

Evaluation of a PCR-amplified IS6110 insertion element in the rapid diagnosis of pulmonary tuberculosis in comparison to microscopic methods in Sudan

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ABSTRACT

Objective: The aim of this study was to evaluate the sensitivity and specificity of a polymerase chain reaction (PCR)-based method (IS6110 insertion site) in the diagnosis of pulmonary tuberculosis in sputum samples in comparison to smears by using culture on Loewenstein-Jensen medium as a standard.

Methods: The study was conducted during the period 1999 through to 2000, at Khartoum Teaching Hospital, Sudan, on 200 sputum samples. The samples were collected from patients suspected of having pulmonary tuberculosis, were examined using a PCR amplified IS6110 insertion element in comparison to Ziehl-Neelsen stained smears in terms of sensitivity and specificity. Culture on Loewenstein-Jensen medium was used as the

standard to control the 2 tests.

Results: Microscope sensitivity was found to be 65.4% and the specificity was 90.5%, whereas sensitivity of the IS6110 was 88.5% and specificity was 98.6%.

Conclusion: The study concluded that though IS6110 sensitivity was 13.1% higher than smear method, it provided a significant improvement in specificity for the diagnosis of pulmonary tuberculosis. Improvement is still needed to increase the sensitivity of the IS6110 methods by decreasing the number of the false negative samples before its use can be at routine levels.

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Tuberculosis (TB) is a major public health threat in many countries including the Sudan. The disease accounts for almost 3,000,000 deaths annually.¹ One million cases of TB occurred in Sub-Saharan Africa in the 1990s.^{2,3} The estimated incidence of TB in Sudan is high: 86 per 100,000 population for new smear positive cases of TB.⁴ Early diagnosis of the disease is a requirement for prompt treatment and for the breakdown of the disease cycle of transmission. In Sudanese laboratories the detection of acid fast bacilli (AFB)

in sputum or biopsy samples is the main method used for diagnosis of TB and tuberculin testing is the only method used, sometimes, for surveys and detection of new non-clinical cases.⁴ Several amplification techniques have been used to replace the time consuming biochemical and physiological tests for clinical routine diagnosis and epidemiological studies of TB.⁵⁻⁹ Microscopy, though, simple and cheap to perform but it provides both low sensitivity and specificity. Many laboratories around the globe are using primers most

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commonly targeting the IS6110.^{7,10-13} IS6110 is an insertion sequences specific for *Mycobacterium tuberculosis* (*M.tuberculosis*) complex.^{14,15} The methods provided from 61.5% sensitivity for smear negative samples up to 87.8% specificity on *M.tuberculosis* culture.¹²

The aim of the present study was to evaluate the sensitivity and specificity of a polymerase chain reaction (PCR)-based method (IS6110 insertion site) in the diagnosis of pulmonary TB in sputum samples in comparison to smears by using culture on Loewenstein-Jensen medium as a standard.

Methods. Sample preparation, smear and culture methods. 200 clinical specimens (sputum) were collected from the Chest Unit, Khartoum Teaching Hospital (El Shaab), Sudan. Specimens were processed according to standard methods.⁶ One aliquot of sputum was subjected to routine direct microscopic examination using Ziehl-Neelsen methods. A second sputum aliquot was digested and decontaminated by N-Acetyl-L-Cysteine-2% NaOH method (NALC-2% NaOH) and concentrated by centrifugation at 3000 g for 20 min. The sediment was used to inoculate 2 Loewenstein-Jensen slants. The second part was used for the PCR. Inoculated LJ media were incubated at 37°C and examined every 2 days during the first 8 days then weekly for up to 6 weeks for the presence of mycobacterial colonies. Cultures were subjected to identification scheme using selected morphological, biochemical and physiological tests.⁶

