Evaluation of the Diagnostic Accuracy of cough collection and analysis using an Aerosol Immunosensor device, for rapid screening and early detection of tuberculosis.

Madhulika A Mistry 1#, Nicol J Murray 2&, Elaine M McCash 2&*, Arun B Mistry 3&

1 Tuberculosis Research Centre, K J Mehta TB Hospital, Amargadh – 364210, India.

2 Rapid Biosensor Systems Ltd, Babraham Research Campus, Babraham, Cambridge, CB22 3AT, UK.

# Current address: Pandit Deendayal Upadhyay (PDU) Medical College, Rajkot - 360001, India.

*Corresponding author

Email addresses:

MAM: madhulika_mistry@yahoo.co.in

NJM: dr.nicol.murray@rapidbiosensor.com

EMM: elaine.mccash@rapidbiosensor.com

ABM: dr_abmistry@yahoo.co.in
Abstract.

**Background:** Tuberculosis is one of the major health problems facing mankind. It spreads via infected aerosol droplets. We describe a novel, aerosol immunosensor for Mycobacterium tuberculosis (MTB,) based on cough collection and analysis, via detection of the Acid Free Bacilli in cough samples.

**Methods:** The TB aerosol immunosensor (Cough Collector “Breathalyser”, Rapid Biosensor Systems Ltd,) which detects the 85B MTB antigen, was field tested at the Tuberculosis Research Center, attached to the Shri K. J. Mehta TB Hospital at Amargadh, Bhavnagar District of Gujarat State, India. Following “wetting” of the respiratory tract, patients coughed into the disposable collection tube. A portable Reader determined the presence/absence of the antigen. Demographic, clinical, X-ray and sputum smear data were recorded.

**Results:** 251 tests were carried out on 230 subjects. The procedure was well tolerated and completed in ~5 minutes. 92 (45%) of the 203 screening subgroup were TB positive; 62 tested Breathalyser positive, all of whom were early stage; the remaining 30 were well-established and/or relapsed. Only 47 of the TB positive patients were sputum positive, 29 of whom were Breathalyser positive; whilst of the 45 sputum negative-TB positive patients, 33 were Breathalyser positive.

46 of the 75 X-ray positive TB positive patients were Breathalyser positive; of the 17 X-ray negative-TB positive patients, 16 were Breathalyser positive.

8 respiratory tract infection patients tested positive using the Breathalyser and were found to be sputum microscopy tests/X-ray positive on follow up. 12 Breathalyser positive tests were negative with X-ray/sputum, but exhibited clinical symptoms. If these 12 tests are treated as false positives a sensitivity of 73.5% and specificity of 91.11% was estimated with Positive Predictive Values of 80.6% and Negative Predictive Values of 87.2%. If well-established patients are expected to give negative breathalyser results the sensitivity is found to be well in excess of 90%.
Breathalyser immunoassays performed on sputum smear samples of known bacterial loading, as determined from sputum microscopy, indicated a detection limit of ~50-75 AFB.

17 tests were for technical assessment. 21 patients undergoing Anti-Koch Treatment (AKT) were tested to investigate their infection status.

All results were unaffected by the presence of HIV and/or other respiratory conditions.

**Conclusions:** The Biosensor Breathalyser demonstrates clear potential for screening and diagnosis of early stage and infectious TB patients, even before sputum smear and/or X-ray results become positive. The Breathalyser test is not generally as useful in well-established/relapsed TB, patients who are not releasing free AFB’s into the atmosphere. They also show potential for evaluating the progress of an AKT regimen.

Key words: Mycobacterium tuberculosis, breathalyser test, clinical examination, AFB and X-ray
Introduction.

Tuberculosis is a world-wide pandemic and global emergency. Current methods of screening for the disease are slow, inaccurate and/or costly and do not detect the condition in its early stages, at which point the spread of the disease could be limited and the treatment regime would be less extreme. This study reports the first detailed investigation of the potential of a cough collection and analysis system that has been devised with the aim of fulfilling the need for a simple, rapid, safe, sensitive, specific, and economic test for the screening and diagnosis of infectious pulmonary TB.

Background.

Since Robert Koch discovered the tubercle bacillus over 100 years ago, tuberculosis has remained one of the major health problems facing mankind, particularly in developing countries. Today, TB infects about a third of the world population and in terms of infectious diseases, it is the second greatest contributor to adult mortality. It is estimated that currently there about 10 million new cases of tuberculosis every year world-wide, with 3 million deaths occurring annually.

