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ASSESSMENT OF DETECTION EFFICACY OF *MYCOBACTERIUM TUBERCULOSIS* IN SPUTUM SAMPLES BY REAL TIME PCR BASED METHOD

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ABSTRACT
Tuberculosis (TB) is a major public health problem in Nepal and ranks as one of the most prevalent communicable diseases throughout the country. In Nepal, 45% of total population is infected with TB and 40,000 people get TB every year. Twenty thousand new sputum positive cases are seen every year and 5000-7000 people die each year from TB. Thirty sputum samples were collected from Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu, Nepal and the comparative study of Acid-fast Bacilli (AFB) test and Real time PCR were conducted separately with the culture test which is regarded as gold standard by WHO. Detection of *Mycobacterium tuberculosis* through use of Real time PCR was found to be higher as compared to AFB and culture. Real-time PCR test showed higher sensitivity (100%) and specificity (94.11%) as compared to AFB test with sensitivity of 84.61% and specificity of 88.24%. Positive predictive value was found to be 84.61% and 92.86% for AFB and Q-PCR respectively. Negative predictive value was found to be 88.24%, and for Q-PCR, it was found to be 100%. Our statistics clearly show that TB diagnosis by Q-PCR is highly efficient and reliableover conventional methods of diagnosis and here we recommend its use in the hospitals and clinics of Nepal.

Keywords: Acid Fast Bacilli test, Real time PCR, TB

INTRODUCTION
Tuberculosis (TB), one of the oldest recorded human afflictions, is still one of the biggest killers among the infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics. Tuberculosis, MTB or TBis a common and in many cases lethal infectious disease caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis* [1]. Tuberculosis usually attacks the lungs but can also affect other parts of the body. It is spread through the air when people who have an active MTB infection cough, sneeze, or otherwise transmit their saliva through the air [2].

In Nepal, Direct Observed Treatment Short course (DOTS) strategy was introduced by the National tuberculosis program (NTP) in 1996 and since then the number of deaths from tuberculosis has reduced but still 5,000 to 7,000 patients die every year due to tuberculosis [3]**.** These and increasing number of death every year is mainly due to delayed diagnosis and hence late start of treatment. This delay in the diagnosis and treatment of tuberculosis cases cause

spread of infection in the community thus increases severity of the disease and hence results in higher risk of mortality.

In Nepal, the laboratory diagnosis of tuberculosis is mainly done on the basis of Acid fast bacilli staining and AFB positives are confirmed by culture. These conventional tests have many drawbacks. AFB staining test, though being a popular and much cheaper technique, has its inability to distinguish *Mycobacterium* species as *M. smegmatis* as well as *M. tuberculosis* both are take up primary stain and appear red. Secondly, the staining test cannot distinguish between viable and non-viable organisms leading to a false positive cases and hence a wrong diagnosis of the disease.

On other hand, culture method is considered as the gold standard for both diagnosis and drug sensitivity testing. Although it is one of the reliable techniques it is not a quicker method of diagnosis as it may take 6-8 weeks from the day of inoculation which hampers timely detection of the disease.

Various PCR techniques have been shown to work efficiently in diagnosis of the tuberculosis diseases. We aim to detect the 12.7 Kb of *IS6110* gene fragment that is specific to *M. tuberculosis* hence differentiating between *M. bovis* and other *Mycobacterium* complexes [4].

MATERIALS AND METHODS

Subjects and setting

Hospital based cross-sectional study was conducted during the month of March, April and May with oral informed written consent. Thirty sputum samples were collected from patient screened for tuberculosis symptomslike: chest pain, night fever and cough complain at ShukraRajTropical and Infectious Disease Hospital, Teku, Kathmandu, Nepal**.** Patients were instructed to take three deep breath and expectorate were collected in sterile specimen cup.Each patient was asked to submit three sputum specimens namely:Spot specimens on first visit, early morning collection by patient on next day and Spot specimen during second visit. These samples were tested microscopically using ZiehlNeelsen staining method at Shukra Raj Tropical and Infectious Disease Hospital, Teku, Kathmandu, Nepal. For DNA extraction and Real time PCR, samples were transported at 4°C to Intrepid Nepal laboratory and stored at -20 $^{\circ}$C.

AFB (Acid Fast Bacilli test) staining/ smear Microscopy

Acid Fast Bacilli were observed under microscope following the ZeihlNeelsen staining technique. Uniform and consistent smear were prepared with loop taking purulent portion of sputum sample and heat fixed. Staining was performed as per standard protocol of Ziehl-Neelsen staining method [5]. The results were noted down as per observation under microscope.

DNA extraction and Real Time PCR (Q-PCR)

Sputum samples were pretreated with Sodium Hydrooxide followed by DNA extraction using Spin column Genomic DNA extraction kit (ShineGene Molecular Biotech Inc., China). During extraction, pretreated sputum samples were incorporated with 4 µL of internal extraction control DNA.

