



## SEROLOGY VERSUS SPUTUM MICROSCOPY AFTER THE INTENSIVE PHASE OF DOTS THERAPY:

### EVALUATION OF A MPT64 BASED ASSAY

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#### ABSTRACT

Fast, sensitive, specific yet inexpensive methods for detection of tuberculosis remain elusive. In spite of its limitations sputum smear microscopy remains the most widely used method for its detection. Various commercial antigen based serological tests are being promoted as an alternative to sputum microscopy. These tests offer an economically viable pragmatic alternative to smear microscopy. Few studies exist evaluating the performance of serological tests after the intensive phase of DOTS. We believe to be an effective alternative to sputum microscopy; serology must perform reasonably well in all the stages of therapy. We evaluated the sensitivity and specificity of a single serological test after two months of intensive phase drug therapy in previously seropositive and smear positive patients attending the DOTS clinic of a tertiary care hospital. Our results showed a sensitivity of 0.5 (0.17-0.82 95% CI) and a specificity of 0.25 (0.11-0.46 95% CI) taking sputum smear microscopy as the gold standard. We concluded that the present formats of serological tests are not useful in differentiating an active infection from a recently cured infection. Further research must be done on detecting antibodies which reflect the true infection load.

**Key words:** Tuberculosis, MPT64, DOTS, serology, AFB, sputum microscopy

#### INTRODUCTION

Tuberculosis continues to remain a major health problem, especially in the developing world. It is estimated that there were 9.4 million incident cases of tuberculosis worldwide, in 2009 alone with 5.8 million notified cases.<sup>1</sup> Eighty percent of these cases occurred in 22 high-burdens, mainly resource-poor settings. India, with 1.5 million notified cases in 2009 had the highest burden of reported T.B.<sup>1</sup> Early and effective diagnosis of T.B. remains a key element in its control. The Revised National Tuberculosis Control Program (RNTCP) uses the WHO suggested diagnostic algorithm that combines sputum smear microscopy and chest X-ray to detect, classify and treat tuberculosis.<sup>2</sup> Using this method, the estimated case detection rate in 2009 for India was 67% short of the target of 70% sensitivity set by the WHO. In mid-2010, the case detection rate for all forms of TB had reached 55–67%, with a best estimate of 61%.<sup>1</sup>

In addition to sputum smear microscopy, methods superior in sensitivity and specificity such as nuclear amplification techniques, interferon gamma based tests and liquid culture for *M. tuberculosis* exist.<sup>3</sup> The Xpert MTB/RIF (Cepheid), a relatively new technology recently endorsed by the World Health Organization (WHO), provides high sensitivity for detection of TB and drug resistance<sup>4</sup>. However, economic constraints make most of these methods impossible to implement in low- and middle income countries where majority of the patients live<sup>5</sup>.

In spite of the fact, that microscopy is very specific for *M. tuberculosis* in TB-endemic countries<sup>6</sup>. Smear microscopy and chest radiography, the primary tools used in resource limited countries for identifying TB, has often been noticed poorly, especially in HIV co-infected patients<sup>6-8</sup>.

To ensure appropriate care for patients and to improve control of the global TB epidemic, simple, accurate, inexpensive, and, ideally, point-of-care diagnostic tools for TB are urgently needed. Serology seems to offer an economically practical alternative to smear microscopy<sup>9</sup>. The advantages of serology-it requires little physical

infrastructure, technical expertise and provides quick results, make it particularly suitable for developing countries such as India with a vast population and lack of trained personnel. For serology, simple training could easily be imparted to health workers who could commence field visits to detect missed cases of T.B.

However, the WHO has recommended against using serology in India taking into the account the wide differences in sensitivity and specificity between different tests<sup>10</sup>. Analysis has showed that implementing serology can lead to a huge number of false positive tests further increasing the economic burden<sup>11</sup>.

Sputum smear microscopy is performed once before initiation of treatment for detection of the disease and once after intensive phase of DOTS therapy to assess the effectiveness of treatment<sup>2</sup>. We suspected that the antibodies generated against *M. tuberculosis* may persist beyond the intensive phase anti-tubercular therapy of two months making these tests unsuitable for detecting the effectiveness of drug therapy after two months. A literature search revealed the lack of studies regarding the efficacy of serology after the first phase of therapy.

We therefore asked the specific question, what is the sensitivity and specificity of these tests after two months of intensive phase drug therapy when the patient is made to undergo sputum smear microscopy again.

#### METHODS

The study was conducted at the DOTS clinic of Medical College, Kolkata (an urban tertiary care hospital). Approval had been granted by the Institutional ethics committee of the hospital.

Pulmonary T.B. suspects presenting between February and June, 2011 were sent to the DOTS clinic from various departments for examination and evaluation according to RNTCP protocols. Suspected TB cases were defined as those presenting with unexplained cough for at least two consecutive weeks or more.