Currently, more people die from tuberculosis than from any other infectious disease [1.]. Deaths from tuberculosis comprise 25% of all avoidable deaths in developing countries. Nearly 95% of tuberculosis cases and 98% of deaths due to tuberculosis are in the developing countries and 75% of TB cases are in economically productive age groups.

In India, out of a total population of over 1 billion, about 2 million people develop active TB each year, of which approximately half a million die [2.]. It imposes a heavy cost on the economy in terms of current and future output and losses, because of premature deaths and ill health [3.]. In addition to this, Multi-Drug
Resistant Tuberculosis (MDR-TB) is emerging as an increasingly important cause of morbidity and mortality since an MDR-TB patient continues to spread the disease in the society for a prolonged period of time [4.]

Today, there is a resurgence of TB in developed countries due to the migration of people from developing countries [5.] So the control and possible cure of TB remains a serious medical challenge, being further complicated by the emergence of MDR-TB as well as the rising incidence of HIV infection, which tends to mask the TB symptoms in TB-HIV co-infected patients. TB is very infectious in the early stages as the TB bacilli lodged in the upper respiratory tract area are easily transmitted to close contacts from an infected person, by cough in the form of exhaled sputum droplets. Thus it is of paramount importance that an early and a specific diagnosis of TB is made so that an effective and early treatment can be provided, not only improving the patients prognosis but also curbing the transmission of TB infection and disease to other people in the community. Hence the method of active case finding remains the cornerstone for effective control of TB.

Currently, micro-bacteriology laboratories play a vital role in the early and specific diagnosis of TB, using direct sputum smear microscopy to detect acid fast bacilli (AFB) in the sputum smear stained by the Zeihl Nelson staining method and culture of sputum specimens for AFB growth [6.] The Microscopic Examination of sputum smears to detect AFB is rapid and inexpensive, but it lacks sensitivity as there should be more than 10,000 bacilli per ml of sputum present for a positive result. The laboratory culture of sputum samples for AFB is very sensitive as it requires only 10 – 100 viable bacilli per ml of sputum sample to be positive. However it takes a long period of time, about 6 -8 weeks to be done and most clinical and therapeutic decisions have to be made much earlier and before these laboratory results are available. Furthermore, in many settings, facilities for carrying out culture testing are not available, due to cost and staffing constraints.
The Culture of AFB through Radiometric System (BACTEC) and biphasic culture method usually requires less than (2) weeks to confirm the diagnosis of TB. Serology and other new techniques are not widely used due to the high cost, low sensitivity, specificity, or both. Thus, there is a definite need for a rapid, economic and a highly sensitive & specific method for quicker identification of AFB, in order to effect an early diagnosis of TB.

Mycobacterium tuberculosis (AFB) the causative agent of TB is spread from person to person via infected aerosol droplets created by patients suffering from the respiratory form of TB [7.] These bacilli are expelled into the airways following necrosis and destruction of the lung tissue and when released as aerosol droplets, they remain airborne and available for inhalation and infection of a new host. In spite of being the major mode of transmission of AFB, there is little data available regarding exhalation of AFB. The retrospective study of TB contacts suggests that most transmission of AFB occurs within the household prior to the diagnosis and initiation of treatment for TB. A person produces about 3000 droplets of sputum fluid each time he/she coughs or sneezes. The TB infection is spread via these airborne droplets; and the risk of infection depends upon the concentration of droplet nuclei, amount of inhaled air and the body’s defense mechanisms. On average, one infectious patient infects about 10-15 people in a year. But it is not known how early in the infective stage, a patient poses a significant risk of infecting others [8.] There is little data regarding the aerosolation of the bacilli by individual patients. The epidemiological studies show that there is a wide variation in the infectiousness of patients; some patients infect a large number of contacts whereas others fail to pass on the infection to their contacts. [9-11.]

The Rapid Biosensor aerosol immunosensor “Breathalyser” test.

Here we describe a novel, aerosol immunosensor for M.TB, device based on cough test collection and analysis, developed by Rapid Biosensor Systems Ltd., Cambridge, U.K. [12.] This cough analyzing system detects AFB antigen in the cough exudate of human subjects. This will go a long way in fulfilling the need for
a simple, rapid, safe, sensitive, specific, and economic test for the diagnosis of TB. This test system consists of two components:

1. A disposable tube into which the patient coughs, is designed to allow collection of the sample onto the surface of a prism contained within the tube, which has been coated with anti-tuberculosis antibodies primed with fluorescently labeled peptides that are artificially modified subsequences of the T-cell epitope from *M. tuberculosis* Ag85B [13, 14.]

