PERIPHERAL BLOOD-BASED POLYMERASE CHAIN REACTION IN DIAGNOSIS OF PULMONARY TUBERCULOSIS

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Background. The rapid diagnosis of infectious diseases, particularly those that represent a public health problem, like tuberculosis, is a challenging problem. By using nucleic acid amplification techniques like PCR, one may be able to diagnose, the disease on the day of arrival of specimen in the laboratory. For diagnosis of tuberculosis by direct methods like PCR, specimens from site of infection are required. In certain cases it is difficult to get the specimens from site of infection and in such situations; some researchers have tried to detect the DNA of Mycobacterium tuberculosis complex from blood of these patients. The purposive of this study is to determine the diagnostic efficacy of peripheral blood-based polymerase chain reaction for diagnosis of pulmonary tuberculosis. **Methods**. This was a simple descriptive study, carried out in Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi from Jan 2004 to Dec 2004. Sputum and blood samples were collected from 96 suspected patients of pulmonary tuberculosis. Sputum samples processed for ZN staining and AFB culture (gold standard) and blood samples processed for PCR. **Results**. Out of 96 cases, 60 (62.5%) were culture positive. PCR was positive in 14 (14.5%). AFB smear positive were 34 (35.4%). The overall sensitivity and specificity of the PCR assay was 20% and 94.4% respectively and the positive and negative predictive values were 85.71% and 41.46% respectively. The overall efficiency of the test was 47.91%. **Conclusion**. Due to low sensitivity; a negative PCR assay does not rule the disease. However, this test may be helpful in cases where specimens from the site of infection are not available.

Key Words: PCR, peripheral blood, pulmonary tuberculosis

INTRODUCTION

Tuberculosis (TB) is one of the leading infectious disease[1] and is responsible for 2.9 million deaths[2] and 8 million new cases per year in the world.[1] Tuberculosis is one of the common infectious diseases of the developing world, resulting in high morbidity and mortality in these countries.[3] It is estimated that 95% cases occur in under developed world where diagnostic and treatment facilities are rudimentary or non-existent.[4] In Pakistan it is estimated that 2, 68,000 new cases and 64,000 deaths occur due to tuberculosis each year.[5]

Tuberculosis is caused by several species of mycobacteria often described as Mycobacterium tuberculosis complex which include; M. tuberculosis, M. microti, M. africanum, M. bovis and BCG. Out of them Mycobacterium tuberculosis is the most frequent cause of the disease in human. Other members of this complex are rare causes of TB.[6] Initial diagnosis of tuberculosis is usually based on clinical grounds, but definitive diagnosis would require the isolation and identification of the infecting organism. The usual laboratory procedures are Z.N staining and microscopic examination for acid fast bacillus (AFB), isolation of organism by culture and drug susceptibility testing. Because of slow growth rate of most mycobacteria, isolation, identification and drug susceptibility testing can take several weeks.[1]

During the past few years, nucleic acid amplification (NAA) based techniques have been utilized for direct detection of AFB in clinical specimens, species identification, detection of resistance to antimycobacterial agents and molecular epidemiology. By using these techniques one may be able to diagnose, the disease on the day of arrival of specimen in the laboratory.[7] Amplification of specific nucleic acid sequences in specimens is achievable by PCR and related techniques, some of which have become commercially available.[8]

For diagnosis of tuberculosis by direct methods including PCR, specimens from the site of infection are required. In certain cases it is difficult to get the specimens from site of infection and in such situations; some researchers have tried to detect the DNA of Mycobacterium tuberculosis complex from blood of these patients. Studies with blood based PCR assays have suggested that this technique may be helpful in the diagnosis of cryptic TB[9]. We carried out this study to evaluate the role of blood-based PCR for diagnosis of TB. Isolation Mycobacterium tuberculosis by culture from sputa of patients suffering from pulmonary tuberculosis was taken as a gold standard. Simultaneously the blood of these patients was used to detect Mycobacterium tuberculosis complex DNA by PCR.

MATERIAL AND METHODS

This was a simple descriptive study, carried out in the Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi for a period of one year from January 2004 to December 2004. During this time 96 suspected cases of pulmonary tuberculosis referred from various Military and civil hospitals of Rawalpindi/Islamabad, Azad Kashmir and Northern Areas were included in the study.

The patients included were those with; chronic cough more than one month's duration, positive Mantoux test with 5T.U. (area of in duration > 10 mm) and presence of one of the radiographic chest findings consistent with pulmonary tuberculosis i.e. hilar lymphadenopathy, pulmonary infiltrates or cavity formation in the upper lobes or in the apical segments of lower lobes. No discrimination was made on account of age, sex or demography.

The patients and samples excluded from the study were; patients taking anti-tuberculosis treatment, haemolysed or clotted blood, sputum specimens showing less than 10 polymorphs per epithelial cell on Gram stained smear were rejected as saliva and sputum specimens yielding the growth of non-tuberculous mycobacteria (NTM) on culture.

It was a descriptive non interventional study. Sputum and blood samples were collected. Sputum samples were processed for ZN staining and AFB culture. Blood samples were processed for PCR.

