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ORIGINAL ARTICLE

The Diagnostic Accuracy of the Qualitative Polyclonal Antibody Assay for Detecting an Active Pulmonary Tuberculosis, Case Control Study

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Key words:
Tuberculosis; Diagnosis; Sensitivity; Specificity; Follow-up

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ABSTRACT

Background: *The accuracy of diagnostic methods in clinical trials that focus on controlling Mycobacterium tuberculosis is essential.* ***Objective:*** *This study aims to determine the accuracy of the polyclonal antigen cocktail in the diagnosis and follow up the pulmonary tuberculosis.* ***Methodology:*** *The study included 77 patients who had pulmonary tuberculosis (PTB) and 83 individuals who had never contracted tuberculosis. All PTB patients submitted sputum specimens on days 0 and 30 for diagnostic and follow-up assessments, whereas each normal participant provided a single sputum specimen. We performed microscopy with smears, Xpert MTB/RIF, solid culture, and TB antigen cocktail determination with the sputum specimens. We used the sandwich ELISA to detect the tuberculosis antigen. The receiver operating characteristic analysis determined the optimal threshold measurement for antigen absorption.* ***Results:*** *The accuracy of antigen cocktail for diagnosing PTB ranged between 84.0% and 100%. The zone below the ROC curve was 0.987 (98.6% CI 97.2–100). The follow-up process of PTB patients before and after treatment indicated that the antigen cocktails significantly decreased following anti-TB therapy.* ***Conclusion:*** *The current investigation demonstrated that the TB antigen cocktail was faster, inexpensive and a useful way to identify active pulmonary tuberculosis and predict successful therapy during a follow-up.*

INTRODUCTION

Tuberculosis (TB) ranks as one of the most lethal respiratory illnesses globally [1,2]. In 2022, there were 10.6 million cases newly identified and 1.3 million fatalities reported[3]. The restrictions of traditional approaches to diagnosis tuberculosis (such as sputum smear microscopy, PCR, and culture), including prolonged delays in diagnosis, insufficient sensitivity in some populations, and technical difficulties, have been well acknowledged[4,5].

Microscopic detection of bacilli that are Acid-fast (Ziehl-Neelsen stain) analysis in sputum specimens, lung radiography, and cultivation constitute the principal procedures for diagnosing active pulmonary tuberculosis. Acid-fast stains yield positive results only in active and progressed tuberculosis, with a sensitivity rate of 22–78%, whereas cultivation demonstrates a sensitivity of about 95%[6,7]. The AFB test continues to serve as a rapid screening diagnostic tool for tuberculosis, as approved by the WHO in tuberculosis control efforts within endemic nations[8] .

The existing clinical standard Microbiological testing protocols (smear, mycobacterium Cultural practices and the amplification of genes) are inadequate for the inadequate detection and alternative diagnosis of bacterium-negative tuberculosis[9,10].

The serological testing techniques can potentially detect Infection by tuberculosis-associated bacteria (Mtb) by evaluating the immunological response of the host. The place of collection of the hematological specimen is readily available, the procedure is straightforward and expedient, and it has emerged as a significant method for the adjunctive diagnosis of tuberculosis, particularly in cases of bacterium-negative pulmonary tuberculosis, extra-pulmonary tuberculosis, and pediatric tuberculosis[11].

The sensitivity of smear microscopy is limited, and it cannot differentiate among living and non-viable bacilli. The AFB smear's limitations have led to the use of A DNA amplifying test to improve MTB diagnosis. Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) possesses superior sensitivity for identifying MTB and provides results in two hours[12].

However, NAAT is incapable of assessing the viability of MTB, it additionally detects DNA from deceased bacterial tubercles. Therefore, NAAT is inadequate for further diagnostic assessment[12].