The principle of the cough collector is shown in detail in Fig. 1. The respiratory tract is briefly “wetted” using a 0.9% normal saline solution delivered via a modified hand-pumped nebuliser (Easy Air Nebuliser Pump, Cameron Price, Birmingham, UK,) which assists by aiding the cough response and harvesting of the free bacilli, lodged in the upper respiratory tract area. The subject then coughs into the collection system which is in the “extended position”, as shown in Fig. 2. The inner plunger is then inserted into the outer plunger such that a ring of fluid is collected from the walls of the collector. A ‘spoon’ is then used to sweep around the base and collect the sample onto a prism, which is coated with a displacement immunoassay system specific to MTB. The cough collector is shown in use in Fig. 3.

**Fig. 1. The principle of the Rapid Biosensor Cough collector.**
A battery powered, portable photonics Reader instrument which detects any changes in the evanescent wave fluorescent signal at the surface, is shown in Fig. 4. As soon as the sample has been gathered onto the prism, the cough collection tube is inserted into the Reader and signal recording is started. The signal measured at the prism surface decreases if native MTB Ag85B antigens are present as they bind more strongly to the antibodies than do the modified ones and so displace them. The entire test, including preparing the patient, can be conducted in ~5 – 7 minutes.
Materials and Methods.

The study and consent procedure were approved by the Ethical committee of the Shri K. J. Mehta TB Hospital and the testing was conducted at Aditya Vikram Birla Post-Graduate Institute for Medical Microbiology and Laboratory Technology. This institute is affiliated to Bhavnagar University and is housed in the Tuberculosis Research Center, attached to Shri K. J. Mehta TB Hospital at Amargadh, in Bhavnagar District of Gujarat State. All participants provided prior informed verbal consent, which was recorded in the medical records by the attending physician. Written consent was not obtained because of the illiteracy of the participants. Anonymity and confidentiality were assured.

The trial reported here followed a small pilot study in the Shri Mehta Hospital in May/June 2004, when the first tests were carried out using the Rapid Biosensor aerosol immunosensor (Breathalyser) on live human subjects the very first time.

The field trial reported here was conducted in November-December 2005 on human subjects, involving 251 tests on 230 patients, with the aims of evaluating the efficacy and efficiency of the cough test analysis, to determine the range and etiology of TB infected patients for which a positive breath test result could be
obtained. It should be noted that in addition to adherence to patient groupings in the STARD initiative, a number of different criteria outside those of the STARD initiative were applied in order to attempt to fully characterize the nature/stage and effects of other conditions on the detection of TB patients using the aerosol immunosensor.

The subjects selected for inclusion in the trial were adults and children aged 12 years and above. They were classified into the following groups:-

   (a) Pre-diagnosed TB patients whose sputum smear is positive for AFB.
   (b) Pre-diagnosed TB patients whose sputum smear is negative for AFB.
   (c) Pre-diagnosed TB/HIV co-infected patients.
   (d) New undiagnosed chest symptomatic patients with a predisposition towards TB.
   (e) New undiagnosed chest symptomatic patients not having a predisposition towards TB.
   (f) Patients not having symptoms of chest diseases but having symptoms relating to disease of other organs of the body.
   (g) Patients having unknown clinical history.

All of the patients selected, fell into one or more of the following categories as determined by clinical assessment of symptomology and history, by the attending physician:-

   (a) Early stage infectious disease.
   (b) Long term established TB.
   (c) Relapsed TB.
   (d) Treatment Defaulter TB.
   (e) Multi-Drug Resistant TB (MDR-TB).
Following brief “wetting” of the respiratory tract as described above, the patient is asked to cough into a disposable collection tube following the sequence “cough, breathe, cough, breathe, cough.” Once the sample has been gathered onto the prism the tube is inserted into the Reader and the change in signal within a given time period, is recorded.

The cough sample collection was standardized as far as possible and sputum sample collection is avoided in all tests. Typically, 1-2 µl of cough sample was collected into the collection tube, although the total amount and properties of sample (viscosity etc.,) varies significantly from patient to patient.