For culture morning sputum samples of three consecutive days were collected in sterile, wide mouthed plastic bottles. After a standard N-acetyl-l-cystein sodium hydroxide digestion, decontamination[10], sputum was used for microscopy and culture. Radiometric Bactec TB 460 automated system was used for culture[11]. Five ml of treated sputum was inoculated in Bactec 12 B vial and incubated aerobically at 37^0C for up to 8 weeks. Growth of mycobacteria was monitored twice weekly in first two weeks then weekly thereafter. Growth index of 100 or more was taken as positive[12]. Growth identified to be an AFB by utilizing ZN staining was confirmed as Mycobacterium tuberculosis complex by Radiometric p-nitro-α acetylamino-β-hydroxy propiophenone (NAP) test[13].

For PCR 3 ml of blood was collected aseptically in a heparinized container and stored at 4^0C till further processing. DNA purification from whole blood was done by utilizing PUREGENE[TM]. Gentra systems Minnesota USA DNA Purification Kit.

Amplification of PCR products was carried out by using commercial kit manufactured by AcuGen systems, USA. The kit utilizes primers targeting the insertion sequence IS6110, that is present in Mycobaterium tuberculosis complex. Amplification was done by using a Mastercycler gradient, eppendorf, Germany. The amplification parameters included an initial denaturation at 94^0C for 5 minutes followed by 35 cycles each of denaturation at 54^0C for 1 minute, annealing at 68^0C for 1.5 minute, and extension at 72^0C for 2 minutes. The extension step in the 35th cycle was held for 10 minutes before the samples were shifted to 4^0C for storage. Positive and negative controls were run with each batch.

Amplified products detected by electrophoresis on 1% agarose gel followed by staining with ethedium bromide 0.4 mg/L. Sensitivity, specificity, positive and negative predictive values and efficacy were calculated on results obtained.

RESULTS

Sputum and blood samples were collected from 104 clinically suspected patients of pulmonary tuberculosis. Sputum samples were processed for ZN staining and AFB culture (Gold standard) and blood samples were processed for PCR. Eight were excluded from the study; due to contamination (n=6) and growth of NTM (n=2). Out of remaining 96 cases, 56 (58%) were males and 40 (42%) were females. Mean age of the patients was 37 years (range 5 -85 years) and median age was 31 years. Male to female ratio was 2.3:1.

AFB smear positive were 34 (35.4%), 60 (62.5%) were culture positive and PCR was positive in 14(14.5%) (Fig-1).
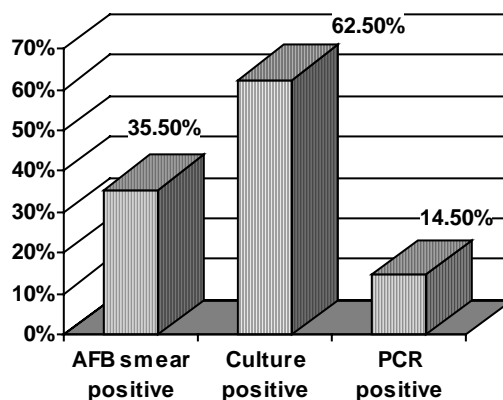

Fig 1: Percentages of AFB smear positive, culture positive and PCR positive

Among the smear positive cases (n=34), PCR assay correctly identify 6 cases which were culture positive. However this assay failed to pick 26 culture positive cases giving a sensitivity of 18.75%. There was no false positive case in these smear positive specimens, giving a specificity of 100% for the test (Table-1).

Similarly in smear negative cases (n=62) this assay correctly identified the 6 out of 28 culture

positive cases. It failed to pick 22 culture positive cases, giving the sensitivity and specificity of 21.42% and 94.11% respectively (Table-2).

The overall sensitivity and specificity of the PCR assay were 20% and 94.4% respectively and the positive and negative predictive values were 85.71% and 41.46% respectively. The overall efficiency of the test was 47.91% (Tables-3 & 4).

Table-1: Results of blood TB PCR assay and sputum culture for Mycobacterium tuberculosis on BACTEC 460 amongst AFB smear positive sputa (n=34)

	Culture Positive	Culture Negative	Total
PCR +ve	6	0	6
PCR –ve	26	2	28
Total	32	2	34

Table-2: Results of blood TB PCR assay and sputum culture for Mycobacterium tuberculosis on BACTEC 460 amongst AFB smear negative sputa (n=62)

	Culture Positive	Culture Negative	Total
PCR +ve	6	2	8
PCR –ve	22	32	54
	28	34	62

Table-3: Results of sputum culture for Mycobacterium tuberculosis on BACTEC 460 and blood TB PCR amongst all specimens (n=96)

	Culture Positive	Culture Negative	Total
TB PCR +ve	12	2	14
TB PCR –ve	48	34	82
Total	60	36	96

Table-4: Performance of PCR in blood as compared to sputum culture for Mycobacterium tuberculosis on BACTEC 460 (n=96)

