

**ORIGINAL ARTICLE****Study comparison of diagnostic methods for Tuberculosis Patients in Iraq****<sup>1</sup>Mohammed Abdul- Imam Almazini, <sup>2</sup>Ahmed Asmer Mankhi, <sup>3</sup>Ekhlas Atia Khalaf Al-Kinani**<sup>1</sup>College of Science-Biology Department-Basrah University-Iraq<sup>2</sup>Center for Chest and Respiratory Disease-Baghdad-Iraq<sup>3</sup>Altalemy Hospital-Missan-Iraq**ABSTRACT**

The study included one hundred –sixty nine (169) samples of patients that attended to Specialized Chest and Respiratory Disease Center/ National Reference Laboratory for Tuberculosis (NRL) in Baghdad. In the period from 1/10/2012-1/8/2013. These samples included (147) sputum samples of pulmonary TB patients and (22) Extra-pulmonary samples. Most of the subjected cases(169)in the study had belonged to the age group(25-34) years with percentage (28.99%), while the lowest recorded age group was observed in (5-14) years with percentage (0.59%), without statistically significant ( $p=0.9222$ ,  $p>0.05$ ). The pulmonary TB samples included (112) samples that belonged to re-treated patients (old case), and (35) samples belonged to new patients. Eighty-eight (88) samples (66 of sputum with 22 Extra-pulmonary TB samples) had been comparatively investigated by different laboratory diagnostic methods, after cultivation on solid media by using (Lowenstein- Jensen media), sputum isolates were taken only from positive cultured on L-J samples as standard and to complete other identifications on isolates; First step was the staining using (Zhiel-Nelsen method), In addition to cultivation in liquid media Middle Brook 7H9 in MGIT tube of advanced technology in liquid culture (Bactec MGT 960 instrument) and by using molecular assay (Gene Xpert MTB/RIF) that achieved the highest percentage of detection rate which were (97.14%) in pulmonary TB samples and (90.91%) in Extra-pulmonary TB samples, while the lowest detection rate were by using staining (53.03%) in pulmonary TB samples and (9.09%) in Extra-pulmonary TB samples. The Bactec MGIT 960 detection rate in pulmonary TB (86.36%) were also higher than Extra-pulmonary (50%).Statistically there were significant differences among the results in pulmonary samples ( $p$ -value=0.000002671, $p<0.05$ ). There were only six samples among 22 Extra-pulmonary samples that showed growth on solid media (L-J) media (27.27%) , and no one exposed growth on(Stone-brink) pyruvate media. For identification and typing of acid fast bacilli, six biochemical typing tests were used in the study ; growth on stone-brink medium .growth on Para-nitro benzoic acid medium (PNB), growth on Thiophene -2-Carboxyl acid hydrazine (TCH)medium, Catalase test, Niacin test, and Nitrate reduction. These tests showed the high percentage of strains belongs to *M.tuberculosis* (90.90%) , and (9.09%) represent the percentage of non agreed results with *M.tuberculosis*.

Key words: comparison, diagnostic methods , tuberculosis , Iraq .

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**INTRODUCTION**

Tuberculosis is an infectious disease caused by various strains of mycobacteria , usually *Mycobacterium tuberculosis* [23]. Approximately 85% of reported TB cases were limited to the lungs . The remaining 15% involved only non pulmonary (extra-pulmonary) or both pulmonary and non-pulmonary sites [16]. Increase in the incidence of TB in the developing countries and its re-emergence in the developed world led the World Health Organization (WHO) to declare TB as a global emergency in 1993 (48), and took a high consideration of this disease [47]. There is an increasing rate of drug resistance strains toward the drugs used for curing of TB in large sectors of risk groups. The problem of emerging multi-drug resistant TB (MDR-TB) is global worldwide [9], especially in crowded places, homeless, and immune compromised patients. Community-based treatment programs such as DOTS-Plus, a MDR-TB-specialized treatment using the popular Directly Observed Therapy –Short Course (DOTS) initiative, have shown considerable success in the treatment of MDR-TB in some parts of the world [36].

**Epidemiology of Tuberculosis:** It is estimated that one third of the world's population (approximately 2 billion people) are infected with tubercle bacilli [52]. The global tuberculosis caseload appears to be growing slowly. Nearly two million persons die from TB each year, [8 ; 40] . The distribution of tuberculosis is not uniformed across the world; about 80% of the population in many Asian and African countries test positive in tuberculin tests, while only 5–10% of the U.S.A population tests positive [23].