All patients who had taken any drug known to be active against T.B bacilli in the past 1 month were excluded from the study. Written informed consent was taken and after enrolment, a brief questionnaire was administered and a physical examination was performed. Two sputum specimens were collected from each patient—a spot specimen at the initial visit and an early morning specimen brought to the clinic the following day. At the laboratory, the sputum specimens were digested and decontaminated with 4% sodium hydroxide and centrifuged. A smear of the concentrated specimen was stained with Ziel-Neelson stain and examined microscopically for the presence of AFB. The sputum smear results were reviewed by the attending medical officer. Patients with at least one positive sputum smear were diagnosed as having smear positive pulmonary tuberculosis.

At the time of handing over reports, 5 ml of blood was collected from all patients with positive smears. It was immediately allowed to clot to separate the serum. One ml of serum was drawn by the provided dropper and the SD TB rapid test (SD Biostandard Diagnostics) was performed according to the manufacturer's instructions. The SD TB rapid test test results were determined by visual confirmation of a band at designated places for IgM and IgG and recorded in a study register.

Results were not made available to clinical and medical officers who were responsible for patient diagnosis and management. At the end of each day, the strips were re-read to verify the results.

The sputum smear results were reviewed by the attending clinical or medical officer. Patients who had at least one AFB-positive sputum smear were diagnosed as having AFB smear positive pulmonary tuberculosis, and treatment was begun following the RNTCP guidelines.

The remaining patients were given a 14 day course of azithromycin (or an equivalent macrolide) and requested to attend clinic after two weeks. On return visit those whose symptoms had cleared were considered to have had an acute chest infection other than tuberculosis. Those with persisting had two additional sputum specimens taken for AFB smear examination again. Those with one or more sputum smears found to be AFB smear-positive on repeat testing were diagnosed as AFB smear positive pulmonary T.B. and treated accordingly.

Symptomatic patients who remained AFB smear negative at either the 2-week assessment had a chest radiograph taken and a report obtained from radiological findings consistent with T.B. were classified as AFB smear-negative pulmonary T.B. and treated following RNTCP guidelines.

The new patients declared as suffering from T.B were asked to donate blood and the SD TB rapid test was performed as mentioned previously.

The patients regularly attended the designated DOTS clinic and were treated strictly according to RNTCP guideline. Defaulters, transferred out patients were excluded from the study. After 2 months of intensive phase therapy patients are again made to go sputum smear microscopy according to RNTCP guidelines. Sputum was collected exactly as mentioned before. After sputum samples had been submitted the patients (who had a positive serological reaction before) were again made to donate blood for a repeat serological test. The SD TB rapid test was performed on the serum samples

exactly as mentioned before and the results were taken for statistical analysis.

For the analysis of results, the test characteristics of sensitivity, specificity and positive predictive value for the SD TB rapid test were calculated using standard definitions.

## RESULTS

A total of 214 T.B. suspects attended the DOTS clinic and were recruited into the study. Of these, patients 40 (18.7%) were diagnosed with tuberculosis, 174 (81.3%) were determined not to have tuberculosis.

Among these patients, 35 (87.5%) were diagnosed with smear-positive T.B. on initial assessment. Following a 14-day course of azithromycin, 165 of the remaining 174 patients responded with amelioration of symptoms.

Among the 9 patients who remained symptomatic 2 (22.2%) were subsequently found to be smear positive. Thus the total number of smear positive cases was 37(92.5%).

Chest radiographs were taken for the remaining 7 patients 4 (57.1 %) had normal chest radiographs. Three patients had a radiographic diagnosis of T.B. made and were classified as smear negative T.B.

The rate of HIV infection was higher among the T.B. patients (16 out of 40) compared with the control group (none out of 174) (40 % vs 0 %).

The subsequent tests were limited to patients with a definitive diagnosis of T.B (both smear positive and smear negative). Overall, 23 out of 40 (57.5 %) of the T.B. patients had a positive SD TB rapid test. The majority 18/23 (78.2 %) of the positive reactions were read as IgG positive, while only 5/23 (21.7%) were read as both IgG and IgM positive.

SD TB rapid test reactivity was related to AFB smear status and HIV serological status Test sensitivity was highest among HIV-negative, smear-positive patients (83.3%, 20/ 24) and lowest among HIV-positive, smear-negative patients (33.3%, 1 /3).

After the two months of intensive phase DOTS therapy tests were again repeated. In this phase 8 out of 35 (22.8%) remained sputum smear positive at the end of treatment. Out of these 4 out 8 (50 %) had a positive SD TB rapid test. All these patients had a positive IgG band only and no IgM band was seen. 27 of the 35 patients who were sputum smear positive initially turned sputum smear negative. 20 of 27(74%) these patients had a positive SD TB rapid test. All these patients had a positive IgG band only and no IgM band was seen even after long term incubation.