Clinical Examination including recording of clinical history, family history of close contacts, occupation, pulse, blood pressure, chest auscultation, height, bodyweight and BMI; Chest X-ray; and 3 sputum samples – spot, overnight, spot; were collected from all patients as per Revised National TB Control Programme (RNTP) guidelines. The sputum samples were examined for AFB by staining their smears with standard Zeihl-Nelson technique and interpreting their results as per RNTCP guidelines. [NB Sputum culture testing was not available.]

A subsequent study was carried out by McNerney et al. in Ethiopia and was reported in reference [15.]

**Results.**

In this trial of 251 Breathalyser tests were carried out on 230 patients; 203 tests are reported for the “screening group”, defined in this work. Of the other 38 patients, 17 were excluded from the screening group, because they were being tested for technical and sample collection issues; and 21 patients were excluded from the screening group because they were already undergoing AKT treatment and so they were being tested to investigate their infection status.
Typically it took 4-6 minutes to perform the test procedure, including explanation to and nebulisation of the patient. The cough sample collected ranges from cough droplets not easily seen by the naked eye to substantial quantity of sputum fluid seen as droplets on the prism.

The Reader for the Breathalyser test measures the change in fluorescent signal at the coated prism surface following deposition of the cough sample. For the trials reported here, the Reader recorded the change in signal over 30 seconds to 2 minutes time period, depending on how large the change in signal was. We established a threshold of -20 as indicating the level at which a positive result was obtained. The total change intensity is an indicator of the quantity of bacilli in the sample, although the relationship is complex.

The correlation of Rapid Biosensor Breathalyser results with sputum smear and X-ray are shown in Tables 1-3. In all the (203) patients of the screening group, a patient was diagnosed as TB positive or TB negative solely on the basis of sputum smear microscopic examination for AFB, as per the Revised National TB Control Programme (RNTCP) guidelines of WHO prevailing in 2005 & radiological evidences when required. In this screening group of (203) patients there were (111) Non –TB Patients and (92) TB Patients as shown in Table 1. Sputum culture for AFB was not carried out for confirmation of TB as the facility for this testing was not available at that time.

### Table 1. Classification of Patients based on RNTCP Guidelines of WHO – 2010:

<table>
<thead>
<tr>
<th>Test Results</th>
<th>Number of Patients</th>
<th>Total</th>
<th>Total Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN/XN/RBS N (TB Negative Tests)</td>
<td>111</td>
<td>111</td>
<td>111</td>
</tr>
<tr>
<td>TB Positive Tests</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Patients</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Previously Treated Patients</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Treated Patients</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>7</td>
<td>17</td>
</tr>
</tbody>
</table>
### Table 2. Comparison of Rapid Biosensor Breathalyser results with sputum status in TB Positive tests:

<table>
<thead>
<tr>
<th>Sputum Status</th>
<th>Total</th>
<th>Breathalyser Test Positive</th>
<th>Breathalyser Test Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum Positive</td>
<td>47</td>
<td>29</td>
<td>18</td>
</tr>
<tr>
<td>Sputum Negative</td>
<td>45</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>92</td>
<td>62</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 3. Comparison of Rapid Biosensor Breathalyser results with X-ray findings in TB Positive Cases:

<table>
<thead>
<tr>
<th>X-ray Findings</th>
<th>Total</th>
<th>Breathalyser Positive Cases</th>
<th>Breathalyser Negative Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray Positive Cases</td>
<td>75</td>
<td>46</td>
<td>29</td>
</tr>
<tr>
<td>X-ray Negative Cases</td>
<td>17</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>92</td>
<td>62</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 2 shows the correlation between sputum smear and the Breathalyser test result for TB positive patients; Table 3 shows the correlation between X-ray and Breathalyser results for TB positive patients; and Table 4 shows the correlation between Xray and sputum smear results for the TB Positive patients.

### Table 4. Comparison sputum smear results with X-ray findings in TB Positive Cases:

<table>
<thead>
<tr>
<th>X-ray Findings</th>
<th>Total</th>
<th>Sputum smear Positive</th>
<th>Sputum smear Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray Positive Cases</td>
<td>75</td>
<td>42</td>
<td>33</td>
</tr>
<tr>
<td>X-ray Negative Cases</td>
<td>17</td>
<td>5</td>
<td>12</td>
</tr>
</tbody>
</table>
Sputum smear tests are more accurate than X-ray findings for Pulmonary TB diagnosis, provided that high standard sputum tests are carried out. However, there are a large number of patients who have TB but are negative by sputum smear tests. In this trial, out of 92 TB positive patients, 47 of them were sputum smear test positive; 75 patients were found to be X-ray positive with a total of 42 patients being positive by both sputum smear and X-ray.

