REAL TIME POLYMERASE CHAIN REACTION (Q-PCR): IT'S COMPARISON WITH CONVENTIONAL TECHNIQUES FOR DIAGNOSIS OF TUBERCULAR LYMPHADENOPATHY

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ABSTRACT
This test validation study was to compare real-time PCR (Q-PCR) with conventional techniques (represented by Acid fast bacilli smear, Histopathology and culture method) in the laboratory diagnosis of tubercular lymphadenopathy in a rural medical college in north India. The study was undertaken on a total of 174 patients of both sexes and various age groups attending the indoor and OPD of tuberculosis and chest disease department. In addition to acid fast bacilli smear and Lowenstein-Jensen culture Q-PCR, histopathological test were used to diagnose tubercular lymphadenopathy. The results show that the sensitivities of the histopathological diagnosis, AFB smear, Q-PCR were 73.3%, 53.3% and 100% respectively and the specificity of histopathological diagnosis, AFB smear and Q-PCR were 94%, 88.1% and 97.6% respectively in comparison to culture that was considered gold standard. Q-PCR can be used for early and prompt diagnosis of tubercular lymphadenitis, which can aid in initiating a timely anti-tubercular treatment and prevent irreversible sequelae associated with morbidity of the disease.

Keywords: Tubercular Lymphadenopathy, Real Time Polymerase Chain Reaction, Histopathology, Culture, AFB.

INTRODUCTION
Tuberculosis (TB) remains one of the world's lethal communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the infection, 360 000 of whom were HIV-positive. Of the estimated 9 million people, more than half (56%) were from the South-East Asia and Western Pacific Regions. India and China alone accounted for 24% and 11% of total cases, respectively. Nonetheless most TB cases and deaths befall men, the burden of disease among women is also high. An estimated 3.3 million cases and 510 000 TB deaths among women were seen in 2013.[1] The incidence of mycobacterial lymphadenitis has increased in parallel with the increase in the frequency of mycobacterial infection globally. TB lymphadenitis (TBL) is seen in nearly 35 per cent of extra pulmonary TB (EPTB) which constitute roughly 15 to 20 per cent of all cases of TB. In HIV-positive patients, EPTB account for up to 53 to 62 percent cases of TB. [2] Diagnosing EPTB remains challenging for the reason that clinical samples obtained from comparatively inaccessible sites may be paucibacillary, thus diminishing the sensitivity of diagnostic tests. Conventional diagnosis of EPTB rests on identification of acid-fast bacilli, histopathological examination of tissues, or culture. These techniques are time-consuming, need expertise, can be nonspecific, and are often delayed.

AIMS AND OBJECTIVE
The aim of the present study was to compare real-time PCR (Q-PCR) with conventional methods in the diagnosis of tubercular lymphadenopathy (TBL).

MATERIAL AND METHODS
Study design and settings:
This test validation study was conducted between December 2012 to December 2013 in the department of Biochemistry, in collaboration with department of Pathology, Microbiology and Department of Tuberculosis and chest diseases in a rural medical college, Mullana Ambala. Haryana. India.

The study was undertaken on a total of 174 patients with clinical suspicion of TB, of both sexes and various age groups attending the indoor and OPD of tuberculosis and chest disease department at M. M. Institute of Medical Sciences and Research, Mullana, Ambala, were enrolled

in the study. All patients were screened to be HIV negative. Informed consent was obtained from all patients to make use of their FNAC samples for this study. Clinical history and examination findings were documented.

The FNAC material obtained was used for:
i) Histopathological diagnosis.
ii) Demonstration of *M. tuberculosis* [Zeihl Neelson staining for acid fast bacilli]
iii) Culture [Lowenstein-Jensen media]
iv) Real-Time PCR.

Histopathological diagnosis.
Presence of tubercular granulomas with or without caseation necrosis on histological examination.

Zeihl Neelson staining
The specimen were examined after Zeihl Neelson staining, which was performed according to standard protocol. All the AFB-stained slides were confirmed microscopically by two independent pathologist, who were unaware of patient's clinical details.

