Title	Immunological Methods for diagnosis tuberculosis in smear negative Iraqi patients.
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Abstract:

Smear-negative tuberculosis (TB) is a common problem in diagnosis by Zehil-Neelsen (ZN). The comparison between the classical methods (ZN test, culture on Lownestien Jenison (LJ) and Middle Brook 7H9 media) and Immunological methods (*Mycobacterium* Growth Identification Test (MGIT) and Rapid Chromatography Immunoassay test) used in the present study and to assessment of tests that show rapidly and accuracy in diagnostic TB. The prospective study done in Ministry of Health / Specialized Center of Chest Respiratory Disease in Baghdad. A total of 110 specimens collected in this center, that include 85 specimens for patients and 25 for control healthy. Specimens collection included sputum and blood .Tuberculosis patients were divided into four subgroups depending on result of ZN test and history of disease. Classical methods showed positive results following ZN 50(58.82%), LJ medium 81(95%) and Middle-Brook 7H9 medium 80(94%). Immunological methods showed positive results following: rapid test 40(47%) and MGIT 81(100%). 35 specimens from all specimens (85) represent for smear negative patients, that showed positive results in LJ media (100%), Middle Brook 7H9 media (94.2%) ; MGIT (100%) and rapid test (48.57%).Methods showed at culture on LJ medium better from ZN test and Middle brook 7H9 medium, high positive results for culture on LJ medium other than low in ZN test. Middle Brook 7H9 medium is rapid and easy to use but requirement of sub culture on solid media for mycobacterial identification and drug sensitivity. Finally detection of MTB by using LJ medium is time consuming, but it still the gold standard for mycobacterial detection. To compare the detection result of immunological methods with classical methods in our study.

Introduction:

Tuberculosis is a contagious and airborne disease. It is a chronic, progressive infection with a period of latency following initial infection. It is a disease of poverty affecting mostly young adults in their most productive years. The vast majority of TB deaths are in the developing world [1, 2].Nearly one third of the global populations are infected with MTB and at risk of developing the disease. More than eight million people develop active TB and about two millions die every year [3]. Diagnosis of active TB has been essentially based on sputum smear microscopy (SSM), simple and rapid, considered a cheap, SSM fails however to detect about half of active pulmonary cases since its sensitivity is compromised by low bacterial loads: only above 10000 bacilli per ml of sputum are detectable by SSM [4]. Definitive laboratory diagnosis and confirmation of sputum smear-negative pulmonary TB (SNPTB) poses a major challenge in the management and control of active pulmonary TB (PTB) in clinical practice, so loss provide enough sensitivity and specifity of TB [5]. Rapid Chromotography Immunoassay Test Device is a qualitative, solid phase, tow-site sandwich immunoassay for the detection of anti-TB antibodies in specimens. During

testing the anti-TB antibodies, if present in specimen react with the particles coated with TB recombinant antigen [6]. *Mycobacterium* Growth Identification Test Device(MGIT)is a chromatographic immunoassay for the qualitative detection of MTBC from an positive result by culture. This product detects *Mycobacterium* protein (MPT64), a *mycobacterium* protein fraction that is secreted from MTBC cell during culture [7].

Aim of study:

The comparative between classical and Immunological methods, and to assessment this teqniques to recommend any test better than others.

Materials and Methods:

Collection of Specimens

The prospective study done in Ministry of Health / Specialized Center of Chest Respiratory Disease in Baghdad. A total of 110 specimens collected in this center, that include 85 specimens for patients and 25 for control healthy which were sent to national reference laboratory of TB; with PTB enrolled in this study. Specimens collection included sputum and blood.

Diagnosis of *Mycobacterium tuberculosis*

A- Classical Methods

1- Zeihl - Neelsen Stain Examination:

The solutions ZN for direct smear microscopy were prepared according to guidelines of laboratory manual work (2009) [8], And After decontamination, smears were stained by the acid-fast staining method. according to the guidelines of laboratory manual work (2009)The slide was examined under oil immersion lens, and reported the results .

2- Culture sputum specimen on Lowenstein Jensen medium.