#### **Tuberculosis in Iraq :**

In the 1950's tuberculosis was widespread among the Iraqi people. At the time 3400-6800 cases of pulmonary tuberculosis and 1400-3100 cases of non-pulmonary tuberculosis were recorded yearly.(38). In the course of the last decade there has been a three-time growth in the incidence of tuberculosis (from 46.1 in 1989 to 131.6 in 2000 for every hundred thousand citizens). New cases 130/100000/year, with about 273000 new case/year, among which 12600 new smear positive pulmonary tuberculosis (PTB)(49). Iraq is located eight in (EMRO) rank according to incidence in 2011,and there were an estimated 15000 incident cases of the all new and relapse cases of TB in Iraq. On other hand ,the incidence of TB in Basrah had been 58.1% from 136 cases in 2001(Rodeen, 2001) , 58.6% from 232 cases in 2004 [2], and 63.8% in 2007[3].

#### **Diagnosis of Tuberculosis:**

**Chest X-ray:**The chest X-ray examination may help to make the diagnosis in respiratory symptomatic patients that are repeatedly negative on direct microscopy sputum examination. It may also help in those individuals that cannot produce sputum for the bacteriological examination [6] .

**Conventional Laboratory Tests :** Conventional methods of diagnosing *M.tuberculosis* are slow because they rely on bacterial growth in culture and on conventional plate methods for identifying species. [14; 42]. Acid fast bacilli (AFB) smear microscopy and culture on Löwenstein-Jensen medium are still the “gold standards” for the diagnosis of active TB, especially in low-resource countries, the only methods available for confirming TB in patients with a clinical presumption of active disease. [21].

#### **Culture in Liquid Media :**

**BACTEC Systems :** In resource poor countries, direct AFB microscopy and Lowenstein Jensen (L-J) media are still the main modalities used for the diagnosis/screening of tuberculosis, but these methods have low sensitivity which also depends upon the number of tubercle bacilli present in the specimen (10), due to lack of diagnostic facilities (The broth based BACTEC 460). Other systems for culture on liquid media, developed to avoid the problem with radioactive disposal, such as MB/BacT, and Mycobacteria Growth Indicator Tube among others [14]. Liquid culture requires only 10 – 100 bacilli per millimeter of sputum, whereas AFB microscopic detection requires 5000-10000 bacilli per Millimeter [41] .

The fully automated, high capacity BACTEC MGIT 960 system is now being used as a rapid diagnostic system for tuberculosis in many developed countries. This system is easy to use, fully automated, non-invasive, non radiometric with high performance. It has an oxygen sensitive fluorescent sensor embedded in silicon base which serves as an indicator of mycobacterial growth [43].

#### **Advanced in Laboratory Diagnosis**

**Gene Xpert (MTB/RIF):**This may represent the biggest advance in TB diagnostics in past decades, as it allows the simultaneous detection of *M. tuberculosis* and (Rifampicin= RIF) resistance in one assay; It requires minimum handling and training, and yields results within 2 hour [44]. This test can diagnose PTB and resistance to rifampicin .In contrast to other techniques (in vitro culture ,Drug susceptibility test and conventional molecular techniques ) the xpert MTB/RIF can be used in peripheral laboratories and does not require sophisticated equipment or highly skill personnel [52]. The assay can be carried out in nearly fully automated manner , including bacterial lysis, nucleic acid extraction and amplification , and amplicon detection. The test runs on the Gene Xpert platform using a disposable plastic cartridge with all required reagents [33]).

#### **Aim of the study :**

**1-Comparing the results of Bactec MGIT 960 TB system and the Gene Xpert assay in the diagnosis and testing drug susceptibility of tuberculosis isolates by comparing the results with conventional diagnostic methods, in pulmonary and extra-pulmonary TB isolates.**

