR tube. 5 ul Positive Control and 5 ul Negative Control was also used into one PCR tube each. Real Time Cycler Rotor Gene Q (Qiagen, Germany) was programmed according to the kit manufacturer's instruction. Specific guidelines were followed for data analysis and result interpretation.

Microscopic examination

Microscopic examination was done at Microbiology department, Apollo Hospitals Dhaka. Microscopy is done directly from specimen without decontamination. A loopful of each specimen was placed directly on a microscope slide and stained with Zeihl Neelsen using standard procedures. Slides were examined at 1000 magnification.

Result and discussion

We received 577 requests for NTM PCR having variety of clinical specimens (table I). Out of these 378 specimens were fluid type and 199 specimens were tissues. We could correlate PCR result of total 168 tissue specimen with histopathology findings as other tissue PCR requests did not accompany with histopathology requests. Out of these tissue specimens 18 cases were MTB positive by PCR (data not shown). So, rest (168-18) 150 cases were compared with histopathology findings (table II).

NTM as well as histologically compatible of chronic granulomatous lesion suggestive of TB. Among these 8 cases 3 were lymph node (NL), 3 were soft tissues, 1 from GIT and 1 from would tissues. 23 were PCR positive for NTM but histologically negative for TB granuloma. Among these 23 cases 18 cases were from GITs indicating high preference of NTM in the GIT. However, whether these NTM in the GIT are simply colonization or infection is not known. As NTMs are environmental pathogen its colonization in the GIT is possible without producing infection. The finding must be correlated with clinical features and other laboratory data. 15 cases were PCR negative while showed histological feature of tuberculosis. Out of these 15 cases 6 were from GIT, 3 were from sinus tracts. It is to be noted that number of bacteria present in the tissues are usually lower than respiratory specimen. That's why PCR sensitivity is lower in non-respiratory specimens than respiratory specimen.[18-20] Also it is important the site of specimen collection and whether disease representative areas are processed both in PCR lab and histopathology lab. Sometimes large tissue is sent to the PCR lab and it is very hard to find out the diseased area for PCR sample processing as for PCR very little amount is needed to extract DNA. Whereas large tissue can be processed by histopathology lab and multiple slides are usually prepared from tissue block. For these reasons PCR and histopathology discordant results can be obtained. In case of such discrepancy DNA can be extracted from the tissue block and a second PCR can be done from extracted DNA from tissue block. In this way we have succeeded to reduce PCR and histopathology discrepancy, however, it involves extra cost, effort and time. Rest 104 cases were negative for both PCR and histology.

Table I: Type of specimens

Specimen Name	Number
Sputum	35
BAL	55
Pleural Fluid	71
Urine	42
CSF	92
Synovial Fluid	27
Bone Marrow	1
Semen	2
Tracheal aspirate	4
Other Fluid (lung, ascetic, breast discharge, scrotal, para vertebral, ear swab, gastric)	49
Gut Tissue	85
Wound Tissue	5
Lymph Node	26
FNAC Aspirate	17
Endometrial Tissue	9
Vertebral aspirate	5
Pus	28
Sinus tract	6
Other Tissue (Bony, Neck swelling, Nasal, Foot)	18
Total	577

Table II: NTM detection correlation between PCR and Histopathology

	PCR +ve N	PCR +ve N	Total
Histopathology positive (Granulomatous reaction suggestive of TB)	8	15	23
Histopathology negative (No granulomatous reaction)	23	104	127
Total	31	119	150

Table III: NTM detection correlation between PCR and AFB staining

	PCR +ve N	PCR +ve N	Total
AFB +ve	1	0	1
AFB -ve	18	180	198
Total	19	180	199

We correlated PCR result of total 215 different verities (table III) of clinical specimen with AFB findings. Among them 16 cases were MTB positive by PCR (data not shown) and 6 of them were AFB positive. So, (215-16) 199 cases were analyzed by PCR and its correlation with AFB findings. NTM was detected in 12 out of 42 bronchial alveolar lavage (BAL), 4 out of 40 pleural fluids. Among 199 cases, only in 1 case (sputum) was positive for both AFB and PCR for NTM. Interestingly, NTM was detected in 6 out of 41 urine samples; this data is not shown here as urine samples were not requested for AFB staining. There was no case that was positive by AFB but negative by PCR. Rest 180 cases were negative for both PCR and AFB.