Lowenstein Jensen medium was prepared according to the guidelines of laboratory manual work (2009) [8]. The specimens cultivated based on Petroff decontamination method that described in laboratory manual work (2009).The procedure of culture which as follows:

1. The sputum specimen was transferred in a 50 ml screw capped centrifuge tube.
2. 4%) NaOH solution was added in a volume equal to the quantity of specimen and leave for 15 minutes.
3. The specimen was centrifuged at a speed of 3000 rpm for 15 minutes at 4 C°, refrigerated centrifugation.
4. Then the supernatant was decanted carefully into a suitable container containing a mycobactericidal disinfectant.
5. Drops of neutralizing solution were added by Pasteur pipette until the color be yellow (neutralization point).
6. Two vials of L.J medium where inoculated with 2-3 drops of the pellet and incubated in slant position and semi-cosed position for 3 days at 37C°.
7. After this period, the vials was tightly closed and incubated vertically at 37C° for 8 weeks, the results were recorded as positive or negative. The positive result means growth of *M. tuberculosis*, but negative result means no growth.

3- Culture Sputum on Middlebrook 7H9 Broth.

a. The specimens preparation according to the guidelines of laboratory manual work (2009) [8]

b. Tow drops were added from inoculum to meduim (7H9 broth medium).
c. The Inoculum was incubated at 37C° for three Weeks.
d. The results were read to meduim , positive (Turbidity) or Negative (no-Turbidity).

B- Immunological Methods:

1-Rapid Chromatography Immunoassay Test Device:

Procedure[6]:

a. Serum specimen was added to the rapid chromatography immunoassay test device by using hanging drop three drops (approximately 75µl) to fall in to the center of specimen well (s) on the test device and then start then timer.
b. Wait for the colored line (s) to appear.
c. The result should be read at 10 minutes.
d. The result was not interpret the after 30 minutes.

2- *Mycobacterium* Growth Identification Test Device (MGIT)

Procedure [7]:Result positive by culture can be tested in the MGIT device. If devices are refrigerated , they must be brought to ambient room temperature in the foil pouch prior to testing.

a. The MGIT device was remove from its foil pouch immediately before testing. The device was placed on a flat surface.
b. One device was labled for each specimen to be tested.
c. (1 ml) from D.W. in can tube was put
d. By loop colonies were taken from culture that appear result positive.
e. The growth of colonies was mixed in can tube to make mixture.
f. By pipett, (100 µl) from mixture of specimen was added in to the MGIT device well.
g. Timer was started for 15 minutes.
h. The result was read at 15 minutes and recorded test result.
i. The test was not interpreted after 60 minutes.

Stastical analysis and sensitivity.

a- Statistical analysis

The Statistical Analysis System- SAS (2012) was used to effect of different factors in study parameters. Chi-square test was used to significant compare between percentages in this study.

b- Sensitivity [9]

Sensitivity relates to the test's ability to identify a condition correctly. Sensitivity of the test is the proportion of people known to have the disease, who test positive for it. Mathematically, this can be expressed as:

Sensitivity= number of true positives/ number of true positive + number of false negative

Results:

- Distribution of patients according to results Ziehl-Neelsen smear test with category patients infected with tuberculosis.

Ziehl-Neelsen test used in the demonstration of acid-fast bacteria belonging to the genus '*Mycobacterium*', which include the causative agent for TB [10]. TB patients were divided into four subgroups; patients who were 25 new sputum smear positive, 25 old sputum smear positive, 22 new sputum smear negative and 13 old sputum smear negative depending on result of ZN test and history of disease.

All specimens that used in our study showed in table (1) that refer to results of ZN test, 50(58.82%) positive results by this test respectively in new and old sputum smear positive category other than 35(40.18%) negative result respectively in new and old sputum smear negative category. The significant differences present between sputum smear positive categories and sputum smear negative categories for TB patients in this test at probability (P≤0.01). The sensitivity for this test showed (58.8%).

Table (1):Distribution of patients according to results Ziehl-Neelsen smear test with category patients infected with tuberculosis.

Category of patient	Total	Percentage of positive (%)
New sputum smear positive	25	(29.41%)
Old sputum smear positive	25	(29.41%)
New sputum smear negative	22	(0%)
Old sputum smear negative	13	(0%)
Total no. of patient	85	(58.82 %)
Control	25	(0%)
Total no. of specimens	110	(45.45%)
Chi-square (χ^2)	---	16.500 **

** (P≤0.01).

The results showed in our study agreed with Al-Kinani in Iraq [11], and agreed with study in America by Wallis [12].The negative results of smear microscopy can be explained that the examination of sputum by ZN test requires bacillary densities of 5000-10,000 cell/ml [4]. Acid-fast staining of sputum is a rapid, inexpensive method for diagnosing TB, sputum examination for the tubercle bacilli is usually conducted for patients clinically and/radiologically suspected of PTB. However, the standard method of sputum examination, that is, ZN test is loss sensitive enough (58.8% sensitivity) and a large number of the suspected cases miss diagnosis. Moreover many cases remain unsuspected and don't seek treatment. The current practices of establishing PTB diagnosis are less sensitive enough to establish the diagnosis of AFB smear negative PTB and over treat people with no -PTB.