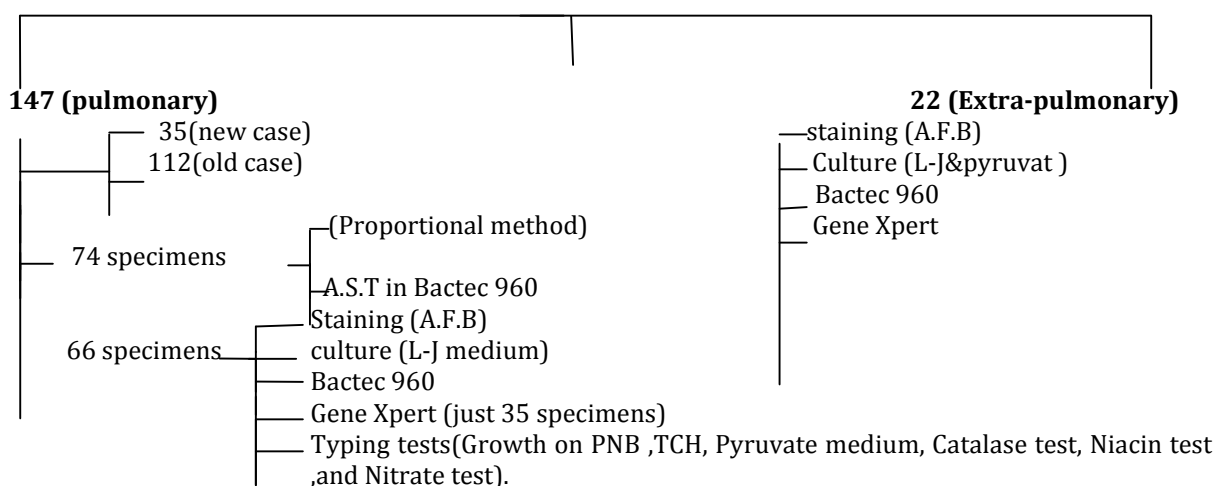
#### **MATERIALS AND METHODS**

❖ The study was implemented at specialized center for chest and respiratory diseases in National Reference Laboratory for Tuberculosis (NRL) in Baghdad. During the period from (1/10/2012...1/8/2013). The samples were selected from (one hundred sixty-nine )TB patients that attended to the center which were , including pulmonary and extra pulmonary TB diseases. Their ages were between (few months-70 year).

- ❖ sputum was collected from patient according to (National Tuberculosis Program in Iraq) three samples were collected from each patient. First one was taken from patient when he just reached the center; second sample collected at early morning before breakfast; and third one was collected at any other time on the day.
- ❖ The samples were divided into two group: pulmonary TB (147) samples and (22) samples extra – pulmonary TB. The pulmonary TB patients (sputum ) divided according to treatment categories into: new cases which were (thirty-five patients) and re-treatment cases (old case/ one hundred twelve patients). The old TB patients divided into four categories (chronic, failure, relapse, defaulter).
- ❖ On the other hand , the extra – pulmonary TB samples were taken included (urine, pleural fluid, fine needle aspiration, gastric fluid, ascitic fluid, lymph node aspirate, epidural abscess).
- ❖ Sixty-six sputum samples and twenty-two of extra-pulmonary were comparatively investigated, first with conventional methods (Ziehl-Nelsen staining and culture on solid media), the results of direct examination appear within less one hour , and on solid culture ( Lowenstein – Jensen media) need (6-8 weeks). Then just positive cultured isolates were taken as standard and to complete other identifications. in addition to the culture in liquid media by using new technology named (Bactec MIGHT 960 system) their result need (1-3 weeks) to appear. and molecular – base (Gene Xpert) assay system ,their results need two hours to appear.
- ❖ In addition, all these specimens were submitted to the identification and typing of bacilli tests such as growth on stone brink medium ,Growth on P-nitro benzoic acid medium (PNB), Growth on Thiophene -2- Carboxyl acid hydrazine (TCH) medium ,Catalase test ,Niacin test, and Nitrate reduction.

**169 (Total specimens)**

All subjected to drug susceptibility test depending on (proportional method)



**(Diagram of the study )**

**Laboratory Investigation :**

**Direct Examination (Ziehl- Neelsen stain):** [21].

**Preparation of Culture Media (50) :**

**Preparation of Lowenstein – Jensen media:**

**Preparation of Sputum for Culture on (L-J media) and Stonebrink Media, (Petroff decontamination procedure). (18).**

- 1- An equal volume to sputum specimen of (4%) sodium hydroxide solution were added to about 4 ml of sputum in appropriate container.
- 2-Homogenizing; by shaking on a shaker to decontaminate for 15 minutes at 37°C if it possible, then let it in cold centrifuge for 15 minutes, on the whole, a relative centrifugal force of about (3000) rpm appears to be adequate to precipitate mycobacteria.
- 3-Pour off the supernatant fluid from the Pellet sediment, and the sediment neutralized by using a drop of (1 N) hydrochloric acid with phenol red (2%), gradually until being converted to yellow (neutralizing point).
- 4-Inoculate two slant of L-J media, each with 2-3 large drop of the Pellet in the above step, by using a pasteur pipette.
- 5-The inoculated media were incubated in slant position for 2-4 days to distribute the pellet sediment

over the media without close the cover completely . After this period, slant media should be in straight position to complete incubation of (6-8) weeks at (35-37C°) with tightly closing cover.

**Bactec MGIT 960 Kit:**

Bactec MGIT 960 system consists of the following, according to [37] :

**A- MGIT medium : (Mycobacteria Grow Indicator Tube)**

The MGIT 960 tube contains 7.0 ml of modified 7H9 broth base. The approximate formula, per1000 ml of purified water, contains:

- 1- Modified Middlebrook 7H9 broth base.... 5.9 gm
- 2- Casein peptone..... 1.25g

**B- Bactec Growth Supplement:**

MGIT growth supplement contains 15 ml of the following Approximate formula/ litter purified water:

- Bovoin albumin.....50 gm
- Dexrose.....20 gm
- Polyoxyethylene Streate(POES).....1.1gm
- Catalase.....0.03gm
- Oleic acid.....0.1gm

**C- MGIT PANTA Vial:**

Each vial of MGIT PANTA (for MGIT 960) contains a lyophilized mixture of the antimicrobials with the concentrations, at the time of production, as follows:

- Polymyxin B .....6,000 units
- Amphotericin B ..... 600 µg
- Nalidixic Acid .....2,400 µg
- Trimethoprim ..... 600 µg
- Azlocillin ..... 600 µg

Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement.

