Original Article

Assessment of Prevalence of Non-tuberculous Mycobacteria in Archival Acid-fast Bacilli Positive Smear Slides by TaqMan Real-time PCR Assay

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Abstract

Background: The emergence of non-tuberculous mycobacteria as clinically relevant pathogens has necessitated us for the study of these organisms in the context of their environment. Differentiation of *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria is important especially when we have a positive smear slide test result. **Aim:** In this study, we planned to survey the prevalence of tuberculosis and non-tuberculous mycobacteria among archival acid-fast bacilli positive smear slides. **Materials and Methods:** A number of 200 acid-fast bacilli positive smear slides were collected from different parts of Sistan and Baluchestan Province, the biggest province of Iran with the highest incidence of tuberculosis. The presence of mycobacterial IS6110 was evaluated in slides' scraped material by TaqMan real-time polymerase chain reaction assay. **Results:** The real-time polymerase chain reaction tests of archival acid-fast bacilli positive smear slides showed that 171 slides from 200 examined slides had *M. tuberculosis* DNA and in the remaining 29 examined slides, *M. tuberculosis* DNA was not found. **Conclusion:** Our findings showed that there was no *M. tuberculosis* DNA in 14.5% of archival AFB positive smear slides, and this finding necessitates us to reviewing our diagnostic and anti- tuberculous protocols.

Keywords: Tuberculosis, nontuberculous, real-time, polymerase chain reaction, PCR

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Introduction

Although success in tuberculosis (TB) elimination efforts has decreased the incidence of TB during recent years,[1] an annual increase in the prevalence of nontuberculous mycobacteria (NTM) have been noted.[2-7]

In previously published medical literatures, it was assumed that NTM observed in patients with preexisting conditions, but Prince and colleagues[8] recognized pulmonary NTM infection in elderly white women without these preexisting conditions. On the other hand, it has been proved that there is no predominant immune phenotype in PNTM infection.[9,10] Clinical and paraclinical studies also showed that since 1980s, the number of patients with NTM pulmonary disease without underlying risk factors have been increased.[11-14]

As NTM are ubiquitous environmental organism and with respect to their contribution in NTM disease, numerous studies in different countries have been done to assess the epidemiology of NTM infection and its related diseases,[15] but there are no nationally representative data regarding the prevalence of pulmonary disease associated with NTM in these countries including United States.[1]

Different risk factors for infection with NTM have been identified; they included cystic fibrosis, HIV, chronic lung disease, working in the mining industry, warm climate, advancing age, and male sex.[15]

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Treatment of NTM infections can be challenging and time consuming since the organism may be resistant to commonly prescribed antituberculosis antibiotics and their prevalence has shown geographic variability.[15]

Lai and colleagues[16] reported that increased isolation of NTM from patients implies that more patients with acid-fast bacilli (AFB) positive samples have received inappropriate or unnecessary empirical antituberculosis treatment.

As in working with archival materials, culture and microbiological investigations are impossible and because in large geographical areas like Sistan and Baluchestan province, immediate availability to advanced laboratory tests is impossible and transportation of clinical samples is not easy, we used this method in studying frequency distribution of tuberculosis and NTM in AFB positive smear slides.

Materials and Methods

The study was approved by the Zahedan University of Medical Sciences Ethics committee and the patients were aware of collection of their samples for diagnosis.

Two hundred AFB-positive smear slides from tuberculosis patients which were collected after quality control by the Tuberculosis Center in Sistan and Baluchestan Province were used in our study.

Each slide was rechecked in the Zahedan Research Center for Tropical and Infectious Diseases, and their slide scores were recorded. Iranian National Union Against Tuberculosis form were filled by sending sector. Archival material of each slide was scraped and collected in a 1.5 ml Eppendorf microtube. DNA was extracted from scraped materials as explained previously[16] and 5 µl of extracted DNA, 1.0 µl of Primer/Probe (Primer Design, UK), 3.0 µl of DDW, 10 µl of TaqMan universal master mix (Applied Biosystems, USA), and 1.0 µl of internal extraction control (Primer Design, UK) were mixed for a 20 µl reaction mixture. Amplification was performed after an initial denaturation at 95°C for 10 minutes followed by 50 cycles of denaturation step at 95°C for 15 sec, both annealing and extension at 60°C for 60 sec (ABI Prism 7500, Applied Biosystems). Minimal bacterial cell numbers allowing detection were determined by using serial dilution of the Kit positive control by 10 fold dilution from 1×10^5 to 1×10^2.