By comparison to this, The Rapid Biosensor test was positive in 62 of the 92 TB patients, and the remaining 30 patients were Rapid Biosensor test negative. Of these 30 Rapid Biosensor test negative patients, 29 were having long term well established TB, who are unlikely to be screened by the Rapid Biosensor test in normal circumstances.

In the trial, 8 patients were being treated for respiratory tract infections. However, in spite of 10 days of treatment with broad spectrum antibiotics, their general condition did not improve. On follow up, Breathalyser tests were done and were found to be positive in all 8 patients. Subsequently, they were diagnosed to have Pulmonary TB as confirmed by sputum microscopy tests, X-ray chest findings and clinical finding.

12 positive breathalyser tests gave negative results for both sputum smear and X-ray. Of these, 3 patients had previously had TB and one was at the end of the treatment process. Of the remaining 8 tests (which included 3 repeats which each confirmed the initial result,) all patients appeared to be starting to present with very early symptoms of the condition.
Rapid Biosensor tests on 21 patients who were on AKT gave a good indication at an early stage about the effectiveness of the AKT, when compared to the usefulness of sputum smear tests for monitoring this. Among them, all 21 were chest X-ray positive for TB, 9 patients were sputum smear positive for AFB and 12 patients were Breathalyser test positive.

In order to assess the absolute sensitivity that the Rapid Biosensor test might achieve, two approaches were adopted.

For the first, for one patient whose Breathalyser test was positive, the prism/cough sample was removed from the collection tube and direct sputum smear microscopy was done on it. The smear test was negative for AFB leading to the conclusion that there were less than 10,000 AFB per ml of sputum sample.

The second approach was to perform the immunoassay part of the Breathalyser tests on 3 sputum samples which had been tested by direct sputum smear microscopy. In these tests, 2-3 µl of each sample was placed on the coated prism, estimated to contain about 50-75 AFB, which could be the smallest limit of AFB detection. The results of these 3 sputum samples are given in Table 5.

**Table 5.** Rapid Biosensor Immunoassay results for Sputum smear samples.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Sputum Smear Report</th>
<th>Signal Change in Breathalyser Immunoassay Test</th>
<th>Interpretation of Breathalyser Immunoassay Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample-1</td>
<td>1+ for AFB</td>
<td>-43 in 4 minutes</td>
<td>Positive test</td>
</tr>
<tr>
<td>Sample-2</td>
<td>Negative for AFB</td>
<td>+6 in 40 seconds (the static)</td>
<td>Negative test</td>
</tr>
<tr>
<td>Sample-3</td>
<td>3+ for AFB</td>
<td>-57 in 2 minutes</td>
<td>Positive test</td>
</tr>
</tbody>
</table>

**Discussion.**
It is clear that the tests currently used for the detection of TB in the field, such as sputum smear/culture and X-ray, detect different patient groupings, and indicate different stages and progressions of the disease for a given individual. This is why only ~50% of sputum producing patients produce positive smear results. One of the main purposes of this study was to explore the categories/groupings of patients that can be detected with the novel aerosol immunosensor breathalyser; and to determine how the results relate to the standard tests currently used and the stage/progression of the disease at which it can be detected in a collected cough sample.

The results revealed that the Rapid Biosensor device detects early stage patients and those who are actively infectious. Only some patients with well-established and/or relapsed TB appear to be detected, if they are actively producing bacilli. Patients who have chronic TB extensively affecting the lungs tend to be very ill and are inappropriate for the Breathalyser screening procedure. However they were included in the tests reported here as it is important to establish the range of patients where TB is detectable using the Breathalyser test, since it may, in future, be appropriate as a diagnostic tool.

Table 6 shows the estimated sensitivities and specificities for the Breathalyser and sputum smear results. It should be noted that the sensitivities and specificities calculated in Table 6 have included the 12 tests that were found to be SN/XN/RBS P, as being TB negative, (i.e. Breathalyser False Positives,) even though there is strong clinical evidence to suggest that these patients were in fact TB positive. Furthermore, the results reported here indicate that the Breathalyser does not detect cases where the condition is well established, and these results have been included as False Negatives. This gives rise to an observed sensitivity for the Breathalyser test of 73.53% compared to 56% for sputum smear: and specificity of 91.11% for the Breathalyser test compared to 96% for sputum smear. The Breathalyser tests gave Positive Predictive Values (PPV) of 80.6% and Negative Predictive Values (NPV) of 87.2% compared to PPV of 89.4% and NPV of 78.8%.
for the sputum results. If only early stage cases are considered, then the sensitivity of the Breathalyser is found to be well over 90%.