Mix until completely dissolved. Add 0.8 ml of this enrichment to each MGIT tube.

**Mycoprep specimen digestion and decontamination kit.[37]:**

**Mycoperp kit:(Test kit No.8809541).**

Mycoperp specimen digestion/ decontamination kit compose of the following:

**1- NALC – NaoH solution (Mycoprep reagent):**

- NaoH ----- 20gm
- Trisodium citrate ----- 14.5 gm

Each sealed ampoule within the bottle contain 0.57 gm NaCl (C<sub>5</sub>H<sub>9</sub>No<sub>3</sub>S).

**2- Mycoprep phosphate buffer:**

Packages of phosphate buffer PH 6.8, approximate formula / 500ml purified water:

- Disodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>) ----- 2.34gm.
- Mono – potassium hydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) ----- 2.27gm.

One package were mixed with 500 ml distilled water and sterilized in autoclave.

**Processing of samples :**

Equal amount of clinical specimens and mycoprep reagent (about 10ml of each) were added in falcon tube, and were mixed by vortex mixer for few seconds, then left at room temperature for 15 minutes. Phosphate buffer was added to continue the mixture to 50 ml, and then the mixture was centrifuged for 15-20 min at 3000 rpm. The sediment was used for culture and direct exam .

**A- Inoculation of MGIT medium :**

- Label MGIT tubes with specimen number.
- Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube.
- Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well mixed processed/concentrated specimen as mentioned in(2.4.2) to the appropriately labeled MGIT tube.
- Immediately re-cap the tube tightly and mix by inverting the tube several times.
- Wipe tubes and caps with a mycobactericidal disinfectant and leave inoculated tubes at room temperature for 30 minutes.
- Work under the biologic safety cabinet for the specimen inoculation.

**B- Incubation**

**Incubation Temperature:** All inoculated MGIT (7mL) tubes should be entered in the Bactec MGIT 960 Instrument after scanning each tube It is important to keep the cap tightly closed and not to shake the tube during the incubation. The instrument maintains 37°C + 1°C temperature. MGIT tubes should be incubated until the instrument flags them positive.After a maximum of six (6) weeks, the instrument

flags the tubes negative if there is no growth.

### **Preparation of Bacillary Suspension from Active Growing Culture ( 11):**

- Put (1-2) drops of sterile distilled water into screw-capped homogenizer (14ml screw-capped tube containing several glass beads).
- Take one loopful of growth, transfer to the homogenizer.
- Homogenize on vortex mixer for few min, then add (7ml) of sterile distilled water, allow large particle to settle.
- Transfer supernatant suspension to sterile tubes and adjust density to that of McFarland (No.1)(0.1BaCl<sub>2</sub>+9.9H<sub>2</sub>SO<sub>4</sub>) with sterile distilled water. The suspension was used for biochemical and susceptibility test.

### **Biochemical Typing Tests :**

It is desirable to identify *Mycobacterium tuberculosis* complex prior to processing further tests such as drug susceptibility test. There were several tests done for identification and typing of TB bacilli such as:

1. Growth on pyruvate (stone brink) media.
2. Growth on P.N.B ( P- nitro benzoic acid) media.
3. Growth on T.C.H ( thiophene-2- carboxyl acid Hydrazide) media.
4. Niacin test.
5. Nitrate reduction test.
6. Catalase test.

### **Stone Brink Medium:**

**Preparation of Pyruvate (Stone- Brink) Medium:** [13].

### **Growth Inhibitors :**

**Growth on medium containing Para-nitro benzoic acid :**

#### **Preparation:**

Weigh 0.5 gm PNB and dissolve in the dimethylformamide ( ~15ml).

Add to 1 liter of L-J fluid, distribute and inspissate once for 50 minutes at 85 degrees. Store in cold room. Inoculate The bacterial suspension as prepared in item(2.4.3) on one slope of L-J medium and one slope of p-nitro benzoic acid (PNB) at a concentration of 500 µg/ml and incubate at 37°C for each set. Read on 28th day. PNB should not be kept for reading at 42 day. It is critical to inoculate with neat suspension prepared for DST and reading should be only on 28th day [45].

