

## Evaluation of diagnostic assays for tuberculosis (TB) and formulation of a feasible testing strategy for rural areas of Pakistan

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### Abstract

Pulmonary tubercle bacillosis (PTB) is a highly prevalent infectious and lethal disease worldwide. To overcome this problem, a proper and feasible diagnostic protocol is required to diagnose the TB-infected persons particularly in hot rural areas wherein the disease incidence is expected to be high, e.g., the east Punjab region of Pakistan. This study was performed to identify the hidden cases so as to avoid further transmission of TB in the area. The most common procedures employed for the diagnosis of TB were Myco-Dot, ZN smear, Fluorescence microscopy, Mantoux test, and GeneXpert. A total of 259 samples of suspected patients were subjected to each of the earlier-mentioned protocols and compared with the gold standard TB culture method. By doing this it was possible to identify a good sensitivity- and specificity-based assay which would be easily available at an affordable cost by the common people. The GeneXpert test showed 81.1% sensitivity and 95.6% specificity, being higher with those determined by the other tests. However, this approach is cost-intensive and out of reach for the Punjab rural communities. Fluorescence microscopy was ranked second in its sensitivity (64.5%) and specificity (96.4%), since this method is relatively cost-effective, but not as much as the other methods already in vogue.

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## Introduction

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis*. It mainly affects the lungs, but can also impact other parts of the body. This disease is transmitted through the air when a person with the disease coughs or sneezes; its symptoms include coughing, fever, fatigue, and weight loss (Kumar et al., 2007; Senarath et al., 2014; Field et al., 2018). Although it is commonly treated with antibiotics, drug-resistant strains have emerged, making the disease treatment more challenging. This is a significant global health concern, with an estimated 10 million cases and 1.4 million deaths worldwide (Gurung et al., 2021). There are several diagnostic strategies for pulmonary tuberculosis (TB) such as sputum microscopy (ZN smear and fluorescence microscopy), that involve examination of a patient's sputum under a microscope to detect acid-fast bacilli, which are characteristic of *Mycobacterium tuberculosis*. Sputum culture is another test that can be used to confirm the presence of TB bacteria and identify drug-resistant strains, but this is a very time-consuming test (Saglam et al., 2005). Molecular tests including nucleic acid amplification tests (NAATs) can detect TB DNA or RNA and provide results more quickly than that by the culture methods. For example, GeneXpert and TB-PCR. Chest X-rays and computed tomography (CT) scans are also used to identify lung abnormalities caused by TB, such as cavities or nodules (Son et al., 2020).

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Other tests, such as the MycoDot, Mantoux tuberculin skin test, and interferon-gamma release assays (IGRAs), can help identify people who have been infected with TB, but may not have active disease (Auguste et al., 2017). These tests detect the immune system responses to TB antigens. However, they cannot distinguish between the latent TB infection and active TB disease, and they may produce false-positive results in people who have received the BCG vaccine or who have been exposed to other mycobacteria (Gilpin et al., 2018). Diagnosis can be challenging, especially in areas where access to resources is limited or when patients have atypical presentations. Newer diagnostic technologies, such as loop-mediated isothermal amplification (LAMP) and whole-genome sequencing, are being developed and evaluated for their potential to improve TB diagnosis (Garg et al., 2022).

Early diagnosis and treatment are critical to controlling the spread of TB. Prompt diagnosis and appropriate treatment can also improve patient outcomes and reduce the risk of developing drug-resistant TB (Nelson and Wells, 2004). Diagnosing tuberculosis in rural and poor areas is particularly challenging due to several factors, i.e., these areas may lack access to advanced healthcare facilities, trained healthcare providers, and laboratory facilities that are necessary for TB diagnosis. Moreover, delays in diagnosis results and treatment increase the risk of transmission and disease progression (Santos et al., 2021). A diagnostic test, economically affordable and less time-consuming with good sensitivity and specificity ratio, may play a crucial role in poor areas where the burden of tuberculosis (TB) is high. Thus, in the current study, different TB diagnostic tests were compared for their sensitivity and specificity as well as cost-effectiveness.

## Materials and Methods

### Sample collection

Each sample was collected according to the guidelines of each protocol such as sputum samples were collected for Ziehl-Neelsen (ZN) stain, fluorescence microscopy, TB culturing, and GeneXpert. The Mantoux tests were performed directly intradermally on the skin of the forearm, whereas serum/plasma of suspected patients was used for MycoDot testing.