Among the smear negative patients none had either a positive smear or positive SD TB rapid test.

## DISCUSSION

At present there are a wide number of serological tests are available in the market most of have a very unreliable performance<sup>12</sup>. It is this consideration, and that the performance of most of these serological tests are yet to be independently verified that forms the basis of WHO's recommendation against the use of serological tests<sup>10</sup>. Still, it must be accepted that serological tests are a pragmatic alternative and further work must be undertaken on them.

The performance and reproducibility of 19 of these commercially available antigen based serological tests has been recently evaluated<sup>13</sup>. Among them, we looked for ones with the highest specificity, since the goal of repeat testing is to conclusively identify those patients who have the infectious process still going on and are in need of prolonging intensive phase. The SD Tb Rapid test with a specificity of

95.95%, was the best locally available. This test uses antigen MPT 64 to detect for the presence of antibodies. MPT64 has been a potential candidate for serological tests for a long time. It has been favorably reviewed with one study suggesting 100% sensitivity and specificity<sup>14-15</sup>.

Among the 214 patients who were recruited into the study, 37 were found to be smear positive. This gives the ratio of suspects examined to smear positive patients diagnosed to 5.7 which was quite below the national average of 8<sup>16</sup>. The ratio of smear positive to smear negative cases was 12.3.

Among the 40 tuberculosis cases identified 23 gave a positive SD Tb rapid test giving a poor sensitivity of 57.5 %. The WHO found a sensitivity of 20.6% while evaluating the same test. This was however lower than the manufacturer's claim of 98% according to the product leaflet. The higher sensitivity we found compared to the WHO was perhaps due to our use of fresh serum while the WHO used frozen serum. After two months of intensive phase therapy 77.5 % of the patients turned smear negative. This national average conversion rate was 90%<sup>16</sup>.

Among these patients who had converted 74.5 % were still seropositive for T.B. This outcome of the study shows that antibodies to the antigen used (MPT64) tend to persist even after the patient has been rendered non-infectious. All the patients who were seropositive at this stage showed only the presence of IgG antibodies. There were 5 cases who were both IgM and IgG positive at the beginning of the intensive phase therapy. They had turned IgM negative.

This conclusively proves that humoral antibodies to MPT64 tend to persist in the circulation even after the patient has been rendered non-infectious. Specificity of all diagnostic tests is very important, but it becomes critical in diseases that warrant treatment or with a social stigma. TB comes under both categories. Because of the low specificity of this antigen after intensive phase therapy a large number of false positives would be generated leading to unnecessary expenses and man days lost, besides the enormous social impact of wrongly classifying a patient to be still suffering from T.B.

India has a wide repertoire of atypical mycobacteria many of which have antigens that are immunologically similar to those present in *M. tuberculosis*. A positive antibody result could be a result of infection with any of these atypical mycobacteria or a result of *Mycobacterium tuberculosis* infection itself. The low sensitivity and specificity of serological tests has often been attributed to the presence of atypical mycobacteria in India. We accept that atypical mycobacteria may be responsible for the false positives generated by the SD Tb Rapid test in our study. However, this essentially proves that in spite of its attractive performance in laboratory based studies the MPT 64 antigen is inadequate to be used on the field.

The implication is that MPT 64 based tests are at this present moment grossly inadequate to be incorporated into the already established anti-TB infrastructure. We agree with the contention of Dowdy *et. al.* that instead of scaling up serology the resources might be used for more expansion of the RNTCP program<sup>11</sup>. In spite of its drawbacks sputum smear microscopy is still the most viable method for detection of T.B.in India. Serological testing not only performed poorly vis a vis sputum smear microscopy but also could not satisfactorily indicate whether the drug regime was working. Therefore serology, must perform comparably with

sputum microscopy in not only detecting the disease for the first time but also in the subsequent stages of therapy.

It is obvious that our study suffered from some deficiencies. We tested only one of the 19 kits evaluated by the WHO. So the present study indicates only the dynamics of humoral response against only one antigen. The high specificity of the kit tested suggests that the remaining serological tests will probably give similar results or at least will not compare favorably to sputum microscopy. Still, other tests utilizing different antigens must be evaluated to get a comprehensive picture.

Immunological detection with appropriate sensitivity and specificity will remain an attractive research option for developing immunodiagnosis of tuberculosis. Certainly the antigen targets have to be properly chosen and, this has not been explored so far. It also remains to be seen if multiple tests can be combined at the same time on a single strip to increase sensitivity and specificity.

At the same time the lesson is learnt that a deeper understanding of the humoral response to various tuberculosis antigens is necessary. Serology at present is a non-viable option. But it remains the most pragmatic in any resource deprived country. We hope that in addition to a continuing search for antibodies with greater sensitivity and specificity further research also concentrates on those that accurately reflect progress of infection in a patient.

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