- Distribution results of culture Lowenstein-Jensen (LJ) in Category patients.

According to table (2) that showed distribution results of culture LJ medium in category patients, 81(95.3%) positive result by this test in all specimens category other than 4(4.7%) negative result respectively in new and old sputum smear positive category. The significant differences present between category patients with probability (P≤0.01). The significant differences present between positive results and negative results in this test with probability (P≤0.01).The result in our study agreed with study in Iraq by Shaker [13], and agreed with study by Lawn [14].

Table (2): Distribution results of culture Lowenstein-Jensen medium in category patients.

Category patients	Total	Number and Percentage for positive culture
New sputum smear positive	25	24 (28.2%)
Old sputum smear positive	25	22 (25.9%)
New sputum smear negative	22	22 (25.9%)

Old sputum smear negative	13	13 (15.3%)
Total no. of patients	85	81 (95.3%)
Control	25	0 (0%)
Total of specimen	110	81 (73.63%)
Chi-square (χ^2)	---	6.048 **

** (P≤0.01).

The study showed high percentage for positive result by this test other than low positive result in ZN test because the culture techniques have been estimated to detect as many as 10-1,000 of viable mycobacteria per ml of specimen in comparison with 5,000-10,000 bacilli/ml needed for microscopic examination [15]. For separation operation of sputum by cool centrifuge important to more concentration number of cells in specimen, increase revolution and decrease time of separation and effect (NaOH) that added to specimen through processing important to have good discarded and low contamination by (NaOH) and this agree with Perera [16]. The negative results in our study showed 4.7%, that refer to some factors such as fail of MBT to live in the culture because the patient under treatment, so the bacteria inability to grow and not active or dead.

-Distribution results of culture Middle Brook 7H9 medium in Category patients.

According to table (3) that showed the distribution results of culture Middle-Brook 7H9 medium in category patients, 80(94%) positive result by this test in all specimens category, more than 5 (6%) negative result. The significant differences between category patients in this test present with probability at (P≤0.01), and present with control healthy with probability at (P≤0.01) The result of culture on Middle-Brook 7H9 medium in our study was agreed with study Egypt by Abdel-Aziz and in Iraq by Al-Kinani [17,11].

Table (3): Distribution results of culture Middle brook 7H9 medium in category patients.

Category patients	Total	Number and Percentage for positive culture
New sputum smear positive	25	23 (27.0%)
Old sputum smear positive	25	24 (28.2%)
New sputum smear negative	22	21 (24.7%)
Old sputum smear negative	13	12 (14.1%)
Total no. of patients	85	80 (94%)
Control	25	0 (0%)
Total of specimen	110	80 (72.72%)
Chi-square (χ^2)		7.025 **

** (P≤0.01).

The detection of MTB by using conventional solid culture methods is time consuming, but it still the gold standard for mycobacterial detection without sub culturing requirement. While liquid media are used for mycobacterial recovery from specimens that it is paucibacillary [18]. So it must not be as alternative for solid media but complement and accessory and this agreement with [19]. Due to the increase in the spread of TB worldwide, the need to early diagnosis and prompt treatment have become important for the control this disease, this agree with American Thoracic Society. Another disadvantage of the liquid culture medium is the requirement of sub

culture on solid media for mycobacterial identification and drug sensitivity.

- Rapid chromatographic immunoassay test device.

The Tuberculosis Rapid Test Device (Whole Blood, Plasma, Serum) is a qualitative, two sites sandwich immunoassay for the detection of anti-TB antibodies (Isotypes IgG, IgM and IgA) in specimens of patients [6].

The test utilizes a combination of recombinant antigen to detect elevated levels of anti-TB antibodies in specimens [6].The results illustrate in table (4) representative all specimens that about our study, positive results by this test showed 40 (47%) patients. The significant different present between category patients with probability at ($P \leq 0.05$). Our study disagree with Al-Ani that showed results high simple than our study [20]. In order to overcome prolong time and diagnostic delay, combination of recombinant antigen to detect elevated levels of anti-TB antibodies in specimens tests have been introduced, negative result does not rule out TB infection because the antibodies to TB may be absent at the time the specimen is taken or may not be present in sufficient quality to be detected at early stage of infection [21].