The development of tests for rapid, sensitive, and specific identification of the causative agent of mycobacterial infections is crucial for successful control of this disease. Diagnosis is often performed by acid-fast bacilli (AFB) staining, culture and histopathological testing. The gold standard method of identifying mycobacteria is a mycobacterial culture. The low yields by smear and culture are attributed to the paucibacillary load in the extra-pulmonary specimens.[18-20] The microscopic analysis of smears on acid-fast bacilli is indeed a quick but also an insensitive and not very specific method: as it is impossible to distinguish between either living or dead bacteria or between MTB and NTM, a culture report guarantees an improvement of sensitivity and specificity. A positive result using solid culture can be expected after about 3 to 4 weeks, a negative assessment however is only available after incubation of 8 weeks. The sensitivity limit of the solid culture is approx.102 bacteria/ml. By using liquid culture systems, in addition to increasing sensitivity the detection time can also be reduced. Therefore, a positive result is already available after 1 to 2 weeks, and safe exclusion is possible after an incubation time of 6 weeks. However, a positive growth in culture cannot distinguish between MTB and NTM as

like AFB staining.[21] Several rapid diagnostic modalities for the identification of mycobacteria have been developed. PCR-based assays for the detection of Mycobacterium approach the sensitivity and specificity of conventional culture but have the added advantage of being rapid.[22] In tissue samples, the histological findings of tuberculosis cannot specify the NTM infection and the same features are also available for other verities of disorders likefungal infections, bacterial infections, sarcoidosis, and rheumatologic diseases. To minimize these gaps, in 2009, the Centers for Disease Control and Prevention recommended using at least one molecular technique when testing for TB in a patient.[23]

We detected 18 NTM positive cases by PCR which were found negative by AFB staining, thus indicating the usefulness of PCR in AFB negative cases in routine mycobacteria diagnostic lab. Also we detected 6 NTM positive and 2 MTB positive cases (data not shown) from urine specimens those were not requested for AFB staining.

In order to know the species of NTM we sent extracted DNA from specimens to Yamagata University, Yamagata, Japan where DNA was sequenced. Although this collaborative work is not yet finished we noted dominance of mycobacterium chelonae, mycobacterium valbaalenii, mycobacterium abscessus so far. Sequencing of large number of samples is needed to know the prevalence or incidence of NTMs in the community.

Conclusion

This is the first report about the existence of NTM in this country. NTM was found in respiratory specimens and in non-respiratory specimens such as GIT, genito-urinary tract, lymph node, surgical wound and sinus tract. Early identification of the species of mycobacterium causing illness in a patient by PCR would have significant clinical impact. Patients with AFB positive specimens, especially respiratory samples, are generally presumed to be infected with Mycobacterium tuberculosis because the smear cannot determine the species of mycobacteria detected on microscopic examination and thus are treated with antituberculosis agents and placed in isolation rooms. Increased isolation of NTM causing mycobacterial diseases implies that more patients with AFB-positive samples have received inappropriate or unnecessary empirical antituberculous treatment. Overall rapid identification and differentiation to species level by molecular assay may help in targeted therapy and management of infections caused by different mycobacterial species and indirectly, it will also help in reducing the developing of antimicrobial drug resistance among NTM isolates in the community.

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References

1. Katoch VM. Infections due to non-tuberculous mycobacteria (NTM).Indian J Med Res. 2004; 120(4):290-304.

2. Guglielmetti L, Mougari F, Lopes A, Raskine L , Cambau E. Human infections due to nontuberculous mycobacteria: the infectious diseases and clinical microbiology specialists' point of view. Future Microbiol. 2015; 10:1467-83.

3. Shenai S, Rodrigues C, Mehta A. Time to identify and define non-tuberculous mycobacteria in a tuberculosis-endemic region. Int J Tuberc Lung Dis. 2010 Aug.14(8):1001-8.

