

## Protein antigen b (Pab) based PCR test in diagnosis of pulmonary & extra-pulmonary tuberculosis

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**Background & objectives:** Diagnosis of tuberculosis (TB) is largely based on microscopy and culture examination which are either less sensitive, or time consuming. In the present study a PCR (polymerase chain reaction) test based on DNA sequence coding for a 38-kilodalton protein antigen b (Pab), specific for *Mycobacterium tuberculosis* was compared with Ziehl-Neelsen (ZN) stained AFB (acid fast bacilli) smear examination, culture based on conventional Lowenstein-Jensen (LJ) medium and radiometric BACTEC 460 system for the diagnosis of TB using clinical samples obtained from pulmonary and extra-pulmonary cases of TB.

**Methods:** Clinical samples obtained from 168 patients of suspected TB (pulmonary and extra-pulmonary) were subjected to ZN smear examination, LJ culture, radiometric BACTEC culture and a PCR test by amplifying 419bp sequence coding for Pab, a glycoprotein of molecular weight 38 kDa.

**Results:** A significant difference was seen in the sensitivity of different tests, the figures being 74.2 per cent for PCR test, 53.4 per cent for BACTEC culture, 47.1 per cent for LJ medium based culture and 35.2 per cent for ZN smear examination ( $P<0.05$ ). However, there was no significant difference between different tests as far as specificity was concerned. PCR test sensitivity in pulmonary and extra-pulmonary clinical samples were 74.3 and 71.5 per cent respectively, being significantly higher ( $P<0.05$ ) when compared with sensitivity of other tests. The mean detection time for *M. tuberculosis* was 24.0 days by LJ media culture, 12.8 days by BACTEC culture and less than 1 day by smear examination and PCR test.

**Interpretation & conclusion:** PCR test is more sensitive than ZN smear examination, LJ medium culture and BACTEC culture for diagnosing TB in pulmonary and extra-pulmonary clinical samples.

**Key words** BACTEC - LJ culture - *Mycobacterium tuberculosis* - polymerase chain reaction - ZN staining

The laboratory diagnosis of tuberculosis (TB) is based primarily on microscopy or on the culture of the clinical samples. These procedures either lack sensitivity or are time consuming<sup>1</sup>. Even, the newer tests like the radiometric BACTEC system, serological tests, or the nucleic acid hybridization tests are either not cost-effective or have technically difficult protocols<sup>1-3</sup>. The development of a rapid, sensitive and specific test for the detection of mycobacteria in clinical specimens has been a long standing need.

Nucleic acid amplification techniques, notably the polymerase chain reaction (PCR) have revolutionized investigative microbiology by facilitating the direct detection and identification of infectious agents in clinical specimens in a very short time<sup>2</sup>. A large number of different regions/sequences of the mycobacterial genome have been targeted, *e.g.*, IS6110, IS986, 65kDa and 38kDa antigens coding region in the PCR test<sup>2-5</sup>. Most of the studies have targeted IS6110 sequence of *Mycobacterium tuberculosis* genome<sup>2,3</sup>. However, absence or the presence of a few copies of this sequence has already been reported in some isolates<sup>6-9</sup>. Das *et al*<sup>10</sup> also found that a large number of clinical isolates (40%) of *M. tuberculosis* from south India had either a single copy or no copy of IS6110. Another nucleotide sequence (419 bp) of protein antigen b (*Pab*) gene of molecular weight 38 kDa (total gene size of 1993 bp), specific for *M. tuberculosis* complex has been shown to be a useful target for detection in studies from abroad<sup>11-13</sup>. No study is available from India using this region of 38kDa (*Pab*) gene for the detection of *M. tuberculosis*. However, Dwivedi *et al*<sup>5</sup> used a 38 kDa based PCR test targeting a different region, *i.e.*, 239 bp sequence of 38 kDa gene for detection of *M. tuberculosis*.

We undertook this study to explore the utility of PCR test for the detection of *M. tuberculosis* in different clinical samples obtained from patients with pulmonary and extra-pulmonary TB by amplifying 419 bp sequence of *Pab* gene and comparing the

result with those of smear examination, conventional culture using Lowenstein-Jensen (LJ) medium and radiometric BACTEC culture.