Sensitivity	Specificity	PPV	NPV	Efficiency
20%	94.44%	85.71	41.46	48%

PPV= Positive predictive value, NPV= Negative predictive value

DISCUSSION

Tuberculosis is a persistent problem in the developing world and the major cause of mortality due to rudimentary diagnostic and treatment facilities. A rapid and accurate diagnosis of tuberculosis is a cornerstone of tuberculosis control strategies.[9,14]

Microscopy for acid fast bacilli (AFB) is cheap and simple and detects most of TB cases.[6] It is less sensitive, because a large number of bacilli must be present in a specimen for the smear to be positive. It fails to differentiate between dead and living mycobacteria.[15]

Serological tests are used as an additional diagnostic tool among the investigations done for tuberculosis. The main problem in serology is antigen cross reactions, which result from epitopes that Mycobacterium tuberculosis shares with many environmental mycobacteria.[16]

Isolation of Mycobacterium tuberculosis by culture techniques is taken as gold standard for diagnosis. This bacterium grows very slow in culture and may take several weeks for visible growth on conventional solid media.[14] Radiometric liquid (BACTEC) and biphasic (MB Chek) culture systems have reduced the detection time significantly, but these systems still cannot influence initial bedside decision making.[15]

Traditional diagnostic approaches like sputum smear examination, sputum culture and chest radiography have been virtually unchanged for many years. However, there is an urgent need for rapid and accurate diagnosis of tuberculosis. HIV infection, institutional outbreaks and multidrug resistant disease underscore the urgency of early identification and treatment.[17]

A rapid and sensitive test for diagnosis of tuberculosis is still required.[18] Direct detection of mycobacterium by nucleic acid amplification techniques represents the most dramatic improvement in its diagnosis. By using these techniques, the disease can be diagnosed on the day of the arrival of the specimen in the laboratory.[7]

PCR based tests have shown guarantee for the detection of mycobacterium in clinical samples. The test is rapid and can detect fewer than 10 organisms in clinical specimens. Although the specificity of this assay can be high, the sensitivity is less than that of culture.[14] In certain cases, like, disseminated and exrapulmonary tuberculosis, the specimens from the original site are not available, peripheral blood seems to be a specimen of choice. Earlier studies with blood-based PCR suggested that this test may be useful in immunocompromised patients.[17]

More data is required to determine the effectiveness of this test especially in immunocompetent patients.

The sensitivity and specificity of PCR in our study is 20% and 94.44% respectively. 0ur results in this record are contrary to those of Ahmed N et al,[9] who reported, a much higher rate of positive PCR with blood samples. However, the patients included in their study were already diagnosed cases of pulmonary tuberculosis. The specificity of our assay was compareable to those of Pfyffer et al.[7]

There were two patients who were PCR positive without culture confirmation. The reasons for these false-positive cases were not exactly known. An important observation in these patients was that both of them were of 5 years old and were BCG vaccinated one of the causes of false positive PCR. Secondly they may be infected by Mycobacterium bovis as PCR

assays based on IS6110 cannot distinguish between Mycobacterium tuberculosis and Mycobacterium bovis. The possibility of carry-over contamination is minimum because we retested the positive samples and routinely included positive and negative controls. Separate rooms were dedicated for the process of DNA extraction, amplification and electrophoresis to rule out the problems of contamination.

By considering both smear positive and smear negative cases together, PCR assay correctly identified 12 out of 60 culture positive cases. It also identified 2 culture-negative cases as positive. Forty eight patients with negative PCR assays had positive sputum cultures for Mycobaterium tuberculosis, we counted them as false negatives. The high false negative results may be due to low levels of infection or early stages of disease. It is also possible some of these patients may be infected with strains of Mycobaterium tuberculosis that did not carry the insertion sequence IS6110. Such strains are uncommon, but do exist.[18] Moreover M. tuberculosis resides in macrophages in the blood. We carried out the PCR using whole blood. Concentrating the white blood cells by using the buffy coat as sample instead of whole blood increase the yield of such a PCR.

The positive and negative predictive values vary with the prevalence of the disease in a given community. With the very low prevalence of tuberculosis in a population, the test would provide a good negative predictive value for exclusion of the disease, while a positive result would be less helpful. In contrast, if used in a high prevalence setting, the high positive predictive values of the test would make a positive test result useful in strengthening the clinical suspicion, but a negative result would be less useful. This was true for our study as well; a positive predictive value of 85.71% makes this test very useful in our high prevalence setting.

A significant number (34/96) of suspected cases turned out to be negative both by culture and PCR assay despite the strict criteria of clinical suspicion that we had laid down. Two reasons could have been responsible for this. Firstly a large percentage of population is tuberculin positive due to latent TB infection. The other possibility might be that the apparent radiological findings were of past, healed tuberculosis and the individuals were not actually suffering from TB. Lastly the possibility of antimicrobials could not be considered, because such patients were already excluded from study.

CONCLUSIONS

Due to low sensitivity; a negative PCR assay from the blood does not rule out pulmonary TB. However, this test may be helpful in cases where specimens from the site of infection are not available.

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