A positive MTB culture is the recognised and optimal criterion for diagnosing active tuberculosis. Moreover, the time to detection (TTD) in culture solutions and the colony count on solid medium indicate the load of viable MTB bacilli. Cultural investigation is crucial for the accurate examination of sensitive tuberculosis and for determining the effectiveness of

anti-tuberculosis treatment, particularly in patients with multidrug-resistant tuberculosis (MDR-TB)[13]. Still, access to cultural investigations can be difficult in several countries that are developing. Moreover, culture investigation is difficult because of the slow-growing features of MTB and findings can't be acquired rapidly[10].

Still, access to cultural investigations can be difficult in several countries that are developing. Moreover, culture investigation is difficult because of the slow-growing features of MTB[14]. The traditional cultivation technique using Lowenstein-Jensen medium continues to be considered the gold standard for diagnosing the bacteria that cause TB and evaluating drug sensitivity, although this method is time-intensive, requiring several weeks or months[15].

The World Health Organization (WHO) has developed multiple novel methodologies in laboratories to speed up the detection and determination of drug-resistant strains. These plans use both phenotypic and genetic methods, such as the MGIT 960 liquid culture and the molecular PCR method through the GeneXpert systems and the line probe assay[16,17]. While microscopy and culture are essential to therapeutic evaluation, the global adoption of quick molecular testing and automated cultivation techniques is rising, leading a number of nations to discontinue smear microscopy for diagnostic purposes[9]. However, due to their high costs, only a small number of diagnostic centres can use them[18].

Consequently, it is necessary to establish an inexpensive and speedy assay capable of identifying *Mycobacterium tuberculosis* and distinguishing it from non-tuberculosis mycobacteria (NTM) in instances of contamination by rapidly proliferating NTM[19].

Furthermore, it has become essential to quantify *M. tuberculosis* for evaluating the curative effectiveness of anti-mycobacterial agents.

Therefore, this study aims to develop a rapid, cost-effective, and highly sensitive assay for diagnosing pulmonary tuberculosis by employing a sandwich ELISA to detect TB antigenic determinants in sputum specimens. We use sandwich ELISA wells pre-coated with polyclonal antibodies specific to *M. tuberculosis* antigens

METHODOLOGY

Studied population:

The examined population consisted of 160 individuals, classified into two categories. The first group was 83 suspected TB patients served as control group, while the second group was 77 confirmed active pulmonary TB. The first morning sputum specimens were collected from the patients attending to the Ramadi respiratory diseases clinic, Anbar\Iraq.

Selection of participants:

The individuals with active tuberculosis were detected via a positive acid-fast bacilli test, Löwenstein-Jensen culture and confirmed by gene expert. The control group was the patients suffering from pulmonary infections caused by another infectious agents' bot no TB. The pulmonary TB was excluded by a negative result of AFB, culture and gene expert.

Ethical approval:

The study was approved by Research Ethical Committee at the University of Anbar (protocol No. IN13-004) All individuals have requested permission to participate in this study.

Inclusion criteria:

All pulmonary TB cases who were more than 18 years.

Exclusion criteria:

PTB Patients treated with standard anti-TB treatments for more than 6 days were determined ineligible to prevent misleading negative results in microbiological investigations. The individuals in the non-PTB group tested negative for hepatitis C and B viruses, as well as HIV.

Sample preparation for ELISA test:

The sputum specimens were homogenized by adding 4X phosphate buffered saline (PH 7.2-7.4) with ratio (1:1) and incubated 15 min at a room temperature. Then the specimens undergo repeated freezing and thawing to release the intracellular components. The sediment collected carefully after centrifugation for 20 min at 2000-3000 rpm. The sediment suspended with 0.5 ml phosphate buffered saline and then 50 uL from the mixture was added to the sample well.

Sandwich ELISA procedure:

The procedure of ELISA test was carried out according to instruction of the manufacturing company (Human\Germany) which provided as paper leaflet with the kit. The wells of the ELISA kits were pre coated with polyclonal antibodies specific to detection of *Mycobacterium tuberculosis*.

Specificity and sensitivity:

The variables were determined by the maximum method. Youden's index based on the following formulas: sensitivity = TP/(TP+FN) and specificity = TN/ (TN + FP); where TP = True Positive, FN = False Negative, TN = True Negative and FP = False Positive (Alvarez et al., 2012).