### Material & Methods

A total of 168 clinical samples obtained from patients of pulmonary and extra-pulmonary TB with a strong clinical/radiological/histopathological evidence of TB referred from different hospitals of Delhi like [GTB (Guru Teg Bahadur), RML (Ram Manohar Lohia), AAA (Aruna Asaf Ali), VPCI (Vallabhbai Patel Chest Institute), AIIMS (All India Institute of Medical Sciences)] between January and December 2003 were included in the study. All the necessary clinical details were obtained from the hospitals' records in the format developed for the purpose.

*Inclusion criteria:* New suspected cases of tuberculosis referred to National Institute of Communicable Diseases (NICD), Delhi, for mycobacterial culture, accompanied with appropriate clinical/radiological/histopathological information on a predesigned performa indicative of tuberculosis which were further confirmed by their response to antitubercular therapy (ATT) on follow up were included in the study. In addition, 10 cases of pulmonary tuberculosis who were receiving ATT for less than 4 wk duration were also included in the study for comparison.

*Exclusion criteria:* Smear negative patients with pulmonary and extra-pulmonary TB not responding to ATT after four weeks of treatment were excluded from the study.

*Break-up of clinical samples:* The clinical samples (n=168) included 57 sputum samples from 57 suspected cases of pulmonary TB, 18 BAL (broncho alveolar lavage) from children with pulmonary TB, 22 skin biopsies from skin TB cases, 17 synovial fluid samples from 17 cases of osteoarticular TB, 6 urine (UTI), 30 pus, 1 pleural fluid, 4 lymph node aspirates, 1 bone marrow aspirate, 1 ascitic fluid,

3 tissue biopsies, 3 CSF sample, 1 endometrial biopsy, 3 menstrual blood, and 1 semen sample. Extra-pulmonary samples were subclassified into two groups, *i.e.*, one containing all the samples except biopsy and other containing only the biopsy material.

In addition, 37 sputum samples obtained from an equal number of individuals with non tuberculous problems (chronic asthmatics, chain smokers, *etc.*) initially screened by AFB smear examination and chest X-ray were also included in the study as negative controls.

*Gold standard:* Response to antitubercular treatment (feeling of well being/weight gain/ disappearance of fever) was taken as gold standard in the study.

*Processing of samples:* All the samples were processed in the Microbiology Department of National Institute of Communicable Diseases (NICD), Delhi. Direct and concentrated smears were prepared from clinical samples after treating with NALC (N-acetyl-L-cysteine) -NaOH (sodium hydroxide) method and other standard methods<sup>14,15</sup>. Briefly, the NALC-NaOH methods involved the decontamination and digestion of the clinical samples (like sputum, pus, BAL, *etc.*) with 2 per cent NaOH (final concentration) in 0.5 per cent NALC and concentrated by centrifugation at 3000 *g* for 15 min. Supernatant was discarded and to sediment, 1-2 ml of sterile phosphate buffer of *pH* 6.8 (1 to 2 ml) was added. Deposits were inoculated into LJ slant and radiometric middlebrook BACTEC 12B vial (Becton Dickinson, USA)<sup>14,15</sup>. Body fluids like CSF, synovial fluid, pleural fluid and ascitic fluid which were collected aseptically and were expected to have no other contaminating bacteria, were inoculated without decontamination procedure. Tissue samples collected aseptically were ground in a preautoclaved mortar and pestle and inoculated as such. Slides were stained by Ziehl-Neelsen method<sup>14</sup>. The mycobacterial isolates obtained were subjected to limited biochemical testing for species characterization by carrying out Niacin test

(Niacin strip by Sigma, USA) and p-nitro- $\alpha$ -acetyl amino- $\beta$ -hydroxypropriophenone (NAP) sensitivity test (Becton Dickinson, USA)<sup>14,15</sup>.

*Sample preparation for DNA extraction:* Different methods were employed for preparation of samples for extraction of DNA from different clinical samples: (i) sputum, pus, BAL, menstrual blood, semen, urine: Pellet obtained during NALC-NaOH method was used for DNA extraction; (ii) body fluids (CSF, pleural fluid, ascitic fluid, synovial fluid): Samples were used as such. 1000 $\mu$ l of specimen was used for DNA extraction; and (iii) skin, lymph node and other biopsy material: Were ground in a preautoclaved mortar and pestle. 1 ml of sample was used for DNA extraction.

*DNA extraction:* DNA was extracted using commercially available Qiagen DNA extraction kit (Qiagen Company, Germany) with one initial modification step of keeping the preliminary processed materials at 80°C for 10 min for inactivation of possible mycobacteria. The material was then processed as per the guidelines of the manufacturer of the kit to obtain the DNA.

