

PCR based molecular detection of *M. tuberculosis* and comparison of its efficacy against AFB based bacteriological detection in diagnosis of Pulmonary Tuberculosis in Nepal.

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Abstract

Introduction: Tuberculosis is high burden infectious disease in Nepal. Diagnosis strategy for tuberculosis includes Chest X-Ray, Ziehl-Nelson staining method and culture. Culture is considered to be a “gold standard” in TB diagnosis; however, it takes almost 3 weeks to do culture which affects rapid diagnosis of TB. This study investigates molecular based TB diagnosis and compares the result with the AFB staining.

Methods: Sputum samples from the National Tuberculosis centre (NTBC), Sano Thimi, Nepal were tested with Real Time PCR (QPCR) based assay for MTB detection at the affiliated laboratory of the Center for molecular Dynamics Nepal (CMDN), Kathmandu, Nepal.

Results: Out of 47 samples, 7 AFB positive samples yielded quantifiable QPCR positive result. However, of the 40 AFB negative samples assessed, 3 were found to contain quantifiable amount of *M. tuberculosis* using TB DNA detection. This amounts to 6.38% under detection using just AFB microscopy alone.

Conclusions: There is added benefits of using PCR in detection of *M. tuberculosis*. This method, if used in conjunction with traditional AFB will provide a more rapid, reliable and accurate diagnosis of *M. tuberculosis*.

Key Words: Real-Time PCR, Molecular detection, Tuberculosis, TB, Comparative analysis, Diagnosis, AFB method

Introduction

Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis in humans which leads to severe infection of the lungs. This disease is the second commonest cause of death worldwide, after HIV.¹ Tuberculosis (TB) remains one of the major public health problems in Nepal, with almost 50% population infected with TB, 60% of those being adults. In Nepal prevalence of all types of tuberculosis cases is estimated at 240 per 100,000 population and incidence is estimated around 173 per 100,000 population.² Every year, 21,827 smear positive infectious TB incidence cases are expected to arise in the country. National DOTS strategy

was introduced to Nepal in 1996 and has achieved nationwide coverage since April 2001. Although introduction of DOTS has already reduced the numbers of deaths, however 5,000 to 7,000 people still continue to die each year. Tuberculosis case detection increased from 30% in pre DOTS era in 1995 to 72% in 2008.² However, national reports on TB depicts the fact that case finding has remained almost static for past few years with a constant gap between estimated and notified cases per 100000 populations every year since 2000. This finding suggests a need for improvement in case detection in order to decrease the transmission of TB, which could be achieved by expanding the services for early diagnosis of Tuberculosis.

PCR based molecular detection

Despite the enormous burden of tuberculosis (TB) and the overall low rates of case detection, sputum smear microscopy remains the key tool for diagnosis of infectious tuberculosis. Although microscopy is simple, specific and rapid, its low sensitivity is a major cause for concern.³ In addition, type of specimen, its quantity, quality, bacterial content and viability of organism considerably influence the sensitivity and the specificity of this method. Mitigation of this influence is difficult in resource constrained country like Nepal. Microbial culture and diagnostic facilities are being provided through a unique public private partnership with an NGO –run laboratory in Nepal.⁴ Hence, culture as a diagnostic test is not easily available in Nepal, in addition it is time consuming and technically demanding. In addition, dependence on smear microscopy and chest radiography for the diagnosis of TB makes it difficult to diagnose Smear-Negative Pulmonary TB and Extra Pulmonary TB. The long process of MTB detection presents many challenges in Nepal. Most of the TB patients live in rural areas where these facilities are not available. Thus, waiting for confirmatory result in the city poses a major financial constraint for them.