Culture
All clinical samples were purified by digestion and decontamination by the N-acetyl-cysteine [NALC]-NaOH method.[3] The decontaminated samples were inoculated on L.J. media an incubated at 37°C for 8 weeks. The culture were inspected every 2 weeks for growth and presence of acid fast colonies.

Isolation of genomic DNA from tissue
Template DNA was extracted from an aliquot of sediment using ExiPrep™ 16 Automated Nucleic Acid Extraction System (Cat. No: A-5010); instrument and ExiPrep™ plus Tissue Genomic DNA Kit (K-4212) was performed according to the manufacturer's instructions. The tissue was cut and transferred into a 1.5 ml test tube.20 μl of proteinase K and 200 μl of tissue lysis buffer were added into the 1.5 ml test tube. The test tubes were incubated at 60^0C overnight with shaking. The test tubes were centrifuged at 13000 rpm for 5 minutes to remove any unlysed tissue. The supernatant was taken out and transferred into a new 1.5 ml test tube. 200 μl of sample was loaded into the sample loading wells.

Q-PCR
The assay used Exicycler™ 96 Real-Time Quantitative Thermal Block (Cat. No: A-2060 BIONEER Corporation, Republic of Korea.) instrument and reagent (AccuPower® MTB Q-PCR Kit). Q-PCR was performed according to the manufacturer's instructions. The MTC-specific primers were used to amplify specific fragments of the insertion sequence (IS) 6110 target. The MTC-specific TaqMan hydrolysis probe is an oligonucleotide labeled with an FAM (6-carboxyfluorescein) fluorophore at the 5′-end and a TAMRA (tetramethylrhodamine) quencher molecule at the 3′-end. PCR was performed on premix reconstitution mixture made by mixing 44 μl

PCR grade water and 1 μl internal positive control (IPC) per reaction.45 μl of the premix reconstitution mixture was added to all premix tubes. For the tube that was serving as negative control, 5 μl non template control was added. For the tube that was serving as positive control (PC) DNA, 5 μl MTB PC was added. 5 μl of nucleic acid extract (specimen) was added to the remaining tubes, making the total volume 50 μl in each tube. Optical film was cut to size and with the help of an applicator the tubes were completely sealed. Samples were vigorously vortexed for 20 seconds. Tubes were transferred into the Exicycler™ 96 Real-Time Quantitative Thermal Block. Cycling conditions were at 95^0C for 10 min, 95^0C for 20 s, and 55^0C for 30s with 45 cycles. Fluorescence measurements were performed in every cycle. The cycle threshold (Ct) is the cycle at which there is a significant increase in fluorescence above the background or a specified threshold. The Ct was determined from a curve generated from a plot of cycle number vs. fluorescence with a manual threshold set above the background fluorescence of the negative control. The test was repeated if the Ct was in the suspicious range. The total time for amplification, detection and analysis is approximately 90 min for 96 samples per run.

Ethical Considerations
The study was approved by the Ethics Committee of Maharishi Markandeshwar Institute of Medical Sciences and Research. The approval was on the agreement that good laboratory practice, quality control ensured, and that every finding would be treated with utmost confidentiality. All work was performed according to the International Guidelines for Human Experimentation in Biomedical Research.[4]

Data Management and Statistical Analysis
During data collection completed questionnaires were checked repeatedly to rectify any discrepancy, logical errors or missing information. The data entry was carried using Microsoft Office Excel worksheet and then exported to statistical software and analyzed using appropriate statistical tests by using Statistical Package for Social Services (SPSS vs 21 for Mac.IBM Inc, Chicago).

RESULTS
A total of 174 patients were studied. The patients' median age was 29 years and 53 (30.4%) were males. The majority of patients (87%) had unilateral lymph node involvement.

Table 1: Comparison of result of Q-PCR with Culture in Tubercular Lymphadenopathy.