4. Hsueh PR, Liu YC, So J, Liu CY, Yang PC, Luh KT. Mycobacterium tuberculosis in Taiwan. J Infect. 2006; 52:77–85.

5. Rapid increase of the incidence of lung disease due to Mycobacterium kansasii in Japan. Chest. 1983 Jun.83(6):890-2.

6. Rapid increase of non- tuberculous mycobacterial lung diseases at a tertiary referral hospital in South Korea. Int J Tuberc Lung Dis. 2010 Aug. 14(8):1069-71.

7. Davies B, Roberts CH, Kaul S, Klein JL, Milburn H. Non-tuberculous slow-growing mycobacterial pulmonary infections in non-HIV-infected patients in south London. Scandinavian Journal of Infectious Diseases 2012 Nov.44(11):815-9

8. Amorim A, Macedo R, Lopes A, Rodrigues I, Pereira E. Non-tuberculous mycobacteria in HIV-negative patients with pulmonary disease in Lisbon, Portugal. Scand J Infect Dis. 2010 Aug. 42(8):626-8.

9. Thomson RM. Changing epidemiology of pulmonary nontuberculous mycobacteria infections., Australia study Emerg Infect Dis. 2010 Oct. 16(10):1576-83.

10.Winthrop KL, Varley CD, Ory J, Cassidy PM, Hedberg K. Pulmonary Disease Associated with Nontuberculous Mycobacteria, Oregon, USA 2011 Sep. 17(9): 1760–1

11.Griffith DE, Aksamit T, Brown-Elliott BA, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007; 175(4):367–416.

12.Lai CC, Lee LN, Ding LW, Yu CJ, Hsueh PR, Yang PC. Emergence of disseminated infections due to nontuberculous mycobacteria in non–HIV-infected patients, including immunocompetent and immunocompromised patients in a university hospital in Taiwan. J Infect. 2006; 53:77–84

13.Maurya AK, Nag VL, Kant S, et al. Evaluation of an immunochromatographic test for discrimination between Mycobacterium tuberculosis complex & non-tuberculous mycobacteria in clinical isolates from extra-pulmonary tuberculosis. Indian Journal of Medical Research. 2012; 135 (6) 901–6.

14.Nasiri MJ, Dabiri H, Darban-Sarokhalil D, Hashemi Shahraki A. Prevalence of Non-Tuberculosis Mycobacterial Infections among Tuberculosis Suspects in Iran: Systematic Review and Meta-Analysis. PLoS ONE. 2015; 10(6): e0129073.

15.Lee HS, Park KU, Park JO, Chang HE, Song J, Choe G. Rapid, Sensitive, and Specific Detection of Mycobacterium tuberculosis Complex by Real-Time PCR on Paraffin-Embedded Human Tissues. J Mol Diagn. 2011; 13(4): 390–4.

16.Maurya AK, Nag VL, Kant S, et al. Prevalence of Nontuberculous Mycobacteria among Extrapulmonary Tuberculosis Cases in Tertiary Care Centers in Northern India. BioMed Research International. 2015; 465403.

17.Saritsiri S, Udomsantisook N, Suankratay C. Nontuberculous mycobacterial infections in King Chulalongkorn Memorial Hospital. J Med Assoc Thai. 2006; 89(12):2035-46.

18.Chakravorty S, Sen MK, and Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. J. Clin. Microbiol. 2005; 43:4357–4362

19.Noussair, L, F. Bert, V. Leflon-Guibout, N. Gayet and M. H. Nicolas- Chanoine. Early diagnosis of extrapulmonary tuberculosis by a new procedure combining broth culture and PCR. J. Clin. Microbiol. 2009; 47:1452–1457.

20.Pai, M. and D. I. Ling. Rapid diagnosis of extrapulmonary tuberculosis using nucleic acid amplification tests: what is the evidence? Future Microbiol. 2008; 3:1–4.

21.Early Diagnosis of Mycobacteriosis - Hain Lifescience www.hainlifescience.de/

22.Amato RFD, Wallman AA, Hochstein LH, et al. Rapid diagnosis of pulmonary tuberculosis by using Roche AMPLICOR Mycobacterium tuberculosis PCR test. J Clin Microbiol. 1995; 33(7): 1832–4.

23.Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. Centers for Disease Control and Prevention (CDC). 2009 Jan 16. 58(1):7-10.