#### **Growth on Medium Containing Thiophene-2- Carboxylic Acid Hydrazine :**

Prepare 0.2 gm/ml Thiophene carboxylic acid hydrazide (TCH) (weight 2gm of TCH in 20 ml sterile distilled water). Transfer 0.5 ml of this solution to a tube with 4.5 ml of distilled water .Then repeatedly add 0.5 of these solution to 4.5 D.W. Add 4.8 ml from final solution to 240 ml of LJ medium. Distribute in tubes and coagulate with inclination, at 80°C for 45 minutes.

#### **Procedure :**

0.1-0.2 ml of Mycobacterial suspension was prepared as in (2.4.3) were inoculated on two L-J slopes. One L-J slope contain 2 µg/ml TCH (prepared in 2.5.2.1) and another slope without TCH. Both slopes were incubated at 37 °C for 2-3 weeks. The suspected organism were recorded resistant to TCH when the growth on TCH containing media was equal to or greater than 1% of that which was observed on the TCH free L-J media (43).

**Heat labile catalase test (at pH 7.0 and 68°C) :** [34].

**Niacin paper strip test ( 46):**

**Nitrate reduction test:** (46)

**2.6- Gene Xpert MTB/RIF assay system procedure:** (Test kit No.71001696).

#### **Processing of Samples for Xpert procedure:**

1. The pulmonary samples (sputum )were processed as in (2.3.2.2.).
2. The extra-pulmonary samples were processed as in (2.4.2).

#### **Gene Xpert Procedure:**

As documented by (17).

- ◆Labeling each Xpert MTB/RIF cartridge with the sample ID .
- ◆Transferring at least 0.5 mL of the processed sample pellet (2.6.1) to a conical,screw-capped tube for Xpert MTB/RIF using sterile transfer pipette.
- ◆Storing re-suspended sediments at 2–8 °C if they are not immediately processed for Xpert MTB/RIF.
- ◆Adding 1.5 mL of Xpert MTB/RIF Sample Reagent (SR) to 0.5 mL of resuspended sediment sample using a sterile transfer pipette and shake vigorously 10 – 20 times.
- ◆Incubating the specimen for 15 minutes at room temperature, at one point between 5 and 10 minutes

of the incubation, shaking the specimen vigorously again for 10 – 20 times. then put 2ml of these specimen into the cartridge. The computer was turned on ,and then the Gene Xpert instrument was turn on. The Gene Xpert shortcut icon was double-clicked. The Gene Xpert System software was logged on using the user name and password.

◆Create Test icon was clicked. The Scan Cartridge Barcode dialog box appears. The barcode on the Xpert MTB/RIF cartridge was scanned. Start Test icon was clicked. The cartridge was loaded. Left until the system releases the door lock at the end of the run.



Fig (2-2) Gene Xpert Apparatus

Fig.(2-3) Cartridge of Gene Xpert on right, and the reagent on the left.

### Statistical Analysis

All data were analyzed statistically using SPSS 17 (Statistical Package for Social Science), with significant level ( $p < 0.05$ ).

### RESULTS

Frequency of tuberculosis cases in Baghdad and other governorates.

**Table ( 1 ) :** The percentages of distribution of TB disease in Baghdad and other governorates.

| <i>Governorate</i> | <i>Total No.of patients</i> | <i>No.of resistant patients</i> | <i>Total No.of extra-pulmonary</i> |
|--------------------|-----------------------------|---------------------------------|------------------------------------|
| Baghdad            | 87(51.47%)                  | 56                              | 17                                 |
| Basrah             | 12(7.1%)                    | 11                              | -                                  |
| Diala              | 13(7.69%)                   | 10                              | 2                                  |
| Salah aldeen       | 9(5.32%)                    | 8                               | -                                  |
| Deqar              | 7(4.14%)                    | 7                               | -                                  |
| Kut                | 7(4.14%)                    | 7                               | -                                  |
| Mousil             | 6(3.55%)                    | 5                               | -                                  |
| Karbalaa           | 6(3.55%)                    | 6                               | -                                  |
| Najaf              | 5(2.95%)                    | 5                               | -                                  |
| Arbil              | 4(2.36%)                    | 4                               | -                                  |
| Anbar              | 4(2.36%)                    | 2                               | 1                                  |
| Qadisiyah          | 2(1.18%)                    | 1                               | -                                  |
| Sulymaniah         | 1(0.59%)                    | 1                               | -                                  |

Relationship of Tuberculosis with Age and Gender .