Real-time PCR was performed as per instructed by the PrimerDesign Ltd., U.K , Mycobacterium tuberculosis standard Kit. The assay adopted by kit is based on Taqman principle constituting of pathogen probe tagged with FAM (6-carboxyfluorescein) fluorescent dye at 5' end as a reporter dye. In the same assay, internal DNA extraction control primer/probe mixtagged with VIC fluorescent reporter dye was also used. Endogenous ACTB control tagged with FAM (6-carboxyfluorescein) fluorescent reporter dye was performed as separate assay for each sample.

Each sample was processed for QPCR using Eppendorf Real Time Thermal Cycler platform. APCR mixture (20 µL) contained 10µL of PCR master mix (2X reaction buffer, 0.025 U/ µL*Taq* Polymerase, 5 mM $MgCl_2$, 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 1:20 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 1 minute of Amplification and Detection at 60°C.

External TB DNA Standards were provided by the PrimerDesign Ltd., U.K. The standard provided was 2.0E+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 C_t value versus amount (copies/ µL) was plotted generating a linear line. Concentration of unknown Mycobacterial DNA in sample was calculated by comparing the C_t value obtained from logarithmic phase of the fluorescence curve with the linear plot generated from standard curve.

Culture

Lowenstein-Jenson media was used for detection of the *Mycobacterium* spp. in the sputum samples.The media was prepared according to the protocol of NEOGEN ® Corporation and culture was carried out [6]. Culture was performed by decontamination of samples inside a tube was done by treating samples with twice the amount of NaOH.Pellet was resuspendedin little amount of remained supernatant.

Inoculation was done by using 10µL of this sample as inoculum and poured slowly over the surface of media in an inclined position. Tubes were tightly secured. Sample was distributed homogenously over the surface of media by rolling of the tubes. These tubes were incubated at 37°C in inclined position for 4 hours followed by incubation at 30°C for 4-6 weeks. The growth of the *Mycobacterium* was observed every alternate day.

RESULTS AND DISCUSSION

Out of the 30 sputum samples analyzed (Table 3), 43.33% samples were AFB positive and 56.67% samples were AFB negative, 46.67% samples were PCR positive and 53.33% were PCR negative, 43.33% samples were found to be culture positive and 56.67% samples were negative.

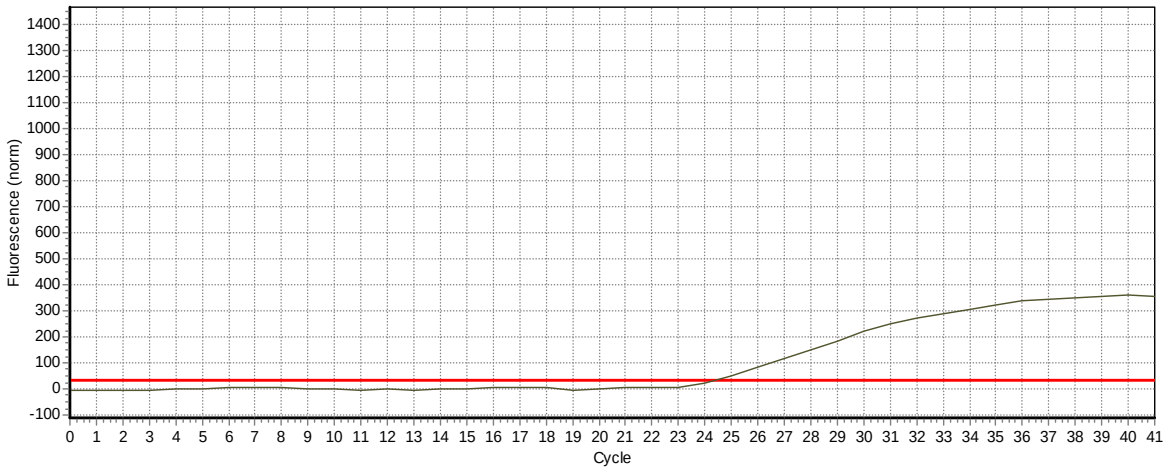

Fig1: Showing QPCRresult of sample M014

13.33% AFB negative samples were found to be PCR positive, 6.67% AFB positive samples were found to be PCR negative, 43.33% samples were found to be culture positive and 56.67% samples were found to be culture negative, 6.67% culture negative samples were found to be PCR positive, 3.33% culture negative samples were found to be AFB positive and 6.67% culture positive samples were found to be AFB negative.

Validity measurement for QPCR.

		culture		
		+	-	total
QPCR	+	13	1	14
	-	0	16	16
	Total	13	17	

Table 1: True Positive and True Negative calculation between QPCR and Gold standard (culture).