Table 6. Sensitivity and Specificity of RBS, and Sputum Test Results:

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>FN</th>
<th>Sensitivity (95% CI)</th>
<th>TN</th>
<th>FP</th>
<th>Specificity (95% CI)</th>
<th>PPV (95% CI)</th>
<th>NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Results</strong></td>
<td><strong>RBS</strong></td>
<td>50</td>
<td>18</td>
<td>123</td>
<td>12</td>
<td>91.11% (84.7-95.1)</td>
<td>80.6% (68.2-89.2)</td>
<td>87.2% (80.3-92.1)</td>
</tr>
<tr>
<td><strong>Total Sputum</strong></td>
<td>Results</td>
<td>42</td>
<td>33</td>
<td>123</td>
<td>5</td>
<td>96.09% (90.7-98.6)</td>
<td>89.4% (76.1-96.0)</td>
<td>78.8% (71.4-84.8)</td>
</tr>
</tbody>
</table>

Key:
- TP – True Positive
- FP – False Positive
- TN – True Negative
- FN – False Negative
- PPV – Positive Predictive Value
- NPV – Negative Predictive Value
- CI – Confidence Interval
- RBS – Rapid Biosensor System’s Breathalyser Test

In the McNerney study [15], which was carried out after the work reported here, using the same version of the prototype device on 60 subjects in Ethiopia, a sensitivity of 74% and specificity of 79% were found; which is an excellent correlation with the sensitivity, but much better specificity was found in the study reported here.

There is no evidence to show that the Rapid Biosensor test was compromised by co-existing medical conditions e.g. respiratory tract infections, COPD, pneumonias, HIV infection, extra-pulmonary tuberculosis and cancer; the range of patients tested within the test group, included individuals with each of these conditions. In HIV patients where the infection tends to “mask” the symptoms of TB the Rapid Biosensor test for diagnosis of TB is reliable.
Clinically, as the disease progresses and the infection becomes deep rooted, it settles in the middle and lower parts of the lungs, the Breathalyzer tests become negative; we postulate that this is due to the “free” bacilli becoming locked up in the sputum so they are not coughed out in the lung exudate.

The sputum results also become negative with the progression of the disease, but they do this at a much more advanced stage. In such instances a positive sputum smear microscopy test can occur only if the patient is either a treatment defaulter or a relapsed case of TB. In MDR-TB, patients having sputum smear positive for AFB tend to be highly infectious until 2nd line AKT drugs begin to work and decrease the bacterial load of AFB.

A positive sputum smear test result of (1+) is seen for a low bacterial load of AFB at an early stage of TB infection. If the patient is left untreated at this stage, then the bacterial load in the sputum would become high and the sputum smear result would increase to (2+) or (3+). With commencement of standard RNTCP DOT therapy, this sputum smear result decreases to (1+) or negative just after taking 3 to 4 doses of DOT AKT.

Some patients, who have negative chest X-ray findings as well as negative sputum smear tests, may still have TB, as seen in 12 patients in the trial (although we have treated these as False Positives for the purposes of sensitivity and specificity estimates, they clearly demonstrated clinical indications of the development of the disease.) These patients had positive Breathalyzer tests as indicated by the detection of free AFB, those that were initially lodged in the upper respiratory tract area were easily available to be collected as a cough sample in the collection tube. However, these free AFB were not present in the trachea or in the upper part of the lungs in all patients, particularly those who were not producing sputum and thus sputum smear tests in such patients were found to be negative for AFB.
Recent communication from the WHO expresses considerable concern about the incidence of such TB patients who are highly infectious to close contacts but are sputum smear negative. In such types of patients, Breathalyser tests would prove to be very useful to detect TB at this early infectious stage and this would enable an early start of their AKT and thereby prevent transmission of TB infection to their close contacts.

The potential for monitoring the progress of treatment was demonstrated by the tests on 21 patients who were receiving AKT. These Breathalyser tests can be done quickly and at much more frequent intervals than sputum smear tests, while monitoring a TB patient on AKT. Even in MDR-TB cases on second line drugs, having 4 such patients in our field trial, Breathalyser tests gave a good indication about the progress of their treatment.

