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PCR as a Diagnostic Tool for Extra-Pulmonary Tuberculosis

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ABSTRACT

Introduction: Extra-Pulmonary Tuberculosis (EPTB) accounts for approximately 40% of the tuberculosis cases. Though it is not communicable, it is a significant cause of morbidity. This study was conducted to know the efficacy of the Polymerase Chain Reaction (PCR) as an additional tool, along with the conventional methods, in the diagnosis of EPTB.

Materials and Methods: Clinical samples were collected from suspected cases of EPTB. The Ziehl-Neelsen staining (ZNS), culture on the Lowenstein-Jensen medium (LJM) and PCR testing with the use of a commercial kit were performed on the homogenized samples.

Results: A total of 182 samples which were received for the molecular diagnosis of EPTB were also tested by ZNS and culture on LJM for the presence of *Mycobacterium tuberculosis*. Of these, 22 were positive by at least one of the tests which were used. PCR detected the maximum number of cases of EPTB, followed by culture. The results of PCR and the conventional tests were analyzed by using McNemar's test for the correlated proportions-the exact method of 'IBM SPSS Statistics 20'. The analysis showed a statistical significance.

Conclusions: Whenever they are feasible, using all the available tests in combination increases the laboratory detection rates of *M. tuberculosis* from clinical samples. PCR must be included in the diagnostic panel of EPTB.

Key Words: Tuberculosis, Extrapulmonary tuberculosis, PCR, Mycobacterium

INTRODUCTION

Tuberculosis is a major public health problem. Every year, more than 9 million new cases are identified and approximately two million deaths are recorded worldwide [1]. Effective drugs have been available for treating this dreaded disease since more than 50 years. This statement may only be partially true because of the escalating problem of drug resistance. Human Immunodeficiency Virus (HIV) has worsened the problem to a very serious level. The Stop TB Partnership aims at saving at least an additional 14 million lives between 2006 and 2015 [2].

Extrapulmonary Tuberculosis (EPTB) accounts for one fifth of all the cases of tuberculosis in immunocompetent patients. The EPTB rate is very high in HIV-positive individuals, accounting for more than 50 per cent of tuberculosis cases which are associated with it [3]. The term 'EPTB' includes lymphatic, pleural, meningeal, pericardial, skeletal, gastrointestinal, genitourinary and miliary TB. Lymphadenitis and pleural effusions are the most common presentations [3]. Radiography provides useful information in the diagnosis of EPTB. The availability of newer radiological tests like computerized tomographic scans and magnetic resonance imaging, as well as laparoscopy and endoscopy, help in a more accurate anatomical localization of EPTB.

Microbiological investigations provide direct evidence about EPTB. The conventional microbiological methods of diagnosis are less reliable, and the diagnosis is often delayed for up to many weeks in the conventional culture method [4,5]. Since the disease usually responds to the standard antituberculosis therapy, an early and an accurate diagnosis is crucial.

A molecular approach to the diagnosis appears to be a better solution. Although molecular methods, in general, have not lived up to the expectation in the diagnosis of tuberculosis, Polymerase Chain Reaction (PCR) is certainly a very useful tool in the diagnosis of EPTB and it can also be used to identify the drug resistant strains. PCR is reported to have high sensitivity, specificity and speed in the diagnosis of this condition [6].

Here, we are presenting our observations regarding the role of PCR in the diagnosis of EPTB at our centre, over a period of two years.

MATERIALS AND METHODS

The present study was conducted at SDM College of Medical Sciences and Hospital, Dharwad, India, between July 2009 and June 2011.Clinical samples like pus, urine, endometrial biopsies, lymph nodes, vertebral discs, pleural fluid, peritoneal fluid, pericardial fluid, cerebrospinal fluid, blood and other tissues from clinically suspected cases of EPTB were received at our laboratory in sterile containers. A total of 193 samples from suspected EPTB cases were received for PCR testing of *M. tuberculosis*, of which 11 were excluded from the comparison, as the quantity was insufficient for the conventional tests. This left 182 tested samples for the analysis. The study was mainly laboratory-based and the institutional ethical committee clearance was obtained to conduct the study.

The samples were transported immediately after their collection, to the laboratory.

The tissues were homogenized by using a tissue homogeniser

(Remi Electrotechnik Limited, Vasai, India). N-Acetyl-L-Cysteine (NALC) plus 2% NaOH method was used to homogenize the viscous samples. The homogenized samples were used for the Ziehl-Neelsen staining (ZNS), culture on the Lowenstein-Jensen Medium (LJM) and PCR. The fluids were centrifuged at 3000g for 20 minutes and the deposits were used for the processing. DNA extraction was done by using the QIAamp DNA mini kit (QIAGEN) by the spin column method as per the manufacturer's instructions.