As a response to these challenges, The Nepal Government National TB program is now aiming to achieve universal case detection, to further shorten diagnostic and treatment delays in order to cut transmission and prevent complications and deaths. Diagnostic technology that has high specificity and sensitivity and is rapid in detecting *M. tuberculosis* within 2-3 days would be very effective in delivering treatment in timely fashion. Every year 44,000 people develop active TB and 20,000 of them have infectious pulmonary form of TB.² By introducing diagnostic methods that could not only help identify the MTB bacteria at the species level, but also reduce detection time of MTB by few days as opposed to microbial culture would assist early drug intervention. Such diagnostic technology with high specificity and sensitivity could then be utilized as confirmatory test for TB diagnosis in major diagnostic centers in the country, while at the same time, be used to accurately diagnose Smear-Negative TB.

Genomic method of Nucleic Acid Amplification Tests (NAATs) such as PCR has been assessed as a possible method of detecting *M. tuberculosis*.⁵⁻⁷ A study showed that multiplex PCR could be used to detect and distinguish *M. tuberculosis* from *M. bovis*, both closely related bacterial species, based on a 12.7kb fragment unique to the former.⁸ Although NAAT also require specialized laboratory infrastructure and expert human resource, it still has a major advantage in detection of MTB detection due to its speed as well as its enhanced overall sensitivity and specificity.³ A study on comparative assessment of different methods of extracting MTB DNA from sputum samples for

Real Time PCR assays has also identified NAAT method as being efficient in *M. tuberculosis* detection.⁹

This is the first ever real time PCR based molecular assessment study carried out to detect TB in human samples testing positive for AFB staining in Nepal. A previous study using loop mediated isothermal amplification (LAMP) method has reported the successful detection of pulmonary tuberculosis in patients visiting a hospital.¹⁰ This research is intended to assess the molecular method based on Real Time PCR technique in MTB detection in suspected pulmonary TB cases and compare the results with the DOTS-based bacteriological Acid Fast Bacilli (AFB) staining.

Methodology

Baseline cross sectional study was conducted from February 2009 to June 2009. A total of 56 samples were obtained from National TB centre based on clinical symptoms including chest pain, coughing period, weight loss, chest X-Ray, while excluding relapse cases and patients on medication. With informed consent taken, the suspected TB patients were provided with a collection vial and asked to submit early-morning-deep sputum sample the next day. Samples were used to perform AFB staining test at National TB Centre and remaining transported directly to Intrepid Nepal Laboratory, Thapathali, Kathmandu, Nepal and stored at -20°C.

Out of 56 samples, 47 samples were processed while others were excluded due to inappropriate nature of the submitted sample. Sputum samples were digested and decontaminated followed by DNA extraction using Genomic DNA extraction kit (ShineGene Molecular Biotech Inc., China).

Primers and probes used detected and amplified 12.7 k.b. fragment specific for *M. tuberculosis* which is not present in *M. bovis* (PrimerDesign Ltd., UK). The assay was based on Real Time PCR. The 12.7-k.b segment is labeled with 3' quencher end and a FAM (6-carboxyfluorescein) fluorescent dye labeled at 5' end. An internal DNA extraction control was used during extraction to detect PCR inhibition in the extracted sample which was detected using VIC fluorescent dye. Endogenous ACTB control was used to check the validity and satisfactory presence of the biological template in the sample. Detection was performed using FAM (6-carboxyfluorescein) fluorescent dye.

Each sample was processed for QPCR in duplicate using Eppendorf Real Time Thermal Cycler. The PCR mixture (20 µl) contained 10µl of PCR master mix (2X reaction, 0.025 U/µl Taq Polymerase, 5 mM MgCl₂, 200 µM each dNTP), 1 µl Primer/probe mix for pathogen *M. tuberculosis*, 1 µl Primer/probe mix for Internal extraction control DNA and 3 µl of molecular grade water and 5 µl of each diluted samples were

prepared. Water was used for the “No template” control. Cycling conditions used were as follows: 10 minutes of Enzyme Activation at 95°C followed by 50 cycles of 10 seconds, denaturation at 95°C and 60 seconds of Amplification and Detection at 60°C.