On the basis of our findings we have devised a Text Expectations picture, shown in Fig. 5, which compares the expected results for the Breathalyser test in comparison with sputum, X-ray and PCR tests. NB this figure does not include an analysis of the time taken to obtain a result – the Breathalyser test, taking 5-7 Minutes to perform is significantly faster than the other tests currently available.
Fig. 5. Test Expectations picture for the Breathalyser test compared to Sputum smear culture, X-ray and PCR. (NB ~50% of TB patients are non-sputum producing.)

<table>
<thead>
<tr>
<th>Patient Category</th>
<th>Sputum producing?</th>
<th>RBS Breathalyser</th>
<th>AFB smear</th>
<th>culture</th>
<th>X-ray</th>
<th>NAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>Yes</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Negative</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
</tr>
<tr>
<td>Early stage - asymptomatic, highly infectious</td>
<td>Yes</td>
<td>Positive</td>
<td>Possible but unlikely</td>
<td>Possible but unlikely</td>
<td>Negative</td>
<td>Not recommended for this patient type</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Positive</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Negative</td>
<td>Not applicable to this sample type</td>
</tr>
<tr>
<td>Establishing in the lungs - infectious</td>
<td>Yes</td>
<td>Positive</td>
<td>Will be positive once AFBs start to be contained in sputum</td>
<td>Will be positive once AFBs start to be contained in sputum</td>
<td>Unlikely to detect at this stage but not impossible</td>
<td>Positive, but reliable for smear-positive only</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Positive</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Negative</td>
<td>Not applicable to this sample type</td>
</tr>
<tr>
<td>Well-established in the lungs - still infectious (incl relapse to this state)</td>
<td>Yes</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Probably will see small nodes pos on both lobes</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Positive</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Probably will see small nodes pos on both lobes</td>
<td>Not applicable to this sample type</td>
</tr>
<tr>
<td>Well established but metastasised deeply in lung &amp; surrounding tissue etc. - no longer infectious (inc relapsed to this state)</td>
<td>Yes</td>
<td>Negative</td>
<td>May give pos - may have metastasised deeper and is no longer in sputum</td>
<td>May give pos may have metastasised deeper and is no longer in sputum</td>
<td>Lesions may be detectable - dep on expertise of assessor</td>
<td>Positive, but reliable for smear-positive only</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Negative</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Lesions may be detectable - dep on expertise of assessor</td>
<td>Not applicable to this sample type</td>
</tr>
<tr>
<td>Undergoing treatment (e.g. AKT) but not (or not yet) cured and still infectious</td>
<td>Yes</td>
<td>Positive</td>
<td>May be positive if viable cells are present</td>
<td>May be positive if viable cells are present</td>
<td>Lesions may be detectable - dep on expertise of assessor</td>
<td>Not recommended for use for this patient type</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Positive</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Lesions may be detectable - dep on expertise of assessor</td>
<td>Not applicable to this sample type</td>
</tr>
<tr>
<td>Undergoing treatment (e.g. AKT) which is succeeding; no longer infectious</td>
<td>Yes</td>
<td>Negative</td>
<td>May be positive if viable cells are present</td>
<td>May be positive if viable cells are present</td>
<td>Lesions may be detectable - dep on expertise of assessor</td>
<td>Not recommended for use for this patient type</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Negative</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Lesions may be detectable - dep on expertise of assessor</td>
<td>Not applicable to this sample type</td>
</tr>
<tr>
<td>Post treatment once cured (non-infectious)</td>
<td>Yes</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Scarring may indicate past or present disease</td>
<td>Positive up to 12 months after culture negative</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Negative</td>
<td>Not applicable to this sample type</td>
<td>Not applicable to this sample type</td>
<td>Scarring may indicate past or present disease</td>
<td>Not applicable to this sample type</td>
</tr>
</tbody>
</table>

Key:
- Negative result reliably expected
- Positive result reliably expected
- Positive result expected for some patients only
- Positive result not applicable or recommended

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The findings for the direct tests that were carried out in order to gain an estimate of the absolute sensitivity that might be achieved by the immunoassay test revealed that approx. 50-75 AFBs could be the lowest limit of detection. The findings are in keeping with the estimates for sensitivity shown in Table 5. Among the (45) sputum smear negative patients, there were (33) Breathalyser test positive patients which indicates that the Breathalyser test is more sensitive than sputum smear in detecting higher numbers of TB patients. In sputum smear test the sensitivity is low (56.0%) as there should be more than 10,000 bacilli per ml of sputum sample, for it to be positive; whereas in case of RBS test the sensitivity is high (73.53%) as only approx. 50-75 bacilli per 2-3 µl of cough sample, is required for a positive result.