Statistical analysis:

Comparative analyses of the different groups were carried out by a Student's t-test with SPSS version 22 software.

RESULTS

A prospective investigation was carried out with 77 patients diagnosed bacteriologically with Active Pulmonary Tuberculosis (PTB) and 83 patients with

other respiratory infections, designated as suspicious controls (SC). Patients with pulmonary tuberculosis (PTB) included in the study were positive for acid-fast bacilli (AFB) smear and either nucleic acid amplification test (NAAT) or culture for Mycobacterium tuberculosis (MTB). Figure 1 shows the inclusion method and the findings of the microbiological examination. In patients with PTB, the positive rates of sputum smear, Xpert MTB/RIF, and culture dramatically diminished during the anti-TB treatment period (Figure 1).

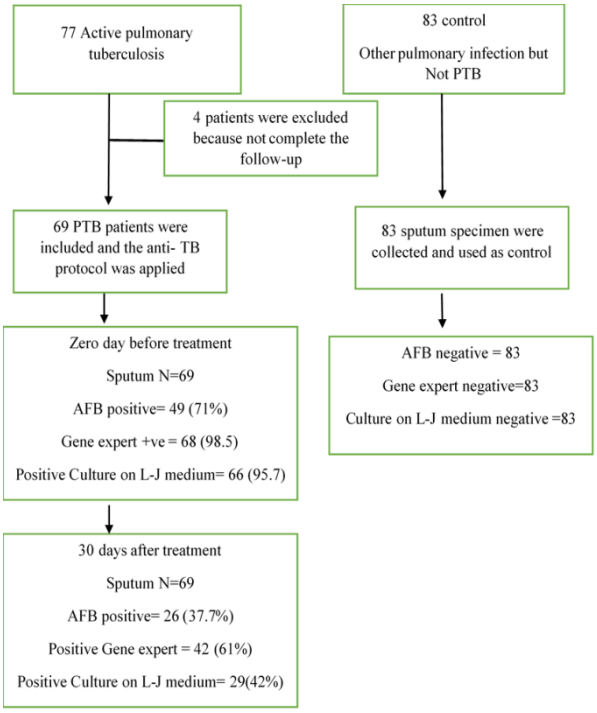

Fig. 1: Process of participant's enrolment and the diagnosis procedure

The presence of TB antigen was evaluated in the 2 groups, APTB and SC (NO TB). The detection of TB antigen was statistically significant higher in APTB support for the group of suspected control (SC) ($p < 0.001$). The result showed that an average of optical density values indicated a noticeable disparity between APTB (1.3448) and SC (0.573) ($P< 0.05$). The median values of the TB antigen were substantially higher in individuals with APTB at 1.4 (interquartile range [IQR] 1.14 to 1.52) compared to SC group at 0.43 (IQR 0.37 to 0.87). The p-value is less than 0.0001 (Figure 2).

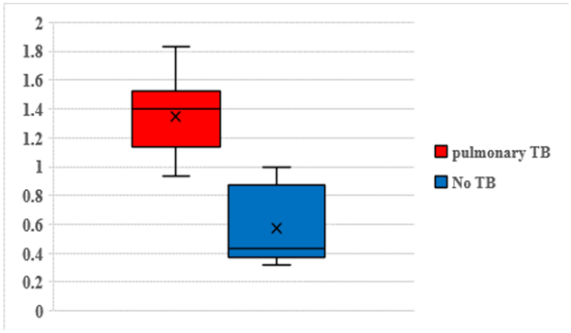

Fig. 2: The absorption measurement of TB antigen in both APTB and SC patients: "The data is visualized through box plots, which display the first through third quartiles as boxes, the median as lines inside the boxes, and the minimum and maximum values (excluding outliers) as lines outside the boxes."