**Table (2):** The distribution of pulmonary and extra-pulmonary TB isolates according to age, and gender.

| <b>Age group</b> | <b>Male</b> |          | <b>Female</b> |          | <b>Total</b> |
|------------------|-------------|----------|---------------|----------|--------------|
|                  | <b>NO.</b>  | <b>%</b> | <b>NO.</b>    | <b>%</b> |              |
| <b>0-5</b>       | 1           | 0.90     | 1             | 1.72     | 2(1.18%)     |
| <b>5-14</b>      | 1           | 0.90     | 0             | 0        | 1(0.59%)     |
| <b>15-24</b>     | 19          | 17.12    | 10            | 17.24    | 29(17.16%)   |
| <b>25-34</b>     | 30          | 27.03    | 19            | 32.76    | 49(28.99%)   |
| <b>35-44</b>     | 22          | 19.82    | 13            | 22.42    | 35(20.72%)   |
| <b>45-54</b>     | 21          | 18.92    | 8             | 13.79    | 29(17.16%)   |
| <b>≥ 55</b>      | 17          | 15.31    | 7             | 12.07    | 24(14.20%)   |
| <b>Total</b>     | 111         | 100      | 58            | 100      | 169(100%)    |

Fisher exact test 2.46, P-value=0.922, P-value>0.05.

Diagnosis of selected isolates of pulmonary TB patients.

**Table (3):** Frequency distribution of pulmonary TB patients (old cases and new cases) according to the laboratory diagnostic methods .

| Lab Methods  | Old case     |              | New case     |             | Total No. | Total percentage |              |
|--------------|--------------|--------------|--------------|-------------|-----------|------------------|--------------|
|              | +ve          | -ve          | +ve          | -ve         |           | +ve              | -ve          |
| Z-N staining | 26<br>47.27% | 29<br>52.72% | 9<br>81.18%  | 2<br>18.18% | 66        | 35<br>53.03%     | 31<br>46.97% |
| Bactec 960   | 47<br>85.45% | 8<br>14.54%  | 10<br>90.90% | 1<br>9.09%  | 66        | 57<br>(86.36%)   | 9<br>13.64%  |
| Gene Xpert   | 28<br>100%   | 0<br>0%      | 6<br>85.71%  | 1<br>14.82% | 35        | 34<br>97.14%     | 1<br>2.86%   |

Chi Square=25.67, Degrees of Freedom=2 ,p-value=0.00002671

Sensitivity of Z-N staining= 53%,Sensitivity of Bactec 960= 57%,Sensitivity of Gene Xpert= 97%

Diagnosis of selected TB isolates of pulmonary and extra-pulmonary patients.

**Table (4) :** Numbers and percentages of negative results of Z-N staining , (solid , liquid) culture and Gene Xpert of positive pulmonary and extra-pulmonary TB patients.

| Type of TB         | No. of samples | Z-N staining (-ve) | No. of samples | Bactec 960 (-ve) | No. of samples | Gene Xpert (-ve)      | Culture on L-J medium |
|--------------------|----------------|--------------------|----------------|------------------|----------------|-----------------------|-----------------------|
| Pulmonary TB       | 66             | 31<br>46.96%       | 66             | 9<br>13.64%      | 35             | 1<br>2.86%            | +ve                   |
| Extra-pulmonary TB | No. of samples | Z-N staining (-ve) | No. of samples | Bactec 960 (-ve) | No. of samples | Culture on L-J medium | Gene Xpert (+ve)      |
|                    | 22             | 20<br>90.9%        | 22             | 11<br>50%        | 22             | 16<br>72.72%          | +ve                   |

Biochemical typing tests.

**Table(5):**Results of the Biochemical typing tests of the pulmonary TB specimens.

| Tests                        | <i>Mycobacterium tuberculosis</i>         |   |
|------------------------------|---|---|
|                              | Agreed results with <i>M.tuberculosis</i> | Non agreed results with <i>M.tuberculosis</i> |
| Niacin test                  | 66(+ve)<br>(100%)                         | 0(-ve)<br>(0%)                                |
| Catalase(68°C) test          | 64(-ve)<br>(96.97%)                       | 2 (+ve)<br>(3.03%)                            |
| Nitrate reduction test       | 66(+ve)<br>(100%)                         | 0(-ve)<br>(0%)                                |
| Resistance to TCH            | 65(+ve)<br>(98.48%)                       | 1(-ve)<br>(1.52%)                             |
| Sensitivity to PNB           | 65(-ve)<br>(98.48%)                       | 1(+ve)<br>(1.52%)                             |
| Growth on stone-brink medium | 34(+ve)<br>(51.52%)                       | 32(-ve)<br>(48.48%)                           |
| Percentage                   | (90.90%)                                  | (9.09%)                                       |

\*Identification of *Mycobacterium tuberculosis* complex:-

| Pathogen               | Niacin | Nitrate | Catalase 68C° | TCH | PNB | Pyruvate |
|------------------------|--------|---------|---------------|-----|-----|----------|
| <i>M. tuberculosis</i> | +      | +       | -             | R   | S   | -        |
| <i>M. africanum</i>    | +/-    | +/-     | -             | R   | S   | -        |
| <i>M. bovis</i>        | -      | -       | -             | S   | S   | +        |
| <i>BCG</i>             | -      | -       | -             | S   | S   | +        |
| <i>MOTT</i>            | -      | +/-     | +             | R   | R   | -        |