	Culture +ive	Culture -ive	Total
Q-PCR +ive	90	2	92
Q-PCR -ive	0	82	82
Total	90	84	174

Pearson Chi-Square = 166.165; df = 1; **p < 0.0001.**

In this study, out of 90 culture positives 90 (100%) were also positive by Q-PCR, and out of 84 culture negative samples 2 (2.38%) were positive by Q-PCR (Table 1).

Among 90 culture-positive samples, 66 (73.3%) were positive by cytology, and among 84 culture negative samples 5 (5.95%) were positive by cytological examination. (Table 2).

Table 2: Comparison of result of Histology/Cytology with Culture in Tubercular Lymphadenopathy

	Culture +ive	Culture -ive	Total
Histology/ Cytology +ive	66	5	71
Histology/ Cytology -ive	24	79	103
Total	90	84	174

Pearson Chi-Square = 81.668; df = 1; **p < 0.0001.**

As shown in Table 3, among 90 culture-positive samples, 48 (53.3%) were positive by AFB staining and among 84 culture negative samples, 10 (11.90%) were found to be positive by AFB staining. Statistically highly significant correlation was also found between culture and Q- PCR (**p < 0.0001**), culture and cytology (p **< 0.0001**) and culture and AFB stain (p < 0.0001).

Table 3: Comparison of result of AFB with Culture in Tubercular Lymphadenopathy

|	Culture +ive	Culture -ive	Total
AFB +ive	48	10	58
AFB –ive	42	74	116
Total	90	84	174

Pearson Chi-Square = 33.557; df = 1; **p < 0.0001.**

Table 4: The diagnostic attributes of each test in all samples.

	Histology/ Cytology	AFB	Q-PCR	Culture
N	174	174	174	174
Positive	71	58	92	90
Negative	103	116	82	84
Sensitivity (%)	73.3%	53.3%	100%	100%
Specificity (%)	94%	88.1%	97.6%	100%
Positive predictive value (%)	93%	82.8%	97.8%	100%
Negative predictive value (%)	76.7%	63.8%	100%	100%
Accuracy (%)	**83.34%**	**70.11%**	**98.85%**	**100%**

Table 4 shows the diagnostic attributes of each test in TB lymphadenitis. Among the three methods tested and compared to culture, Q-PCR had the highest sensitivity

(100%) while AFB stain had the lowest (53.3%) sensitivity. The specificity of Q-PCR was the highest (97.6%) followed by cytology (94%) and AFB stain was the least specific (88.1%). Positive predictive value (PPV) of Q-PCR (97.8%) was the highest and that of AFB staining (82.2%) was the lowest. Negative predictive value (NPV) of Q-PCR was the highest (100%).
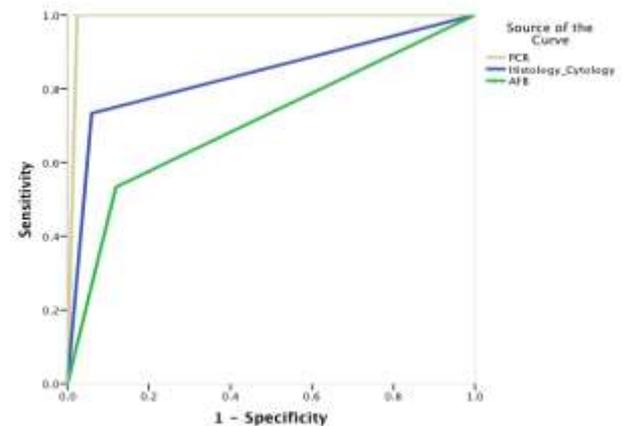
Figure 1: ROC showing performance of AFB, Q-PCR AND histopathology in the diagnosis of tubercular lymphadenopathy.

DISCUSSION

TB a primordial scourge of human civilization is still a leading public health challenge in a number of countries today. TB rose at a snail's pace, noiselessly, seeping into homes of millions, like an eternal miasma once arrived, it never went away again. Year after year, century after century, it tightened its unrelenting hold, worsening whenever war or famine reduced the population's resistance, infecting nearly everybody, curiously sparing some while destroying others, bringing the young down onto their sickbeds, where the flesh slowly fell from their bones and they were consumed in the years long fever, their minds brilliantly alert until, in apocalyptic numbers, they died, like the fallen leaves of a dreadful and premature autumn.[5] TBL patients present with enlarged, usually painless lymph nodes, mostly in the cervical region. Systemic symptoms, like fever, weight loss, malaise may or may not be present at an initial stage.