ZN test representative more test common than other tests for diagnosis of TB in third of the world's population furthermore but no specific for diagnosis of TB [22], therefore rapid chromatography immunoassay test representative specific for MTB that caused TB, non require sophisticated tools, cheap, non give any result false positive in cases vaccinator by BCG vaccine, therefore must accentual diagnosis of TB by this test and especially in smear negative patients and patients TB in children that representative challenge cases in be taken specimens, in this cases must collect between ZN test and rapid chromatography immunoassay test and give evidence and diagnosis early for TB then treatment [23].

As a conclusion rapid test device utilizes used to detect elevated levels of anti-TB antibodies in specimens, positive results 40 (47%) that representative low results than classical tests that no specific for diagnosis of TB such as ZN test , negative result does not rule out TB infection because the antibodies to TB may be absent at the time the specimen is taken or may not be present in sufficient quality to be detected at early stage of infection. therefore rapid test representative specific for MTB that caused TB, non require sophisticated tools, cheap, therefore must accentual diagnosis of TB by this test and especially in smear negative patients and patients TB in children that representative challenge cases in be taken specimens, in this cases must collect between ZN test and rapid chromatography immunoassay test and give evidence and diagnosis early for TB then treatment.

Table (4): Distribution of results by Rapid chromatography Immunoassay test with category patients

Category patients	Total	No. of positive and percentage (%)
New sputum smear positive	25	14 (16.47%)
Old sputum smear positive	25	9 (10.5%)
New sputum smear negative	22	11 (12.9%)
Old sputum smear negative	13	6 (7%)
Total no. of patients	85	40 (47%)
Control	25	0 (0%)
Total no. of specimen	110	40 (36.36%)
Chi-square (χ^2)	---	5.017 *

* ($P \leq 0.05$)

- Distribution of *Mycobacterium* Growth Identification Test with category patients.

Mycobacterium tuberculosis and non- *Mycobacterium tuberculosis* are clinically different; so prompt detection, isolation and discrimination are essential for appropriate management. The MPT 64 protein is highly specific for *MTBC*, including *M. tuberculosis, M. africanum and M. bovis* [24].

From results about our study that illustrate in table (5) for this test showed rise positive results in all category as 81(100%) patients. The significance difference present between all category patients with probability at ($P \leq 0.01$).

The results our study agreed with previous study by other researches in Iraq [13], and agreed with previous study in America [25]. The test depended on the qualitative detection of MTBC from a positive result by culture on LJ. This product detects mycobacterium protein (MPT64), a protein fraction that is secreted from MTBC cell during culture. When positive result showed in the test , MPT64 antigen binds to anti-MPT64 antibodies conjugated to visualizing particles on the test strip [7].

Table (5): Distribution results of *Mycobacterium* Growth Identification Test with category patients

Category patients	Total	No. of positive and percentage (%)
New sputum smear positive	24	24 (29.6%)
Old sputum smear positive	22	22 (27.2%)
New sputum smear negative	22	22 (27.2%)
Old sputum smear positive	13	13 (16%)
Total no. of specimen	81	81 (100%)
Chi-square (χ^2)	---	6.048 **

** ($P \leq 0.01$).

A Conclusion, the test showed high sensitivity (100%). The test is also good enough to confirm the tuberculosis bacteria or not when the mycobacteria specimen is cultured. The kit showed detection limit of bacteria 10^{-5} CFU/ml, therefore it is good enough to confirm the tuberculosis bacteria using colonies of solid culture media that is identified by naked eye, and the test is for confirming the result in 15 minutes without any equipment.

Comparative positive results by different tests with category patients.