## DISCUSSION

### Frequency of tuberculosis cases in Baghdad and other governorates.

The majority of TB cases were more from Baghdad city with percentage (51.47%) than other governorates at percentage (48.53%) as shown in Table (1), so the high percentage of TB disease were from Baghdad may be related to the high density of population associated with crowded conditions, and because of the availability of more diagnostic methods facilities than other governorates that enable to record more TB cases among suspected TB patients, with difficulties for patients to reach Baghdad. The high percentages of resistant cases among little of governorates such as (Basrah, Diala, Salahaldeen) may be probably, these are large cities with crowded populations than other governorates, may increased the possibility to develop TB to higher level of drug resistance.

### Diagnosis of selected isolates of pulmonary TB patients.

Among one hundred forty-seven of sputum specimens referred to pulmonary TB cases, sixty-six sputum specimens were subjected to conventional tests as

well as the beneficial advantaged technology in cultivation (Bactec MGIT 960 system) and new molecular assay (Gene Xpert) as a comparative investigation, first they were subjected to direct smear microscopy (Z-N staining) for diagnosis of TB bacilli, most cases of TB can be correctly diagnosed by this simple, cheap, and rapid methods that giving result within one hour, which

are generally available at peripheral consultant TB clinic. Although direct smear microscopy and Lowenstein-Jensen (L-J) culture remain the cornerstone of the diagnosis of TB, but these traditional bacteriological methods possess several disadvantages, they are either slow or their sensitivity is quite low, especially with clinical samples that contain small number of organisms [30].

Conventional microscopy is the most commonly used technique in the routine diagnosis of TB. However, sensitivity of sputum Ziehl-Neelsen (Z-N) staining does not exceed 60% to 70% as compared with sputum culture according to report of [22, 27]. A delay in the diagnosis together with misdiagnosed TB cases contributes to *Mycobacterium tuberculosis* (Mtb) transmission and mortality [28], so that were also shown in the results of this study in Table (3) which presented that Z-N staining were not reliable because of high percentage of negative results (46.97%), where as the positive rate were (53.03%) with sensitivity rate 53%, these percentages were high especially with the new TB patients (81.81%) than re-treated patients (old case) (47.27%), who found the less positivity detection rate were by using Z-N staining (44%) in comparison to other diagnostic methods such as solid media (70%), and with (35), (28% of Z-N staining) compared with (39%) of solid media.

According to culture on solid media by using the egg-based media, such as Lowenstein-Jensen (L-J) medium, have been used for cultivation of Mycobacteria for several decades. Solid media (L-J) is most popular media in clinical laboratories, allows the determination of characteristic features of colony morphology, and pigment production but it takes very long time (6-8) weeks. In 1958, Middlebrook and Cohn described an agar-based medium to permit more rapid detection of mycobacterial growth [15] However, it still required an average of 3-4 weeks to recover mycobacteria from clinical specimen, two hundred sputum specimens in this study were cultured on L-J media, only 66 sputum specimens showed growth on L-J media, most of them were rough, tough and creamy colony, within approximately 4-5 weeks, and these positive cultured were taken as standard to complete other identifications and to observe the true positive/false negative results of other laboratory diagnostic methods. Culture techniques have been estimated to detect as many as 10-1,000 of viable mycobacteria per ml of specimen in comparison with 5000-10,000 bacilli/ml needed for microscopic examination [7]. So it capable to detect fewer bacilli in the same specimens, even in small quantities of sputum.

This system is easy to use, fully automated, non-invasive, and non radiometric with high performance. [43]. Due to the increase in the spread of tuberculosis worldwide, the needs to early diagnosis and prompt treatment have become important for the control of this disease [4]. The advancement in development of new diagnostic methods is an important element of the 'Global Plan to Stop TB' and the WHO new global "Stop TB Strategy" (31), such as Bactec MGIT 960 system which used in present study, that showed higher positive result than AFB staining at percentage (86.36%) while the negative result were (13.64%) with sensitivity 57% as shown in Table (3), and Bactec 960 cannot detected bacilli in all positive cultured specimens, this disagreed with [25] who found higher recovery rate of Bactec 960 (100%) in compared with L-J media (82%), a report of (1) stated similar findings (89.5%) of Bactec 960 and (74%) for solid media, and [5] declared that the Bactec MGIT 960 system detected (94%) than L-J media (75.8%). It is found in this study that, the Bactec 960 in combined with conventional solid medium increases the overall recovery of mycobacteria in culture, and can expedite the recovery of TB bacilli in culture. So, there were no clear answers for these difference between cultivation in liquid and solid media results, expected reasons for lower positive rate of Bactec 960 than L-J medium could be explained due to inoculum size number of bacterial population within inoculum