*PCR test:* PCR was performed on extracted DNA samples obtained using primers (synthesized in NICD, Delhi) Pabf (5'-ACCACCGAGCGGTTTCGCCTGA-3') and Pabr (5'-GATCTGCGGGTCGTCACAGGT-3') to amplify a 419 bp sequence coding for the Pab 38 kDa antigen coding gene<sup>11,12</sup>. Briefly, a 25  $\mu$ l reaction mixture was set up containing 10.7  $\mu$ l of double distilled H<sub>2</sub>O, 2.5  $\mu$ l of 10X buffer, 1.5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 300  $\mu$ M (each) of the four deoxyribonucleoside triphosphate (available in Gene Amp PCR core reagent kit supplied by Applied Biosystem of Roche Company, USA), 2.5 U of Taq polymerase (Perkin Elmer), forward and reverse primer at final concentration of 10 pmol, and 5  $\mu$ l of DNA sample. Positive control DNA from H<sub>37</sub>Rv and negative control (distilled water) were used for amplification. Amplification cycle used for PCR included 3 min at 95°C, 34 cycles of 1 min each at 94°C, 1 min at 63°C,



**Fig.** PCR amplification of 419 bp region in 38 kDa gene of *M. tuberculosis* on 1.5 per cent agarose gel. Lane 1, 4-6: positive, lane 2: Positive control, lane 3: Negative control, lane M: 100bp DNA marker.

and 72°C for 1 min, one cycle of 72°C for 1 min. PCR products were electrophoresed on a 1.5 per cent agarose gel in 0.5X TBE buffer containing ethidium bromide at 10 µg/ml concentration; 3 µl of 100 bp ladder was also loaded. The samples were run at 80 V for 1 h. Samples showing the presence of 419 bp band under ultraviolet transillumination (Biometra, Germany) were considered positive.

**Statistical analysis:** The difference between sensitivity rates for various clinical samples by different tests were compared using the  $\chi^2$  method with Yates modification.

## Results

Of the 168 samples tested, 7 samples were contaminated in both LJ (4.1%) and BACTEC 12 B middlebrook media. In addition, two more samples were found contaminated in BACTEC culture leading the contamination rate of 5.3 per cent. Therefore, in this study, the results of 159 clinical samples of pulmonary and extra-pulmonary TB along with 37 sputum samples from negative control group were analysed.

The ZN smear examination detected AFB in 56 samples with a sensitivity of 35.2 per cent. For LJ culture, sensitivity was 47.1 per cent by isolating *M. tuberculosis* bacilli in 75 samples. For BACTEC culture this figure was 53.4 per cent by isolating bacilli in 85 samples. In comparison, PCR test showed a much higher sensitivity of 74.2 per cent by showing positive result in 118 clinical samples (Fig.). All the culture isolates obtained were confirmed as *M. tuberculosis*.

All the 37 negative control samples of sputum showed a negative result in all the tests, thus giving 100 per cent specificity for all the tests used.

**Pulmonary TB samples:** Of the 71 samples from pulmonary TB cases, 35 (24 sputum and 11 BAL) were positive for AFB smear examination (49.2%), while 55 (44 sputum and 11 BAL) samples were positive for mycobacterial DNA by PCR assay (77.5%). PCR test identified mycobacterial DNA in all 35 smear positive pulmonary samples. Of the 30 smear negative sputum samples, 20 were positive by PCR test (Table I).

**Table I.** Comparison of PCR test and smear examination sensitivity in suspected cases of TB

Clinical samples tested	Smear+ve no. (%)	PCR +ve no. (%)	Smear+ve PCR +ve no. (%)	Smear+ve PCR -ve no. (%)	Smear -ve PCR +ve no. (%)	Smear-ve PCR -ve no. (%)
<i>Pulmonary samples:</i>						
Sputum (54)	24 (44.4)	44 (81.4)	24 (44.4)	-	20 (37.03)	10 (18.5)
BAL (17)	11 (64.7)	11 (64.7)	11 (64.7)	-	-	6 (35.2)
Total 71	35 (49.2)	55 (74.3)	35 (49.2)	-	20 (28.1)	16 (22.5)
<i>Extra-pulmonary samples:</i>						
Biopsies (24)	5 (20.8)	19 (79.1)	5 (20.8)	-	14 (58.3)	5 (20.8)
Other than biopsies (64)	16 (25.0)	44 (68.7)	15 (23.4)	1 (1.5)	29 (45.3)	19 (29.6)
Total 88	21 (23.86)	63 (71.59)	20 (22.7)	1 (1.1)	43 (48.8)	24 (27.2)