### Diagnostic Tools

#### *Direct smear method*

For Ziehl-Neelsen (ZN) staining, a thin smear of the suspected patient's sputum sample was prepared on a clean glass slide. Then the smear was fixed by passing it through the flame of a Bunsen burner, and subsequently the slide was covered with carbon fuchsin, a red dye that stains acid-fast bacteria. Then the slide was heated by passing it through the flame of a Bunsen burner for 5 minutes to enhance the staining. Thereafter, the slide was rinsed with water to remove excess stain. Acid alcohol as a decolorizer was used to remove the stain from non-acid-fast bacteria. The slide was again washed with water and counterstained with methylene blue for 1 minute to stain non-acid-fast bacteria. In the end, the slide was washed with water and allowed to dry to examine under a microscope using an oil-immersion lens. Acid-fast bacteria such as *M. tuberculosis* appeared pink, while non-acid-fast bacteria appeared blue. The presence of acid-fast bacilli in the sample indicates a presumptive diagnosis of tuberculosis (Hooja et al., 2011; Dzodanu et al., 2019).

#### *Fluorescence microscopy*

Fluorescence microscopy is a laboratory method used for the rapid detection of acid-fast bacteria, including *Mycobacterium tuberculosis* (Rieder, 1999; Gurung et al., 2018). A thin smear was prepared of the sputum sample of each suspected TB patient on a clean glass slide and fixed the smear by passing it through the flame of a Bunsen burner for a few minutes. After that, the slide was covered with auramine, a fluorescent dye, that binds to the acid-fast bacteria. The slide was washed with water to remove excess stains. The slide was counterstained with potassium permanganate for 1-2 minutes to quench the background fluorescence; thereafter, the slide was again washed with water and allowed to dry. The slide was examined under a fluorescence microscope using a 40x to the broad field or by 100x oil immersion lens and a blue excitation filter. Acid-fast bacteria such as *M. tuberculosis* appeared bright yellow-green against a dark background, while non-acid-fast bacteria did not fluoresce.

#### *GeneXpert*

The GeneXpert system is based on a real-time polymerase chain reaction (PCR) assay that detects the DNA of *Mycobacterium tuberculosis* in the patient's sputum. The system uses a disposable cartridge that contains all the reagents required for the PCR assay, including primers and probes specific to the target DNA sequence of *M. tuberculosis*. The sputum sample was mixed with the appropriate reagents and introduced into the cartridge, where the PCR amplification and detection occurred. The results were

automatically interpreted by the GeneXpert software, which provided a readout of positive or negative for TB (Ejeh et al., 2016).

### **Manual TB culture**

The bacterium that causes tuberculosis (TB), involves the growth of the organism on a solid culture medium in the manual procedure of TB culturing. Sputum specimens were collected from suspected patients and decontaminated the specimens using a chemical solution to kill any contaminants and release the MTB cells from the patient's cells. The specimens were centrifuged to concentrate the MTB cells to remove the decontamination solution, then suspended the pellet in a small volume of sterile saline which was inoculated onto a solid culture medium such as Lowenstein-Jensen (LJ) or Middlebrook 7H10 agar using a sterile loop or needle, and incubated the plates at a temperature of 35-37 °C for up to 6 weeks. The plates were examined for the growth of MTB colonies, which appeared as smooth, raised, and buff-colored colonies with a dry appearance. This was confirmed by the identity of MTB using the acid-fast staining (Shi et al., 2018).

### **MycoDot**

This test is an immunochromatographic assay based on the detection of antibodies specific to *Mycobacterium tuberculosis* in the patient's serum or plasma. The test uses a combination of recombinant antigens (MPB64, CFP10, ESAT-6) that are highly specific to *M. tuberculosis*. The antigens are immobilized on a nitrocellulose membrane, and when a sample containing TB-specific antibodies is added, a visible line appears on the membrane, indicating a positive test result. The patient's serum or plasma allowed clotting and it was transferred to a clean tube and centrifuged to remove any remaining cells or debris. An aliquot (50 µl) of serum or plasma was dropped on the sample pad of the MycoDot test cassette and 3-4 drops of buffer were also added to the sample pad to initiate the test. The test result was noted after 20 minutes. A visible line on the membrane at the test line region indicated a positive result, while the absence of a line indicated a negative result with a control region which indicated that the test was performed correctly (Simsek et al., 2010).