External TB DNA Standards were provided by the PrimerDesign Ltd., U.K. The standard provided was 2.0×10^7 copies per μl which was further diluted to 2 copies per μl as per manufacturer’s instructions. Standards were run in the Eppendorf Real-Time Thermal cycler with the PCR master mix and thermal cycler conditions similar to that of sample. The graph of Ct value versus amount (copies/ μl) was plotted generating a linear line. Concentration of unknown Mycobacterial DNA in sample was calculated by comparing the Ct value obtained from logarithmic phase of the fluorescence curve with the linear plot generated from standard curve.

Results

Results from real time PCR assay showed that overall 10 samples (21.3 %) were positive for MTB, whereas AFB staining showed that 7 (14.9%) were Mycobacterium tuberculosis positive (Fig. 1). Of the 47 samples processed at CMDN by the method of Quantitative Real-time PCR, 10 samples were positive (21.3%) for M. tuberculosis, while remaining 37 (78.7%) samples were negative for M. tuberculosis (Figure 2). The average DNA copies per ml sputum for all 7 AFB+/QPCR+ samples was 3.474×10^8 (Standard Deviation of the Mean (SD), 5.19×10^8). This number for QPCR+/AFB- samples (n=3) was 4.28×10^6 (SD, 5.33×10^6) (Table 1). This equates to about 100 fold difference between bacterial load counts between the two types of results.

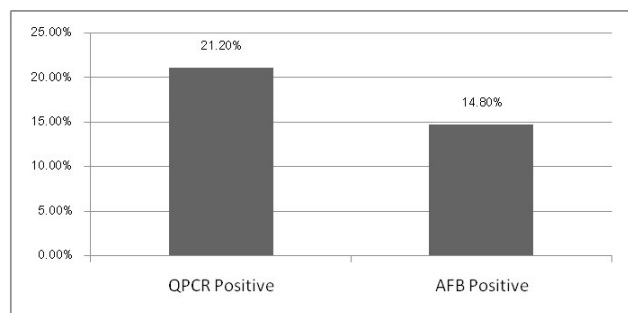


Fig. 1: Graph showing relative percentage of MTB detection in all samples by AFB staining as compared to QPCR method of detection. The percentage of samples testing positive in both instances are indicated on top of each corresponding histogram. The percentage was calculated by taking overall positive results obtained using the two different methods of detection on all 47 samples.

Table 1: Comparison of AFB microscopy results with Real Time PCR results for 10 samples that were Real Time QPCR positive. (Among the remaining samples (n=37), all were tested as being Mycobacterium tuberculosis negative by both AFB and Real Time QPCR methods. “neg” denotes Negative results; “pos” denotes Positive results.)

Internal Patient Code	AFB result	QPCR value (DNA copies/ml sample)
M005*	neg	1.5×10^5
M009	pos	2.0×10^6
M013	pos	3.3×10^6
M028*	neg	2.4×10^6
M033*	neg	10.3×10^6
M036	pos	10.3×10^7
M037	pos	8.1×10^7
M046	pos	4.1×10^9
M049	pos	1.0×10^9
M054	pos	1.2×10^9

*Average QPCR Value for positive samples = 2.44×10^8 copies/ml

The same samples, when assessed using the method of AFB staining had earlier confirmed 7 (14.9%) as being M. tuberculosis positive, 40 negative (85.1%). Comparatively, 3 (6.38%) AFB negative samples showed QPCR positive for Mycobacterium Tuberculosis. The overall M. tuberculosis detection was therefore higher using the QPCR method than using conventional AFB staining method (Fig. 2).

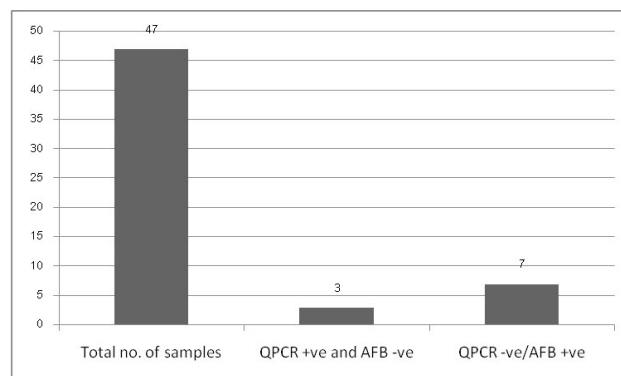


Fig.2: Graph showing comparative differences in MTB detection between QPCR method and AFB method in all samples assessed. The numbers of actual samples are indicated on top of each corresponding histogram. X- Axis indicates the samples and the tests carried out. Y- Axis indicates the number of samples.