**Table II.** Comparison of PCR and Lowenstein-Jensen (LJ) medium culture positivites in cases of pulmonary and extra-pulmonary TB

Clinical samples tested	LJ +ve no. (%)	PCR +ve no. (%)	LJ +ve PCR +ve no. (%)	LJ +ve PCR -ve no. (%)	LJ -ve PCR +ve no. (%)	LJ -ve PCR -ve no. (%)
<i>Pulmonary samples:</i>						
Sputum (54)	25 (46.2)	44 (81.4)	25 (46.2)	-	19 (35.1)	10 (18.5)
BAL (17)	12 (70.5)	11 (64.7)	11 (64.7)	1 (5.8)	-	5 (29.4)
Total 71	37 (52.11)	55 (74.34)	36 (50.7)	1 (1.4)	19 (26.7)	15 (21.1)
<i>Extrapulmonary samples:</i>						
Biopsies (24)	11 (45.8)	19 (79.1)	11 (45.8)	-	8 (33.3)	5 (20.8)
Other than biopsies (64)	27 (42.1)	44 (68.7)	26 (40.6)	1 (1.5)	18 (28.1)	19 (29.6)
Total 88	38 (43.18)	63 (71.59)	37 (42.0)	1 (1.1)	26 (29.5)	24 (27.2)

**Table III.** Comparison of positivities of PCR tests and BACTEC culture in cases of pulmary and extra-pulmonary TB

Clinical samples tested	BACTEC +ve no. (%)	PCR +ve no. (%)	BACTEC+ve PCR +ve no. (%)	BACTEC +ve PCR -ve no. (%)	BACTEC -ve PCR +ve no. (%)	BACTEC -ve PCR -ve no. (%)
<i>Pulmonary samples:</i>						
Sputum (54)	33 (61.1)	44 (81.4)	33 (61.1)	-	11 (20.3)	10 (18.5)
BAL (17)	9 (52.9)	11 (64.7)	9 (52.9)	-	2 (11.7)	6 (35.2)
Total 71	42 (56.75)	55 (74.34)	42 (59.1)	-	13 (18.3)	16 (22.5)
<i>Extra-pulmonary samples:</i>						
Biopsies (24)	14 (58.3)	19 (79.1)	14 (58.3)	-	5 (20.8)	5 (20.8)
Other than biopsies (64)	29 (45.3)	44 (68.7)	29 (45.3)	-	15 (23.5)	20 (31.2)
Total 88	43 (48.86)	63 (71.59)	43 (48.8)	-	20 (22.7)	25 (28.4)

Of the 71 pulmonary TB samples, 37 (52.1%, 25 sputum, 12 BAL) were positive for growth on LJ medium. PCR test was positive in 36 of these samples and found to be negative in 1 LJ positive BAL sample. PCR test was also positive in 19 LJ culture negative sputum samples (Table II).

In case of BACTEC system, 42 (33 sputum and 9 BAL) samples were positive for *M. tuberculosis* (56.7%). PCR test was positive in all these samples.

PCR test was also positive in 13 BACTEC culture negative samples (11 sputum, 2 BAL) (Table III).

On comparing the results, the PCR test was found to be much more sensitive than AFB smear examination, LJ culture or BACTEC culture ( $P < 0.05$ ).

*Extra-pulmonary samples:* Of the 64 extra-pulmonary samples other than tissue and other biopsy, only 16 (25%) were detected as AFB positive

by smear examination. LJ culture showed positive result in 27 samples (42.1%). BACTEC culture showed positive result in 29 samples (45.3%). Except for one sample of pus having positive result by smear examination and LJ culture but showing negative result by PCR, all the samples showing positive result by the conventional test used were found to be positive for the amplification of mycobacterial DNA.

Of the 24 biopsy samples (20 skin biopsy, 3 other biopsy, and 1 endometrial biopsy), AFB was demonstrated in 5 (3 skin biopsy, 2 other biopsy) samples by smear examination. LJ culture was found to be positive in 10 skin biopsy and 1 other biopsy sample. BACTEC culture showed positive result in 14 (11 skin biopsy, 2 other biopsy, one endometrial biopsy) samples. PCR test was found to be positive in all the samples showing positive result by one or the combination of the conventional test mentioned (Tables I-III).