PCR was performed by using In Vitro Diagnostics (IVD) approved commercial kits [Real-TM (Sacace) with the primers targeting IS6110, and Seeplex MTB ACE (Seegene) with the primers targeting IS6110 and MPB64] as per the manufacturers' instructions. All the samples which were received for the PCR testing were also subjected to ZNS and culture on the LJ media (four slopes per sample). The cultures were observed for a period of eight weeks before they were declared as negative for growth.

STATISTICAL ANALYSIS

The kappa statistics was applied to assess the reproducibility and the agreement levels between the different diagnostic tests which were used in the study. The data were also analyzed by McNemar's test for the Correlated Proportions – Exact test (Two-tailed) with the use of 'IBM SPSS Statistics 20'.

RESULTS

The overall positivity for EPTB was 12.1 % (22/182) in the tested samples, as shown in [Table/Fig-1].PCR was positive in 20 cases, culture in nine and ZNS in six cases. Among the tests, PCR alone was positive in 10 cases, whereas PCR could not detect two EPTB cases which were picked up by the conventional methods. All the tests which were used were positive simultaneously in only three cases. The PCR positivity was highest in the pus samples [Table/Fig-2].

Approximately 93% of the tests i.e., conventional (LJM + ZNS) and

Tests	Numbers	Percentage
PCR only	10	45.5
PCR & Culture	6	27.3
All positive	3	13.6
ZN only	2	9.1
PCR & ZN	1	4.5
Total	**22**	**100**

[Table/Fig-1]: Tests detecting *M. tuberculosis* in clinical samples

Sample	Total tested	PCR	Positivity rate
Tissue	46	6	13.0
Pus	31	8	25.8
CSF	30	2	6.7
Pleural fluid	19	1	5.3
Urine	19	1	5.3
Synovial fluid	13	1	7.7
Blood	12	0	0.0
Other fluids	7	0	0.0
Peritoneal fluid	5	1	20.0
Total	**182**	**20**	**11.0**

[Table/Fig-2]: Distribution of extrapulmonary samples and PCR positivity

PCR, showed concordant results. As culture is a less than perfect 'gold standard' for the diagnosis of tuberculosis, we applied Cohen's kappa coefficient (k) to measure the level of agreement between culture and PCR. In our study, there was an 'intermediate' level of agreement between culture and the PCR tests, with a kappa value of 0.59. The Z-value yielded a significance of $p<0.001$. ($Z=7.2$; $p=2.9E-13$).

The incremental numbers of the EPTB cases which were detected by PCR, the new rapid test which was used in the study, were significantly more ($p=0.001$) as compared to those which were detected by ZNS, a conventional rapid test. Similarly, the incremental numbers of EPTB cases which were detected as positive by PCR were also statistically significant ($p=0.001$) as compared to those which were detected by culture on the solid medium- LJM (a traditionally known sensitive test). The same was true when PCR was compared against the combination of both the conventional methods ($p=0.039$, significant).

DISCUSSION

In the present study, a total of 22 out of 182 (12.1%) clinically suspected cases of EPTB were confirmed by the different diagnostic methods. In our country too, ZNS is used primarily because of its affordability, in spite of its low sensitivity [7]. In the present study, 3.3% of the samples tested for EPTB were found to be positive by ZNS. Culture is the established gold standard for diagnosing tuberculosis, though it lacks sensitivity, specificity and speed, especially in the detection of EPTB [7]. In the present study, only 5% of the clinical samples from the suspected EPTB cases yielded growth on LJM.

PCR and other NAATs have very high specificities and variable sensitivities [8]. Whenever it was properly executed, PCR has proved to be a very sensitive test in EPTB [9-12]. In the present study, PCR could detect *M. tuberculosis* in 11% of the tested cases. The reported positivity rates of PCR in the tested non-respiratory samples ranged from 8.6-63% [11-15]. Among the laboratory confirmed cases in the present study, PCR detected the maximum number of cases i.e. 90.9%. The conventional methods together could detect 54.6% of the laboratory-proved cases. All the LJM-positive samples were also found to be positive by PCR.