Statistical analysis

Statistical analysis was performed with SPSS 14 software. Continues variables were compared using the Student's *t*-test.

Results

Positive control of DNA extraction

Positive control of DNA extraction produced expected signals for internal extraction control of the kit, which had a VIC fluorochrome on its 5′ end and TAMRA on its 3′ end with approximately Ct 32.

TaqMan real-time polymerase chain reaction (PCR) of archival AFB positive smear slide. Fluorescent curves of the standard dilution series were used to calculate the concentration of *Mycobacterium tuberculosis* DNA in unknown samples. The linear regression coefficient was 0.998 and efficiency of PCR was 95.8% [Figure 1a].

M.tuberculosis positive control

A positive control for TaqMan real-time PCR was constituted by using extracted DNA from *M. tuberculosis* H37Rv and clinical strain CDC1551 and confirmed by the observation of expected signals which has been plotted by red color [Figure 1b]. Minimal bacterial cell numbers allowing detection were determined by using serial dilution of kit fluorescent curves resulted from TaqMan real-time PCR of tested materials [Figure 1b].

Negative control

A negative control for TaqMan real-time PCR obtained by observation of no amplification signal except for internal amplification of the kit which has been plotted by straight red color [Figure 1b].

The product of TaqMan real-time PCR was electrophoresed in an agarose gel electrophoresis and stained with ethidium bromide [Figure 2].

TaqMan real-time PCR of archival AFB positive smear slides showed that 171 slides from 200 examined slides had *M. tuberculosis* DNA, and in remaining 29 examined slides no *M. tuberculosis* DNA was found.

Discrepant analysis results of specimens by TaqMan real-time PCR system and demographic, clinical, and laboratory findings showed that there was no significant relationship between age, history of tuberculosis in the family, bloody sputum, and history of treatment, but there was significant relationship between AFB slide score and C_t of TaqMan real-time curves (data not shown).

Also, all of the TaqMan PCR results were in accordance with response to treatment. In this study, from 29 specimens with positive smear staining and negative real-time PCR results, 6 specimens had AFB score grade 3, 6 specimens had grade 2, and 17 specimens had grade 1.