DNA isolation. The procedure applied followed protocols commonly practiced at the Institut fur Mikrobiologie und Immunologie, Krankenhaus Zehlendorf, Berlin.⁸ The basic steps of the method and modification in view of simplifying the procedure were as follow: One aliquot of the sediment obtained after NALC-2% NaOH decontamination was either stored for 3 days at 4°C until processed or kept frozen at -20°C for longer periods, if necessary. 50 µl of 10 mM Tris and 3 drops of acid-washed glass beads (100 µm, Sigma) was added to the PCR aliquot. The mixture was vortexed vigorously for 5-7 min, centrifuge for a few seconds and then heated for 20 min at 95°C in order to decrease or destroy inhibitors. 5 µl of this mixture was used for each 50 µl PCR reaction mix. DNA from cultures followed the same procedure after inactivating the cell at 80°C for one hour and washed twice with Tris buffer.

Polymerase chain reaction-based amplification. The target for the PCR assay was the insertion element IS6110. The IS6110 was amplified using the primers TB41 and TB42.^{14,15} The amplification was carried out with a 50 µl reaction mix containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 µl of each of the

deoxynucleoside triphosphate, 20 pmol of each primer, 1 U of Thermus aquaticus DNA polymerase (Pharmacia), and 5 µl of template DNA. The thermal profile involves 38 cycle as follows: Denaturation at 95°C for one min (95°C for 5 min for the first cycle), annealing and extension at 68°C for one min. The following controls were included in each run: positive control: one pg of M. TB DNA, negative control: reagent mixture without sample.

Detection of polymerase chain reaction products. Amplified products of 123 bp were confirmed by electrophoresis on 1.8% agarose gels stained with ethidium bromide, and examined under UV light. Presence of a single band equivalent to 123 bp is taken as positive results when the negative control gave no reaction. False negatives and doubtful results were repeated.

Statistical methods. The percentage sensitivity and specificity was calculated using the following formulas: 6

$$\% \text{ sensitivity} = \frac{\text{true positives}}{\text{true positives} + \text{false negatives}} \times 100$$

$$\% \text{ specificity} = \frac{\text{true negatives}}{\text{true negatives} + \text{false positives}} \times 100.$$

Results. Out of the 200 samples investigated, growth of *M.tuberculosis* in LJ media was obtained in 52. The analysis was thus carried out on the 52 grown strains and results are summarized in **Table 1**. Smear made from suspected samples showed an overall 34 acid-fast bacilli (AFB) of the 52 true positives. Of these 20 were true *M.tuberculosis* and the method revealed 14 false positive, and 18 false negative. The diagnostic sensitivity of the smear was found to be 65.4% and the specificity was 90.5%. When using primer TB40 and TB41 to amplify the specific *M.tuberculosis* IS6110 sequences in sputum, 46 samples were positive on gel electrophoresis (**Figure 1**). Six culture-positive samples were false negative by IS6110, and 2 were false positive. The diagnostic sensitivity using IS6110 was 88.5% and specificity 98.6%. Fifty of the 52 cultures of *M.tuberculosis* were positive on IS6110 sequences, therefore, given sensitivity of 96.2%, and specificity of 100%. Many organisms remained unidentified or having their confirmed identification not completed by the time of submission of this article.

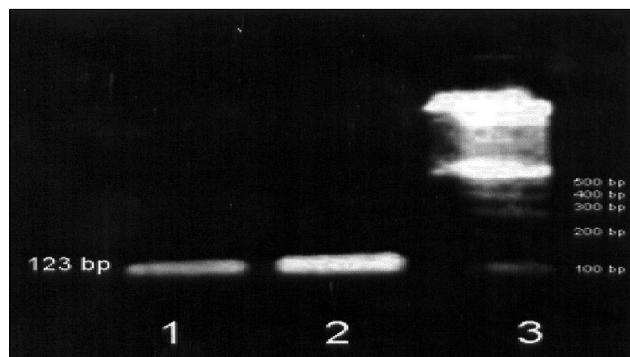
Discussion. Nucleic acid amplification methods to detect *M.tuberculosis* in clinical specimens are increasingly in use as a tool for TB diagnosis.⁷ Application of molecular methodology for routine diagnosis in a country like Sudan has to consider a number of elements including the cost, the ease to reach of the diagnostic kits and,

Table 1 - Comparison between smear and IS6110 sequences in the detection of *M.tuberculosis* in sputum of 200 specimens when growth was obtained in 52 samples.