In the present study, PCR proved to be a very effective rapid test, with a significant p value. The difference in the results was also statistically significant when PCR was compared with the culture. The comparison between the conventional tests-in-combination and PCR showed that the additional cases which were detected exclusively by PCR were statistically significant. There was an intermediate level of agreement between culture and the PCR tests ($k=0.59$). In our study, this disagreement (less than an excellent agreement) had occurred, probably because of the better performance of PCR than the conventional methods. As a single diagnostic test, PCR was the most successful method for the diagnosis of EPTB in our study.

PCR was negative in two samples that were found to be positive by the conventional tests. The internal controls had amplified properly in both the samples, thus ruling out the possibility of PCR inhibitors in the samples. The negative PCR results may be attributable to the nil or very low number of copies of IS6110 [16]. However, the kit which was used for the above samples had primers which targeted IS6110 as well as the additional *M.*

tuberculosis-specific MPB64 gene, to avoid false negativity. The possibility of variations in the DNA extraction cannot be ruled out as a reason for the false negativity. Various methods are available for DNA extraction. Their sensitivities vary and no single extraction method has been accepted as a 'gold standard', especially for the clinical specimens [17,18]. The theoretical sensitivity of PCR has never been achieved in the diagnosis of tuberculosis so far. This might have prompted the WHO and other health agencies to recommend culture as the gold standard, as of today.

PCR has often been criticized for amplifying dead bacilli. This is only a theoretical limitation. When the test yields a positive result among the samples which are collected from clinically suspected active tuberculous lesions, it is highly unlikely to have amplified sequences from the dead bacilli in the tissue. When the clinician is in need of evidence, this highly specific test will provide a more dependable evidence, especially to end the dilemma of "to begin or not to begin the anti-Koch's therapy".

PCR has become a routine procedure in many settings, because it can reliably detect *M. tuberculosis* in the specimens, one or many weeks earlier than culture [19]. The sensitivity and the specificity of the PCR are usually compared to those of culture. However, there is a need to refer to the sensitivities of the liquid culture systems only. The comparison of the growth on solid media and PCR is inappropriate. LJM is less sensitive for growing mycobacteria from the clinical specimens as compared to the liquid culture systems, and is not used in many laboratories in the developed world [20]. However, it is important to note that most of the laboratories in India still routinely use LJM and not the liquid culture systems for various reasons.

Among the PCR-only positive cases (i.e., 10/20), histopathology was done only for seven samples, the other three being body fluids. The selection of a representative portion of the specimen can be a problem for the histopathological examination. Moreover, granulomatous lesions on histopathology, though they are characteristic, are not specific for tuberculosis only [6]. Four of the seven samples in the present study were negative for histopathological evidences of tuberculosis.

Though we tested 193 samples from clinically suspected EPTB cases, we could include only 182 samples for comparison with the conventional methods. The remaining 11 samples could not be subjected to the conventional testing because of insufficient quantities of the samples. Two of these samples were found to be positive by the PCR test. PCR requires a minimum quantity of only 200 µl of the sample. This could be an added advantage of this test, especially when the sample is collected by fine needle aspiration.

In the present study, out of the 10 EPTB cases which were detected as positive by PCR and as negative by the conventional methods, two cases were outside referrals for the PCR test. Clinical follow ups were possible in the remaining eight cases . All were put on the antituberculosis treatment. All of them improved clinically after 6-12 months of the antituberculosis therapy, except one, where the follow up was lost. As the therapeutic response is a very important gauge of the true disease status, we found a 100% correlation between the molecular and the clinical diagnoses in all the seven cases that could be followed up.

The difficulties in the laboratory diagnosis of EPTB or the non-availability of a gold standard have made researchers to use various combinations of criteria or parameters like a combined reference standard in the evaluation of the diagnostic tests [9]. This is pertinent in this era of an evidence-based clinical practice, where any meaningful evidence which is in favour of the clinical diagnosis, to rule in or to rule out the disease, has significance. Therefore, it is ideal to utilize a combination of all the available tests for the diagnosis, which will enable the laboratory to provide maximum useful information to the clinicians.

CONCLUSIONS

The diagnosis of EPTB is, many a times, a clinico-microbiological dilemma. However, the proper utilization of PCR may give vital evidence in more number of cases as compared to the established conventional methods. Molecular techniques which are being simplified and improved continuously and rapidly, appear to be the future tests of choice for most of the infections, including tuberculosis. As of today, all the available parameters have to be utilized and the results need to be carefully correlated with the clinical findings, to diagnose this disease. Indeed, using all the tests may be an important step in slowing down this scourge of mankind.

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