### **Mantoux test**

The interpretation of the Mantoux test results is based on the size of the induration and the patient's risk factors for TB. In general, an induration of 5 mm or greater is considered positive for TB in individuals with high-risk factors such as HIV infection or recent contact with a TB case. An induration of 10 mm or greater is considered positive for TB in individuals with intermediate risk factors such as recent immigrants from high-prevalence countries or injection drug users. An induration of 15 mm or greater is considered positive for TB in individuals with low-risk factors such as healthcare workers or individuals with no known risk factors. The Mantoux test is a simple and inexpensive tool for the diagnosis of TB, but it has limitations such as the potential for false-positive or false-negative results. It is also not useful for the diagnosis of active TB disease, as it only indicates exposure to the bacterium (Simsek et al., 2010).

The Mantoux test is based on a delayed-type hypersensitivity reaction to purified protein derivative (PPD), which is a protein extract derived from the cell wall of *M. tuberculosis* (Khan et al., 2026). Intradermally injection of PPD elicits an immune response in individuals who have been exposed to TB in the past. The injection site was cleaned with alcohol and allowed to dry. Using a 1 mL syringe and a 26- to 27-gauge needle, 0.1 mL (5 tuberculin units) of PPD was injected into the inner surface of the forearm. The date and time were recorded, and the location of the injection site was marked as a circle with a permanent marker. And asked the patient to return for a test reading after 48-72 hours. The site of the injection was assessed for the presence of induration, which is the raised and reddened area around the injection site. In the positive case, the induration was measured in millimeters.

## **Results**

Depending on the facilities available in the rural areas of Punjab, Pakistan, the pulmonary tuberculosis was diagnosed using the methods such as Myco-Dot, ZN smear, Fluorescence microscopy, and Mantoux test. GeneXpert and TB culture facilities were only available in referral labs at a long distance. A total of 259 suspected patients were subjected to TB diagnosis by different tests to identify a reliable and cost-effective test for those areas where no advanced facilities are available for TB diagnosis. These included 130 male and 129 female patient samples. Out of the total number of samples, 62 were true positive cases which were confirmed by the manual TB culturing on LG media, which included 32 males and 30 females (**Figure 1**). Before the TB culturing, all samples were tested with other methods to compare the results, and their sensitivity and specificity.

### ZN smear (specificity and sensitivity)

Out of 259 samples, 28 (14 male and 14 female) were found to be positive by ZN smear. To find out the true positive and true negative results, all samples were confirmed by the gold standard TB culture test. After confirmation by the TB culture, the results showed 4 false positive and 19 false negative tested by ZN smear (Table 1, Figure 2). The sensitivity and specificity values of ZN smear were compared with those obtained by the TB culture. Our data presented as percentages is comparable with those reported in other studies (Ngabonziza, 2016; Kaso et al., 2021).

### Fluorescence microscopy (specificity and sensitivity)

Out of 259 samples, 39 were reported positive by the fluorescence microscopy which included 18 male and 21 female; 49% were below 35-year in age, and 51% over 35-years. To find out the true positive and true negative results, all samples were confirmed by the gold standard TB culture test. After confirmation by the TB culture, the results showed 8 false positive and 17 false negative tested by the fluorescence microscopy smear (Table 1 and Figure 2) The sensitivity and specificity values of the fluorescence microscopy were compared with those obtained from TB culture. These percent values correlate well with other reported results (Chang et al., 2016).

### GeneXpert (specificity and sensitivity)

Out of 259 samples, 52 were reported positive by GeneXpert. However, to find out the true positive and true negative results, all samples were confirmed by the gold standard TB culture test. After confirmation by the TB culture, the results showed 9 false positive and 9 false negative as tested by GeneXpert (Table 1, Figure 2). The sensitivity and specificity of GeneXpert were evaluated by the comparing them with those obtained by the TB culture test. These percent values coincide well with other reported results (Reechaipichitkul et al., 2016).