The ROC curve was generated by comparing the performance of APTB and SC, it is readily apparent that the AUC considerably varied from 0.5, suggesting that this test may effectively differentiate between APTB and SC as represented in Figure 3.

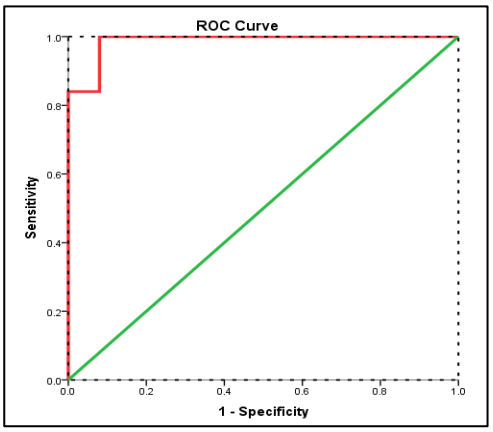

Fig. 3: ROC plots for TB Antigen detection by comparison between Active pulmonary tuberculosis (APTB) and Suspected control (SC).

The cut-off value associated with the maximum Youden's index was established to determine the effectiveness of the test. We calculated the urea under the curve (AUC) and showed it to have statistical significance, along with a 95% confidence interval. The AUC was determined to be 0.987, as evidenced by a p-value of less than 0.001 with standard error was 0.008 and confidence intervals was between 97% to 100% (Table1).

Table 1: Area under the curve of TB antigen detection test.

Asymptotic 95% CI		Asym. sig	SE	AUC
Upper bound	Lower bound			
1.000	0.972	0.000	0.008	0.987

AUC= Area under the curve
SE= Standard Error
Asym. sig= Asymptotic significant
CI= Confidence intervals

The result of the current study showed multiple cut-off values with different specificity and sensitivity. The most powerful point that could differentiate between APTB and SC were 0.915, 0.885, 1.0700 and 0.96. The best optimal threshold values (positive if greater than) determined to be 0.915, with 100% sensitivity and 92% specificity (Table 2).

Table 2: Cut-off values of the sputum TB antigen test.

Specificity %	Sensitivity %	Positive if greater than
92	100	0.915
84	100	0.885
100	84	1.0700
92	86	0.96

The follow-up analysis of polyclonal TB antigen trends in patients with PTB prior to and following TB therapy indicated a significant reduction in Δabs in the PTB group follow anti-TB therapy (Figure 4). Three individuals exhibited consistently elevated Δabs values exceeding the threshold (Δabs = 0.915) and tested positive for both Xpert MTB/RIF and cultures for the duration of the 30-day treatment period. One patient exhibited severe and uncontrollable diabetes mellitus alongside a chronic cough for four months prior to the diagnosis of tuberculosis. Another patient presented with untreated advanced lung carcinoma. The remaining patients were HIV-positive. Three individuals exhibited numerous cavitary lesions identified on chest X-ray.

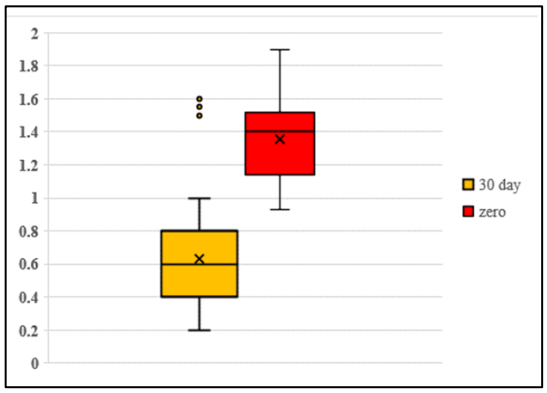

Fig. 4: The TB antigen production before and 30 days after the anti-TB treatment (N=69).