Discussion

The long term health of an individual showing clinical symptoms of pulmonary TB relies immensely on the accurate detection of Mycobacterium tuberculosis for medication intervention. Although the successful DOTS regime has

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contributed to better detection and care of this disease, problems still exist in rapid and sensitive methods of detection.

There is no doubt that microscopy and culture are still the widely accepted methods for laboratory diagnosis of pulmonary tuberculosis.¹¹ However, molecular tools are now being promoted as the preferred method of *M. tuberculosis* detection.³ The main reasons for this are due to the inherent advantages of this method over traditional methods in terms of speed, sensitivity and specificity. This comparative analysis of detection techniques of *M. tuberculosis* in Nepal shows some significant difference in sensitivity and specificity between genomic (DNA) based detection versus microscopy. Even amongst AFB negative samples, the QPCR values were overall lower than average. This suggests that the lower number of bacterial cells may lead to false negative results in AFB staining tests. The overall 6.38% difference between QPCR and AFB staining method using microscopy suggests a trend which may be clearer conducting a larger study with bigger sample size.

There are issues regarding nucleic acid amplification tests (NAATs) such as PCR or Real Time PCR methods. Some reports have suggested that there are high chances of generating false negatives or positives using NAATs.^{6,12} However US Center for Disease Control (CDC) have recommended that diagnosis of pulmonary TB can be presumed in smear positive patients with a positive NAAT result, and in smear negative patients with two subsequent NAAT positive results. Use of PCR targeting IS6110 region has also been recommended as a possible method to diagnose *M. tuberculosis* in smear negative samples.¹³ A recent study has stated that the accuracy of NAATs for MTB detection was higher if IS6110 as amplification target using nested PCR method.^{7,14} Our study has shown that NAAT methodology may detect *M. tuberculosis* in instances where AFB staining suggests otherwise. A published finding based on their study into commercially available amplification tests for the diagnosis of pulmonary TB supports this result.⁶

A limiting factor in our study was the lack of culture results for the samples assessed for MTB. This would have provided a confirmation as to the accuracy of NAAT results from our laboratory against the culture results. However, since this is one of the pioneering molecular assessment studies focusing only on comparison between smear microscopy and QPCR tests, the results will provide a base for future such studies. A comprehensive study involving AFB results, NAAT results and culture results needs to be carried out on large number of samples to assess the efficacy of the methods available in developing countries such as

Nepal.

In addition, comparative culture analysis of the smear negative and QPCR negative samples also needs to be conducted in order to come up with precise sensitivity and specificity data. Bacterial culture is still considered to be the “gold standard” in *M. tuberculosis* detection, although it is cumbersome and takes significant amount of time. We, however, feel there is a growing need for rapid and accurate detection of *M. tuberculosis* in developing countries like Nepal, and genomic based detection would probably be the preferred choice due to its high specificity and sensitivity and also its quick turnaround time for delivering results.

Conclusions

The results from this pilot study show that there are quantifiable benefits of using molecular tool of PC in TB detection as compared to the traditional AFB staining methodology. Our results clearly reflect the low efficiency in TB detection by AFB staining methodology, while direct genome analysis using QPCR method appears to provide better diagnosis. A 6% false negative result based on AFB method as compared to PCR shows that there are shortcomings of the only AFB based methodology in TB detection. Molecular diagnostics are now being accepted as the preferred tools for disease diagnosis (including TB) worldwide, and should now be assessed further for use in TB detection in Nepali context.

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