Table (6) includes tests that used in our study, that include classical methods and immunological methods . Positive results for tests with percentage was ZN 50(58.8%), LJ culture 81(95.3%), 7H9 Broth culture 80(94%), MGIT 81(100%) and Rapid chromatography 40(47%) . The sensitivity for diagnosis PTB of tests in our study was (ZN 58.8%, LJ culture 95.3%, 7H9 Broth culture 94%, MGIT 100% and Rapid chromatography 47%).The significant differences between patients new sputum smear positive category and results of tests present with probability at ($P \leq 0.01$), the significant differences between patients old sputum smear positive category and results of tests present with probability at ($P \leq 0.01$), the significant differences between patients new sputum smear negative category and results of tests present with probability at ($P \leq 0.01$) and the significant differences between patients old sputum smear negative category and results of tests present with probability at ($P \leq 0.01$).The classical methods in our study showed at culture on LJ medium better from ZN test and culture on Middle brook 7H9 medium, with high percentage for positive results by culture LJ medium other than low positive results in ZN test because the culture techniques have been estimated to detect as many as 10-1000 of viable mycobacteria per ml of specimen in comparison with 5,000-10,000 bacilli/ml

needed for microscopic examination [15]. The negative results of smear microscopy can be explained that the examination of sputum by ZN test requires bacillary densities of 5000-10000 cell/ml, that could be due to fact that TB patients were taken medication which decrease the expectoration of TB bacilli in sputum, and this were going with Lim [26]. However, the ZN test is less sensitive. Moreover many cases remain unsuspected and don't seek treatment [27]. The culture in liquid medium is now being used as a rapid diagnostic method for TB in many countries, this method is easy to use [28]. The detection of MTB by using LJ culture method is time consuming, but it still the gold standard for mycobacterial detection without sub culturing requirement. While liquid media are used for mycobacterial recovery from specimens that it is paucibacillary [1,18]. Due to the increase in the spread of TB worldwide, the need to early diagnosis and prompt treatment have become important for the control this disease. So it must not be as alternative for LJ media but complement and accessory[19]. Another disadvantage of the Middle Brook medium is the requirement of sub culture on LJ medium for mycobacterial identification and drug sensitivity [29]. So that were also shown in the results of our study which presented that ZN staining were less detection rate than culture LJ, because of high percentage of negative results (41.2%), that could be due to fact that TB patients were taken medication which decrease the expectoration of TB bacilli in sputum. Less positivity of ZN staining in our study were going with Diacon [27].

The MGIT device From results about our study for this test showed rise positive results in all category as 81(100%) patients. A Conclusion the test showed high sensitivity (100%), and test is also good enough to confirm the tuberculosis bacteria or not when the mycobacteria specimen is cultured. The kit showed detection limit of bacteria 10^{-5} CFU/ml, therefore it is good enough to confirm the tuberculosis bacteria using colonies of solid culture media that is identified by naked eye. The test is for confirming the result in 15 minutes without any equipment. If it is introduced in the clinical microbiology, therefore regarded rapid test.

Table (6): Master table positive for different tests with category patients

Category patients	Total	Z.N. positive result	L.J. positive result	7H9 book positive result	Rapid test positive	MGIT Result positive	Chi-square (χ^2)
New sputum smear positive	25	25 (29.4%)	24 (28.2%)	23 (27.0%)	14 (16.47%)	24 (28.2%)	9.45 **
Old sputum smear positive	25	25 (29.4%)	22 (25.9%)	24 (28.2%)	9 (10.5%)	22 (25.88%)	8.38 **
New sputum smear negative	22	0 (0%)	22 (25.9%)	21 (24.7%)	11 (12.9%)	22 (25.88%)	9.52 **
Old sputum smear negative	13	0 (0%)	13 (15.3)	12 (14.1%)	6 (7%)	13 (15.3%)	6.84 **
No. of patients	85	50 (58.8%)	81 (95%)	80 (94%)	40 (47%)	81 (95.3%)	10.27 **
Control	25	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	NS
Total	110	50 (45.45%)	81 (73.63%)	80 (72.72%)	40 (36.36%)	81 (73.63%)	9.61 **
Chi-square (χ^2)		16.500 **	6.048 **	7.025 **	5.017 *		---
Sensitivity		(58.8%)	(95%)	(94%)	(47%)	(95.3%)	

* ($P \leq 0.05$), ** ($P \leq 0.01$)

To compare the detection result of immunological methods with classical methods in

our study. ZN test representative more test common than other tests for diagnosis of TB, while most of national tuberculosis program in the world's non specific for diagnosis of TB [22]. Therefore rapid chromatography immunoassay test representative specific for MTB that caused TB, it's simple, cheap, rapid than culture medium and not give any false positive in results vaccinator by BCG vaccine, therefore the diagnosis of TB by this test especially in smear negative patients and in children specimens [23].

Our study showed finally, LJ medium consider gold standard for diagnosis TB other than tests.

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