DISCUSSION

The current study used the TB polyclonal antibodies in the ELISA method as a proof of concept for diagnosing active pulmonary tuberculosis and for more evaluation in pre-treated sputum. The TB polyclonal antigens in sputum samples exhibited sensitivity comparable to AFB culture and Xpert MTB/RIF for diagnosing active pulmonary tuberculosis, with an appropriate cut-off value, and TB antigen levels reduced as anti-tuberculosis treatment continued. These findings corroborated the theory of this investigation. The sensitivity of TB Polyclonal-Sandwich ELISA ΔAbs appeared to be greater than that of AFB and comparable to Xpert MTB/RIF and culture. The data indicate that TB antigen during the follow-up period may be effective in projecting MTB culture implications. A noticeable trend was noticed in the reduction of TB antigen levels associated with effective anti-TB treatment, perhaps indicating the response to tuberculosis therapy and reliably predicting culture results at day 30 of treatment.

Recent studies determined MTB-specific antigens, including MPT64, initial secretion antigenic target-6, culture filtrate protein-10 (CFP-10), and CFP-21, in patients sputum samples[20,21]. A research study utilizing the standard sandwich ELISA approach for antigen detection revealed a diagnosis sensitivity of 56% and a specificity of 90% in AFB smear-positive sputum specimens[20]. An additional investigation used an immuno-polymerase chain reaction to identify TB-specific antigens, demonstrating an increase in sensitivity from 56% to 77.5% in sputum Samples that test positive for AFB smear[21,22]. Despite these studies directly measuring tuberculosis-specific antigens in sputum, the sensitivity for detecting active pulmonary tuberculosis cases was inferior to that of the current study.

The novelty of the present research was to evaluate whether an antigen cocktail specific to *M. tuberculosis* could be measured throughout follow-up to evaluate therapy effectiveness. The traditional culture analysis requires several weeks for a positive outcome and about 6–8 weeks to confirm a negative outcome. This postponement hinders the efficacy of treatment efforts for patients. Molecular tests, such as amplifying nucleic acids tests, have a high specificity for finding tuberculosis. However, their sensitivity is still modest and not reliable, especially in cases of smear-negative and extra pulmonary disease[23,26]. Furthermore, these assessments are costly and necessitate specialized equipment and experience[26]. A cocktail of antigens unique to *M. tuberculosis* can be used in ELISA as a quick, easy, and inexpensive alternative to molecular methods for creating a specific test for tuberculosis in places where it is common. Consequently, the current study recognizes the TB antigens-based polyclonal

antibody cocktail as a potential candidate for future application as a supplement to smear microscopy, due to its capacity to accurately identify a significantly high number of smear-negative PTB, HIV/TB, and EPTB patients.

This research exhibited multiple drawbacks. Initially, it excluded patients with multidrug-resistant tuberculosis and included two patients with HIV infection. Future research must assess the efficacy of TB antigen cocktail screening in such groups. Second, a few individuals demonstrated elevated TB antigen levels during the 30-day anti-TB regimen. This could have been caused by a prolonged elevated bacillary burden in individuals with diabetes mellitus and severe pulmonary tuberculosis. Such instances may require a prolonged follow-up period to determine TB antigen compatibility with other common procedures, particularly smear and culture. Third, the evaluation study included a relatively small sample size. Therefore, conducting additional studies with a larger sample size is essential to fully characterize, identify the accurate cutoff, and establish its limits. This study showcased several strengths, such as the minimal use of coenzymes or heating processes to prepare sputum specimens and the ease of use of standard ELISA instruments. Additionally, the control group was suspected TB patients, not healthy persons, which may reflect the excellent specificity of the test.

CONCLUSION

The present research showed the efficacy of antigen detection with sandwich ELISA for diagnosing active pulmonary tuberculosis in sputum samples. In addition, it showed that secreted antigen levels could be used to predict changes in MTB cultures during therapy. The antigenic load in sputum specimens may reflect the viable bacterial loads, which could potentially determine the severity of infection. Identifying the TB-specific antigens from medical samples could enable healthcare providers to evaluate the survival of MTB within one day and may serve as a substitute for culture examinations. This strategy may decrease the duration required to assess the efficacy of the anti-TB drugs and enhance quickly diagnostic assessment.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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