Sensitivity: $\frac{13}{13}$ X 100 = 100 %

Specificity: $\frac{16}{17}$ X 100 = 94.11%

Positive predictive value: $\frac{13}{14}$ X 100= 92.86%

Negative predictive value: $\frac{16}{16}$ X 100 = 100%

Validity measurement for AFB

		culture		
		+	-	total
AFB	+	11	2	13
	-	2	15	17
	Total	13	17	

Table 2: True Positive and True Negative calculation between AFB test and Gold standard (culture).

Sensitivity: $\frac{11}{13}$ X 100 = 84.61 %

Specificity: $\frac{15}{17}$ X 100 = 88.24%

Positive predictive value: $\frac{11}{13}$ X 100= 84.61%

Negative predictive value: $\frac{15}{17}$ X 100 = 88.24%

The sensitivity with respect to gold standard (culture) for AFB was found to be 84.61% while for Q-PCR it increased to 100%; specificity for AFB was found to be 88.24% while for Q-PCR, it increased to 94.11%; Positive predictive value for AFB was found to be 84.61% while for Q-PCR, it increased to 92.86% ; Negative predictive value was found to be 88.24%, while for Q-PCR, it increased to 100%. These statistics clearly show that Q-PCR is highly efficient for the diagnosis of TB.

SAMPLE	AFB Result	QPCR Result(DNA copies/mL)	Culture Result
M002	+	+	+
M008	-	-	-
M009	-	-	-
M010	-	-	-
M012	+	+	+
M013	-	-	-
M014	**-**	**+**	**+**
M015	-	-	-
M016	-	-	-
M017	-	-	-
M018	+	+	+
M021	**-**	**+**	**-**
M022	-	-	-
M023	-	-	-
M024	-	-	-
M025	-	-	-
M026	**-**	**+**	**+**
M027	-	-	-
M028	-	-	-
M029	-	-	-
M031	**+**	**-**	**-**
M032	+	+	+
M033	+	+	+
M034	+	+	+
M035	+	+	+
M037	+	+	+
M038	**+**	**-**	**-**
M039	+	+	+
M040	+	+	+
M044	+	+	+

Table 3 Results of culture, AFB test and Real time PCR method

The study shows that Real time PCR method is more reliable, efficient, specific and faster when conventional process fails to timely detect presence of tuberculosis infection. Even in the lowest load of the *Mycobacterium tuberculosis,* the earliest possible diagnosis can be made by this process. This earlier diagnosis of disease will ultimately decrease the death rates of tuberculosis infected patients. Conventional process fails as only small fraction of sample is being observed for testing and bacillus is not uniformly distributed and due to low amount of presence of bacilli. So there is high possibility of bacilli to be missed in detection of TB by AFB processes and culture. But since the Real time PCR is based on the nucleic acid amplification principle, even the smallest amount of DNA present can be replicated in high amount to be detected using the technology.

Similar studies were carried out by different researchers during different times. But our result was most effective and better. Our study result shows the sensitivity, specificity, PPV and NPV values for PCR as 100%, 94.11%, 92.86% and 100%. Beige *et al*. in 1995 found sensitivity 98% and specificity 70% during his study on TB patients [7].

In 2001, Goel *et al*. compared four conventional methods of diagnosing TB and found that correct diagnosis of TB could be made in 94.87% of cases by combination of the four methods. His study showed sensitivity, specificity, PPV and NPV of PCR as 94.44%, 38.23%, 44.73% and 92.85% respectively when culture alone was considered as gold standard [8]. In 2005, Chakravorty*et al*. used PCR technology on extra pulmonary specimens and showed sensitivity 68.6% and specificity 92.6% [9]. In the other study carried out by Halder *et al*. in 2007, he found sensitivity 95% and specificity 92.9% [10]. This result shows that our research was comparatively better than all other studies. This advancement is achieved by the use of Real-time PCR method. High sensitivity, reliability and advanced technology made all these possible.

In our research, RT-PCR yielded different results. Those were more efficient compared to the results from AFB staining and culture technique. Nevertheless, it is more expensive as different chemicals, reagents and instruments are used. So the feasibility of this technique is limited to the small group of people. Also the unavailability of this technique in most of the hospitals and lack of knowledge of this technique in the health personals has limited its access to the people across the country. From the calculations, the sensitivity for Q-PCR was found to be 100% and other features such as specificity, positive predictive value, negative predictive value were close to the perfect. This may have been entailed due to the unavoidable contamination during the process. In the future, such flaw should be carefully considered prior to the amplification so that we can enhance the accuracy and specificity of this method.Tuberculosis being one of the prominent killers in this century, its identification and detection should be more rigorous especially in developing country like Nepal. As conventional methods such as AFB generally lacks sensitivity and specificity and culture technique requires more time. Our data has shown detection through TB-PCR to be more rapid and accurate diagnosis. So the appropriate technique treatment regimen can be started early. It has a disadvantage of high cost but the disadvantage has been masked by the sensitive and quality results generated by this method.

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