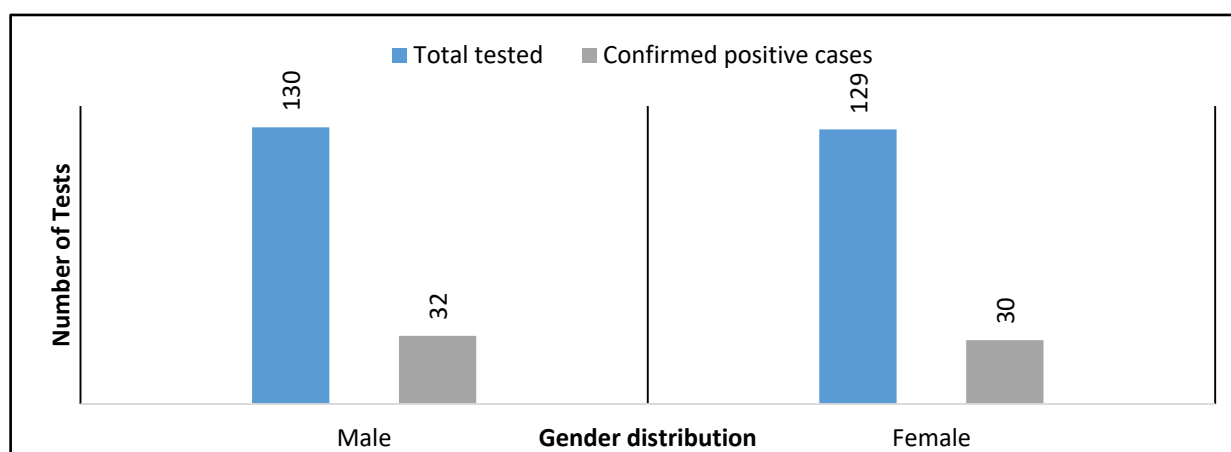


Figure 1. Distribution of TB-positive cases confirmed by the TB culture according to gender

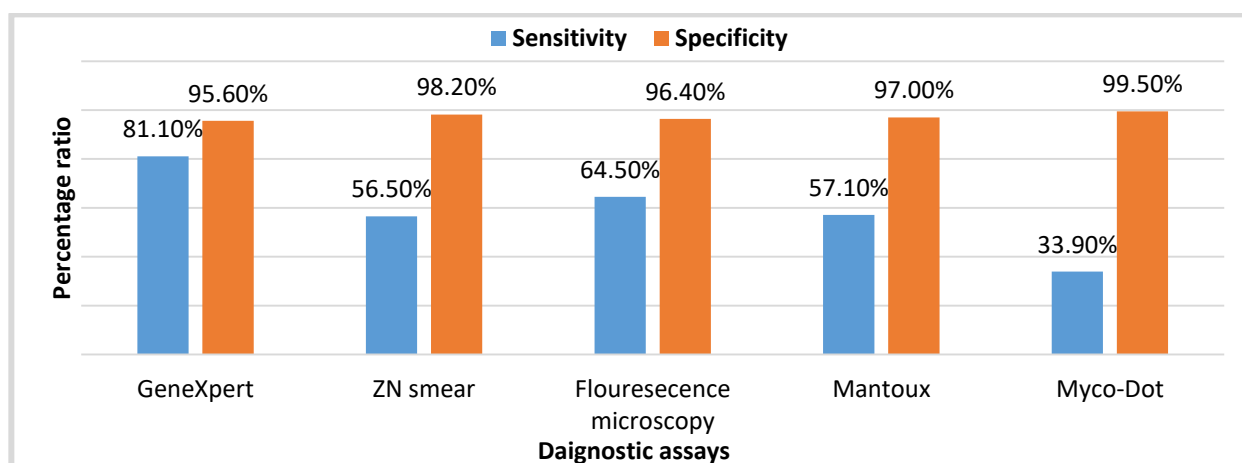
Table 1. Sensitivity, specificity, positive predictive value and negative predictive value of GeneXpert, ZN smear, Fluorescence microscopy, Mantoux, and MycoDot.

Techniques	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<i>GeneXpert</i>	81.1	95.6	82.69	95.17
<i>ZN smear</i>	56.5	98.2	85.71	92.40
<i>Fluorescence microscopy</i>	64.5	96.4	79.49	92.83
<i>Mantoux</i>	57.1	97.0	61.54	97.56
<i>MycoDot</i>	33.9	99.5	95.00	86.59

PPV: Positive predictive value, NPV: Negative predictive value

Table 2. Feasibility of TB diagnostic assays

Assay	Test cost (PKR)	Time duration	Lab requirement	Feasibility
<i>GeneXpert</i>	6000	4 h	Level-III	Expensive
<i>TB culture</i>	5000	6 Weeks	Level-III	Expensive and time consuming
<i>Myco-Dot</i>	800	30 Min.	Level-II	Low sensitivity
<i>ZN Smear</i>	500	1 h	Level-II & III	Low sensitivity
<i>Mantoux</i>	700	72 h	Level-II	Low sensitivity and time consumables
<i>Fluorescence microscopy</i>	600	1 h	Level-II & III	Affordable/rapid & specific



**Figure 2. Cumulative sensitivity and specificity of performed diagnostic tests**

### Mantoux (specificity and sensitivity)

Out of 259 samples, 13 were recorded as positive through the Mantoux test, which included 7 male and 6 female, of which 50% were below 35-year age, and 50% over 35 years. To find out true positive and true negative, all samples were confirmed by the gold standard TB culture test. After confirmation by the TB culture, the results showed 5 false positive and 6 false negative as tested by Mantoux (Table 1, Figure 2). The sensitivity and specificity values obtained by the Mantoux test were compared with those by the TB culture. These percent values are very comparable with those reported elsewhere (Rose et al., 1995).