In the present study, median age of the patients was 29 years which is consistent with the studies conducted by Sreenidhi GM *et al*, [6] Karthikrajan M, [7] Wamala D *et al*. [8] In our study 53 (30.4%) were males while 69.5% were females, indicating disease being common in female, the present study consistent with the studies conducted by Sreenidhi GM *et al*, [6] Wamala D *et al*, [8] Gautam PB *et al*, [9] Chen *et al*.[10] The present study also demonstrated that majority of patients (87%) had unilateral lymph node involvement, same observations was also noted by studies conducted by Wamala D *et al*. [8], Gautam PB *et al*, [9]

In the present study the sensitivity and specificity of diagnosing TBL by ZN staining was 53.3% and 88.1% respectively. ZN staining though rapid, inexpensive, for acid-fast bacilli requires 10^4-10^6 bacilli/ml of tissue or fluid specimens to give a positive result[11], and it cannot be used to distinguish between various members of mycobacteria, and has a sensitivity ranges between 10% and 50%. [12, 13]

In our study the sensitivity and specificity of diagnosing TBL by histopathological examination was 73.3% and 94% respectively. Iwnetu et al. has shown the sensitivity, specificity of histopathology FNAC against culture to be 76%, 88% respectively.[14] Derese et al. in their study, the sensitivity, specificity, PPV, and NPV of FNAC was 81%, 50%, 54.2%, and 78.6% respectively.[15]

Use of culture as the gold standard revealed that Q-PCR had a sensitivity and specificity of 100% and 97.6% respectively similar results were observed in a study conducted by kesarwani RC et al who observed an overall sensitivity, specificity, positive predictive value and negative predictive value of PCR were 97.87%, 100%, 100%, and 94.73% respectively.[16] Linasmita P et al[17] observed a specificity of 96.9%. Gautam PB observed a sensitivity and specificity of 96.7% and 87.5% respectively on PCR targeting IS6110 gene sequence of M. tuberculosis.[9]

To reduced morbidity and mortality associated to EPTB, quick diagnosis and treatment is essential. To achieve early and accurate diagnosis, quick laboratory techniques are vital. Although culture is the gold standard, it still needs 10^1-10^2 bacilli/ml of sample for the diagnostic yield and requires two to four weeks for the growth of *M. tuberculosis*. Diagnosis of tuberculosis from tissue samples is usually made by histopathological examination that depends on the presence of granulomatous inflammation and caseous necrosis. It needs high expertise and the final reporting takes more than one week.[11] A diagnostic method that is less time-consuming and at the same time has high sensitivity and specificity is therefore desirable.

Advantages of Q-PCR
The turnaround time we observed in our study was less than 24 hours for Q-PCR. The advantage of Q-PCR system compared to the conventional PCR system is not only saving the time of post PCR works, but most important it diminishes the chance of generating the cross-contamination risk. In Q-PCR system, the PCR amplification progress is observed by fluorescence signal in the machine without exposing the PCR product to the open environment. The step is vital for limiting the generation of aerosol containing PCR product and cross contaminate the starting material.

Limitation of the present study
The current study included 174 samples, which might not signify the evaluation of a diagnostic assay but considering the cost and the financial condition of the patients that we encountered in a rural medical college of a developing country, this sample size is substantial. This study could be further improved by increasing the sample size and comparing it with currently available routine diagnostic assays.

CONCLUSION
The Q-PCR is an innovative and sophisticated technique and yields high levels of sensitivity, specificity and accuracy. Hence the application of Q-PCR should be encouraged for early and prompt diagnosis of TBL, which can aid a clinician to initiate a timely anti-tubercular treatment and prevent irreversible sequelae associated with morbidity of the disease.

COMPETING INTERESTS
The author declares that there were no competing interests associated with this study.

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