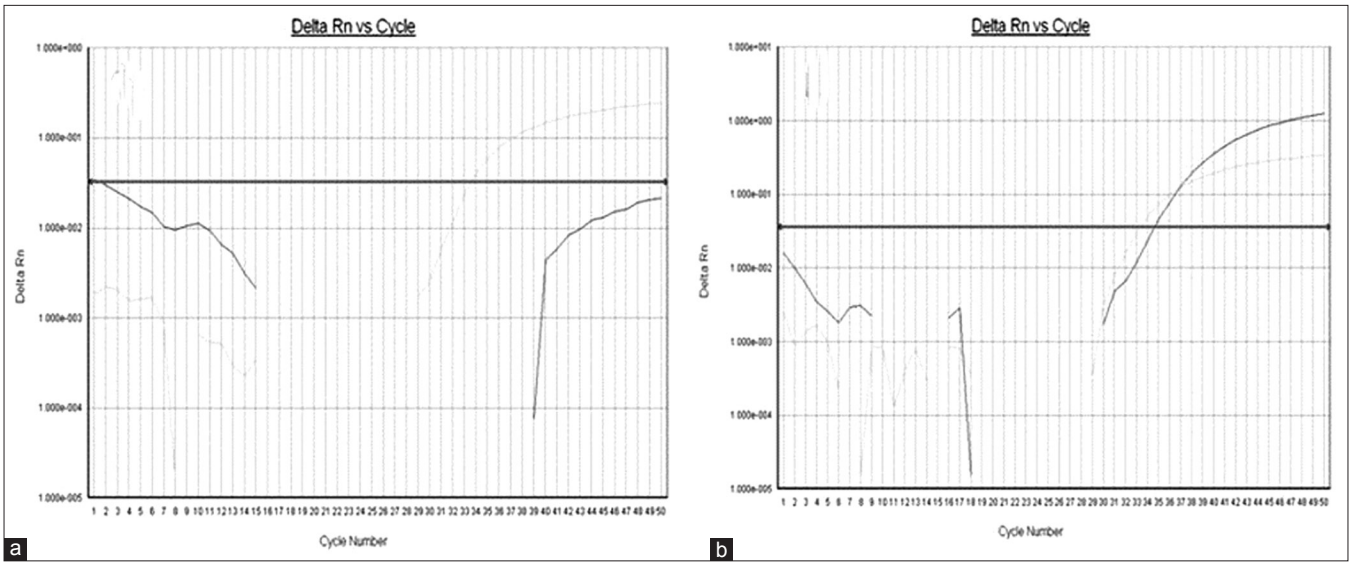

Figure 1: TaqMan real time PCR amplification plot; (a) Internal extraction control; (b) Patient sample

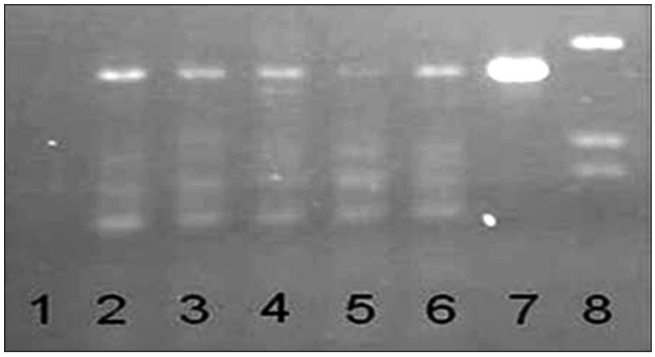

Figure 2: Polymerase Chain Reaction results of Scraping samples from smear positive slides; Line 1: Control Negative; line 2-6 Real time positive slides; Line7: Control Positive (a 166bp band patients, Line), Line 8:1Kbp DNA Ladde

Discussion

Although success in TB elimination efforts has reduced incidence of *M. tuberculosis* related diseases, apparent increase in nontuberculous mycobacterial disease has occurred. In many countries, there are no nationally representative data about the prevalence of NTM related diseases.[1]

Previous studies showed different incidence rates of NTM. Billinger and colleagues reported that during 1981–1983 estimated prevalence of pulmonary tuberculosis was 1–2 cases/100000 persons in the United States.[1] Morras *et al*.[2] reported that there was an average annual increase of 8.4% for the prevalence of NTM in Canada during 1997–2003. Lai and colleagues reported that from January 2000 through December 2008 from total of 23499 specimens with positive mycobacterial culture results, *M. tuberculosis* were isolated from 9204 (3.2%) specimens from 4786 patients.[17]

Our result is in accordance with the findings of Tabarsi *et al*., in which 11.43% of their 105 patients with multiple drug resistance histories were NTM.[18]

Also, Sun *et al*. showed that by IS6110-based PCR, among 104, 59 (56.7%) were positive for *M. tuberculosis* complex and 45 (43.3%) specimens were NTM[19] and Makarova *et al*. showed that 65.4% of their 242 patients were NTM.[20]

These studies showed that a significant number of mycobacterial cultures were NTM and special attention must be made on diagnosis of these mycobacteria. On these aspects, our results are in accordance with Sun *et al*.[19] and Makarova,[20] but their reported incidence is far from our results.

A study by Srisuwanvilai *et al*. showed that from 1417 isolates, 1255 (86%) were identified as MTB and 162 (11%) NTM.[21] These results are in competence with our results. This similarity may be due to closeness of national and cultural conditions.

Although these results confirmed our previous study,[14] the rate of isolation of *M. tuberculosis* was higher than that of our previous study (85.5% and 39.3%, respectively).

Results of this study are in contrast with those of Beqaj *et al*., in which of 13 samples, 12 were positive by real-time PCR. Eleven of their 13 specimens were culture positive. Their culture negative and real-time PCR positive were confirmed by a second PCR method in another reference laboratory.[22] Difference between these results and our findings may lay back in the background of studied populations (i.e., incidence of environmental NTM, immunological status of individuals, economical conditions, and race).

In summary, there is an agreement between our study and the published literature in increasing rate of NTM. Our results showed that a significant percentage of AFB positive smear slides had no extractable *M. tuberculosis* DNA and this finding necessitates us to reviewing our anti-TB diagnostic and therapeutic protocols.

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