All this bodes very well for adopting Breathalyser tests as an alternative to the current direct sputum smear microscopy, for screening of TB patients, as this method is quick and easy to perform whereas the direct sputum smear microscopy is time consuming and needs a skilled microscopist to do it.

Conclusions.

The Rapid Biosensor aerosol immunosensor (Breathalyser) system is outside the established and recognized diagnostic procedures and as such detects a different, but overlapping grouping of presentations of the disease. The Breathalyser is able to detect patients who have contracted TB and are in the very early stages of infection, often, even before their sputum smear test and/or X-ray test become positive for TB. Among (45) sputum smear negative patients, there are (33) Breathalyser test positive patients and among (17) X-ray negative patients there are (16) Breathalyser test positive patients. At this early stage, the patient tends to look normal with/without any typical TB symptoms. The Breathalyser test detects the presence of free and accessible AFB’s that are easily displaced from the upper respiratory tract area by an induced or involuntary cough response. It is these free AFB’s that are released into the atmosphere as sputum droplets and infect the people in close contact with the TB patient.
However the Breathalyser test is not generally as useful in patients who are sputum smear positive for AFB, as some of these patients, following the usual progression of the disease, are not releasing free AFB in the atmosphere. This was seen in the field trial, where 18 tests were Breathalyser negative among the 47 sputum positive tests. These negative results were all for patients with well-established TB.

A TB patient tends to remain infectious until forced, either by circumstances or by debility, to visit a doctor, and this period of being infectious could be about 2-6 months; however, the transmission of TB infection is not well understood, because until now there have been no practicable methods available for studying it. The Breathalyser test demonstrates considerable promise for the investigation of the aerosolisation of bacilli and the associated transmission risk factors for individual patients.

In conclusion, these early field trials were successful in showing that Breathalyser tests are an ideal platform for screening TB patients at the early stage of infection and they also show great promise in evaluating the progress of an AKT regimen that is being followed by a patient. The results of Breathalyser tests show an excellent correlation with clinical findings, sputum smear test and X-ray chest results.

Rapid Biosensor tests carried out alongside direct sputum microscopy would go hand-in-hand in providing economical and easy-to-use tests in the TB Clinics, particularly in rural and semi-urban areas. These tests would be complimentary to each other in covering a wide range of TB patient groupings and so the use of the recently introduced costly PCR based tests could be avoided to a great extent.
Competing interests

MAM & ABM do not have any financial or on-going commercial interest in Rapid Biosensor Systems Ltd or any equity shareholding in the company. They have no conflicting interests in related medical device technology.

EMM and NJM hold equity in Rapid Biosensor Systems Ltd and are the developers of the test device described and inventors of the relevant patent 'Biological Measurement System' (PCT WO 02/084266 A2.) Both this patent and 'Bioassay and Peptides for use therein' (WO2007/072063) are assigned to and owned by Rapid Biosensor Systems Ltd. EMM is a Director of Rapid Biosensor Systems Ltd.

The company’s equity funding is from private individuals (Business Angels) and one Corporate investment from Clement Clarke Holdings.

A full register of shareholders is available on request.

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Authors' contributions

MAM & ABM conceived and designed the trials.

MAM & ABM performed the tests.

MAM, EMM, NJM, ABM analyzed the data.

MAM & ABM wrote the paper.

EMM & NJM provided technical knowledge, protocols and training for the breathalyser test.

All authors read and approved the final manuscript.
Authors Information.

EMM is a Chemical Physicist, Fellow of the Royal Society of Chemistry (FRSC) & Member of the Institute of Physics (MInstP) and is expert in the fields of Surface Science & Spectroscopy.

NJM is a Trauma Surgeon and Pathologist with extensive experience in many and varied global locations. He has considerable interactive expertise in the diagnosis and treatment of life-threatening conditions and is a specialist in communicable diseases.

MAM is at present: Associate Professor; In Charge of Molecular Lab.; Virology, Mycology & Mycobacteriology Lab., Dept. of Microbiology, P.D.U. Medical College, Rajkot, Gujarat, India.

Formerly: Head of Lab Dept. of K.J. Mehta TB Hospital & Professor at Aditya Vikram Birla Post-graduate Institute, TB Research Centre, Amargadh, Gujarat, India.

ABM is a Senior Chest Consultant and expert in the diagnosis and treatment of Tuberculosis.

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References