### Myco-Dot (specificity and sensitivity)

Of 259 samples, 20 were reported positive by Myco-Dot which included 11 male and 9 female; from these samples it was found that 55% were below 35-year in age, while 45% were over 35 years. True positive and true negative results from all samples collected were confirmed by the gold standard TB culture test, and the results showed 1 false positive and 37 false negative as also tested by Myco-Dot (Table 1; Figure 2). The sensitivity and specificity values obtained from Myco-Dot was compared with those from TB culture. The percent values recorded in the present study corroborate well with reported earlier elsewhere (Hussain, 1999).

## Discussion

The most common methods for diagnosing TB are susceptible to human error. For example, during the sample collection or due to unfocused microscopy and other factors beyond control can result in false negatives (Raja et al, 2020). The impact of a false negative in TB diagnosis can have far-reaching consequences and can be very detrimental to the global initiative as it may lead to further spread of TB infections from untreated cases. In our study, fluorescence microscopy (Auramine) showed 10% better sensitivity (64%) than that by ZN smear (56.5%). Serum antibodies-based Myco-Dot showed a very low sensitivity (33.9%) than those by the other techniques, and likewise, a relatively low sensitivity (57%) was observed with skin-based Mantoux test. However, GeneXpert showed the highest sensitivity (81%) as well as specificity (95%), that was followed by fluorescence microscopy (Auramine) compared with AFB culture. Comparable results have been reported in another study wherein GeneXpert showed high sensitivity and specificity than that by ZN smear in pulmonary tuberculosis samples (Agrawal et al., 2016). GeneXpert is a simple benchtop point-of-care diagnostic assay that can be reported within 4 hours, but the arrangement of highly expensive instruments and kits is not an easy task for every rural sector. However, if this technique is adopted by bearing high cost, then this approach is technically more efficient compared with other methods in vogue for the diagnosis of pulmonary tuberculosis. Moreover, fluorescence microscopy does not require special technical expertise and is cost-effective; its time span for exhibiting results is too short compared with that of GeneXpert and other several techniques (Van Rie et al., 2010; Campelo et al., 2021). This study confirms that GeneXpert is a suitable, efficient, and specific method for the diagnosis of TB with much better sensitivity, but it is affordable in basic health units in rural areas. However, if possible, fluorescence microscopy is a very viable option for the rural health care centers in terms of sensitivity, specificity and time duration (Table 2).

Rapid detection of *Mycobacterium tuberculosis* complex is important for patient management in terms of initiating appropriate antimycobacterial therapy as well as controlling the spread of this pathogen. Compared to other used assays, fluorescence microscopy is affordable and is a rapid diagnostic tool even with better sensitivity and specificity. *Mycobacterium tuberculosis* is a major threat

to the health of third-world countries, specifically in rural areas.

Tuberculosis management is always been a great task because of the slow detection rate of *Mycobacterium* and the cost of good quality tests. So, a reliable and feasible testing strategy must be applied in the rural areas, which should be cost-effective and has good sensitivity and specificity.

## Conclusion

GeneXpert is a very specific and sensitive test than other relevant diagnostic strategies in vogue for the investigation of tuberculosis, but this is very cost-intensive, and also it is not convenient for use in rural areas. Moreover, fluorescence microscopy shows a good specificity with a high range of sensitivity; this is also cost-effective compared with several others under use. This technique is a good option particularly in rural areas for the prompt diagnosis of TB.

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## Declaration of Author(s), Editor(s) and Publisher

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No supplementary material is included with this manuscript.

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The authors declare no conflict of interest.

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### Contribution of authors

Designed and carried out the experiment and prepared the draft manuscript: H, A, BA. Guidance and technical support provided: H, BA. Revised the manuscript: H, A, BA.

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This work was approved by Institutional Ethical Review Board/Committee (IERB/C) of the International Islamic University Islamabad, Pakistan, under approval number 501-FBAS/MSBT/S21.

### Handling of bio-hazardous materials

The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discarded to minimize any types of bio-contamination(s).

### Availability of primary data and materials

As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

### Authors' consent

All authors contributed in designing and writing the entire review article. All contributors have critically read this manuscript and agreed for publishing in IJAaEB.

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