On comparing statistically, PCR test was found to be more sensitive than the other three tests for diagnosis of TB in extra-pulmonary clinical samples ( $P < 0.05$ ).

The mean detection time for *M. tuberculosis* was 24.0 days by LJ medium culture, 12.8 days by BACTEC culture and less than 1 day by smear examination and PCR test.

PCR sensitivity was nearly similar in specimens of both pulmonary and extra-pulmonary TB, but the sensitivity of ZN smear examination, LJ culture and BACTEC culture in pulmonary samples were relatively higher than in extra-pulmonary samples ( $P < 0.05$ ).

### Discussion

Culturing of mycobacteria is the cornerstone for the diagnosis of mycobacterial infection. The aim of this study was to develop a rapid, specific and sensitive PCR by using primers based on the DNA sequence of *Pab* gene of molecular weight of 38kDa.

Sensitivity of the PCR using this primer was earlier shown to be 10 fg which is equivalent to two cells of *M. tuberculosis*<sup>11</sup>. Specificity, sensitivity and speed of PCR test in diagnosis of TB shown in this study should encourage the use of this method in routine diagnosis of TB. We compared the performance of various tests in different clinical samples for diagnosis of TB. PCR showed the highest sensitivity as compared to other tests as supported by other studies<sup>1-5</sup>.

We could not detect mycobacterial DNA in one smear and LJ culture positive pus sample and one LJ culture positive BAL sample which could be due to the presence of PCR inhibiting substances in the sample or unequal distribution of AFB in these samples.

In the present study, the high sensitivity of PCR test seen in smear negative samples has also been supported by earlier studies<sup>16</sup>. Some of the clinical specimens (10 sputum samples) that were positive by PCR test but were negative in culture, were obtained from cases who were being treated with ATT at the time of collecting the clinical sample, thereby suggesting that the DNA amplification method could detect even nonviable mycobacteria.

PCR results gave a higher sensitivity in our study than that reported by Sjobring *et al*<sup>12</sup> who showed 42.8 per cent sensitivity by amplifying the same region of *Pab* gene. As per the available information this is probably the first *Pab* gene (419bp) based PCR study in India which evaluated the usefulness of this sequence in diagnosis of both pulmonary and extra-pulmonary TB. PCR sensitivity of 74.3 per cent in pulmonary and 71.5 per cent in extra-pulmonary samples was also comparable with our earlier PCR studies targeting 165 bp region of 65 kDa antigen coding gene showing 74.4 per cent sensitivity in pulmonary and extra-pulmonary cases of TB<sup>17</sup>. Moreover, our PCR results were also comparable to that of Tiwari *et al*<sup>1</sup> showing 75.6 and 61.7 per cent sensitivity in pulmonary and extra-pulmonary samples respectively targeting IS6110 gene.

However, our PCR results showed a lesser sensitivity than that of Dwivedi *et al*<sup>5</sup> who showed 85.6 per cent sensitivity in cases of pulmonary TB using 38 kDa (239 bp gene) antigen coding gene based PCR. Kadival *et al*<sup>18</sup> showed a sensitivity of 45 per cent in sputum samples from patients with pulmonary TB. Our PCR sensitivity of 71.5 per cent in extra-pulmonary samples was comparable to that of Singh *et al*<sup>19</sup> who had amplified 513 bp sequence of *devR* gene showing 68 per cent sensitivity in lymph node biopsy samples. Our PCR sensitivity was higher than that of Ahmad *et al*<sup>20</sup> showing 43.7 per cent sensitivity by PCR targeting 306 bp region of IS1081 but lower than that of Narayanan *et al*<sup>21</sup> showing 91 per cent sensitivity of PCR by targeting 173 bp region of TRC<sub>4</sub> repetitive element.

Our study and those of others suggest that PCR could make a considerable impact in the diagnosis of TB, particularly extra-pulmonary TB which is often be missed by conventional tests producing negative result or causes an unacceptable delay in diagnosis<sup>11</sup>. This is specially true in tuberculous meningitis cases in which early diagnosis is essential for the outcome of the diseases<sup>22</sup>.

In conclusion, PCR has a potentially important role in strengthening the diagnosis of TB both pulmonary and extra-pulmonary. In addition, rapidity of the test allows quick implementation of treatment regimen.

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