Reading	Smear	IS6110 in sputum	IS6110 in culture
True positive	34 of 52	46 of 52	50 of 52
False positive	14	2	0
True negative	134	146	148
False negative	18	6	2
Sensitivity (%)	65.4	88.5	95.2
Specificity (%)	90.5	98.6	100

importantly, the availability of skilled personnel to introduce and to control the quality of such technology.

The present study aimed to assess the value of one of a widely used PCR method, the IS6110 insertion site, in the routine diagnosis of TB and to compare that with microscopy. The number of samples processed for smears, cultures and PCR were 200 and fair degree of agreement in the 3 methods was obtained with published similar studies.¹² Microscopic examination directly from sputum smear after ZN stain, which is the common method for sending rapid report back to clinician in many regional laboratories in Sudan where culture is not common, achieved sensitivity of 65.4% and specificity of 90.5. Microscopic sensitivity and specificity worldwide recorded figures ranging from 84%⁴ to up to 88%.⁹ Many factors may influence the accuracy of this method notably the degree of bacilli shedding in sputum (sensitivity and specificity) and the technical capabilities of the laboratory. Polymerase chain reaction-amplified IS6110 provides 100% specificity on grown *M. tuberculosis* but 2 false negative samples were recorded. The number of false negatives was as expected higher when amplification was performed on sputum (6 cases; resultant sensitivity=88.5%). Two false positive samples were recorded using IS6110 on sputum (resultant specificity=98.6%). The sensitivity of this method reviewed by Roth et al⁷ ranges from 70-90%. Common factors that attributed to the production of false positivity is the contamination that may resulted from the carry over products or cultures containing large amount of target DNA.⁵ There is also reasonable ground for the false negativity noticed in the present study, especially in sputum-IS6110 samples. This could be due to technical reasons such as sample preparation or the presence of PCR inhibitors in the clinical

**Figure 1** - Polymerase chain reaction amplified IS6110 sequences on ethidium bromide-stained agarose gel electrophoresis. *M.tuberculosis* clinical isolate strain SD99 (lane 1); reference *M.tuberculosis* strain H37v (lane 2); DNA marker 100 bp (lane 3).

samples.⁷ One of the inadequacies of this report is that 20 acid fast bacilli other than TB (AFBOTT) remained unidentified (to species level) till the submission of the present article. The study concluded that smear provided low sensitivity and improvement was achieved by using the PCR method, but the specificity of the smear method remained comparatively low. For diagnostic and treatment urgency perhaps laboratory such as those of Khartoum, Sudan, should adopt a knocking out policy. First, all sample should be screened for *M.tuberculosis* specific IS6110. AFB-positive IS6110-negative should be investigated for *M.avium* complex, and slow growing mycobacteria notably *M.xenopi* and *M.kansasi* and for the rapidly growing mycobacteria; namely *M.fortuitum* complex which have been frequently found to cause pulmonary infection.¹⁶ Smear-positive samples (Gram's stain) but AFB-negative are most likely to be considered for fungi or other Gram's +/- bacteria.

According to our observation, around 6-10% of the pulmonary infections in Khartoum Hospital, were due to AFBOTT including *M.species* (spp) *Nocardia* spp. and fungi which routinely treated with first then shifted to second line anti- TB drugs that is of course, without improvement. As of the high cost, the PCR method would be much useful in smear negative samples rather than systematic application. In the present study, a considerable number of smear negative samples have been positively detected using the IS6110-system.

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