as this technique need 10-100 bacilli per ml of specimens, and technical experience. It was found that the Bactec 960 can be used as reliable method especially in combination with culture on L-J medium, and the non-radiometric, fully automated, Bactec MGIT 960 system is rapid in terms of recovery of mycobacteria, and time to detection of TB bacilli as compared with conventional L-J medium. To compare the detection rates of conventional methods and Bactec MGIT 960 with the Cepheid Gene Xpert, MTB/RIF assay for direct detection of *Mycobacterium tuberculosis* in respiratory specimens (pulmonary TB) and non respiratory specimens (extra-pulmonary TB). The performance of the Gene xpert assay was determined for rapid diagnosis of tuberculosis. The new techniques for rapid diagnosis (showing results within 2 hours) of TB and drug resistance have been used in TB centers, to facilitate critical decisions. One of the latest systems, the Gene Xpert MTB/RIF assay integrates DNA extraction, genomic amplification, quantitative detection of *M. tuberculosis*, and rifampicin (RIF) resistance determination in a single cartridge, for DNA extraction and amplification of a 192-bp segment of the rpoB gene. So Gene Xpert assay implemented highest positive rates in pulmonary TB isolates at positive percentage 34/35 (97.14%), as presented in Table (3,14) where as the negative results were very low at 1/35 (2.86%), and positive result (90.91%) in extra-pulmonary isolates of (positive ,negative) smear and( positive ,negative) results of Bactec 960 system which confirmed with the growth on solid media, these results were in correspondence with [38] who found rate of detection (100%) by this assay of positive/negative smear specimens, and [29], who observed the Gene Xpert implemented the highest detection rate (90.6%) among diagnostic methods. These results reflect the fact that Gene Xpert assay can detect even one bacilli in specimens whether viable or not, it so useful in little volumes of specimens/or with few numbers of bacilli in specimens. The present study also demonstrated another advantages that the Gene Xpert MTB/RIF test provided the best detection rate than previous methods within two hours, and represented a simple method, and routine staff with minimal training which can use the system, and the test provided to be as high sensitive 97% with pulmonary TB specimens, and got highest effectiveness tool with extra-pulmonary TB specimens. There were statistically significant differences in the results of diagnostic methods ( $p=0.000002671, p<0.05$ ).

#### **Diagnosis of extra-pulmonary TB isolates:**

Regarding the extra-pulmonary TB (EPTB) specimens, (28) had mentioned that the pulmonary tuberculosis infected lungs mainly, while the extra-pulmonary tuberculosis can present as pleural effusions, tuberculous lymphadenitis, tuberculous meningitis, abdominal tuberculosis and tuberculosis of bones and joints. So rapid, sensitive, and specific method was needed for its diagnosis in order to provide appropriate treatment to the patient. Initial diagnosis is dependent on the smear microscopy for acid fast bacilli (AFB) by Ziehl Neelsen (Z-N) staining and culture are documented as gold standard for isolation and identification of *mycobacterium tuberculosis* (MTB). The Z-N smear is rapid and inexpensive and is widely used in the developing countries for both pulmonary and extra-pulmonary specimens. There are various reports regarding the sensitivity of Z-N smear for extra-pulmonary specimen ranging from as low as 0% to as high as 75% as reported by [19], these findings were clear in this study, as a total of twenty-two extra-pulmonary specimens which were diagnosed with conventional staining and solid culture, in addition were subjected to Bactec 960 system, and Gene Xpert assay diagnosis, the Z-N staining, showed lowest positivity rate (9.09%), as results presented in Table [7]. The fact concerning the comparative investigations was different from that of pulmonary TB specimens, this result were less detectable than culture on solid media (27.27%). Both were less than culture in liquid media (Bactec 960) of a positive percentage (50%), as shown in Table (4,7), these results going with [30] who observed the detection rate of Z-N staining (20.25%) were less than culture on L-J medium (46.38%), on the same line in accordance with [25] who stated approximate results, (22.9%) for AFB staining and (59%) for Bactec 960. And these result of the study not in consistence with [15], who reported high positivity rate for Bactec 960 (88.8%) and (69.3%) for solid media, nor with [4] who stated that Bactec 960 percentage was (91.6%) as it most sensitive test for extra-pulmonary TB diagnosis, and get (58.3%) in solid media in his reaserch. So, this limitation has been reported to be ascribed to inadequacy and the paucibacillary nature of specimen. (53) state that AFB smear positive of fluids is rare. Specimens which are fluids require large quantity of up to 1 liter to be centrifuged before being subjected to AFB smear and culture. However it was found that AFB smear although rapid but insensitive for extra-pulmonary specimens. Low sensitivity of cultivation in Bactec 960 with high negative result (50%) system and in L-J media of (72.73%) of negative result, was also due to paucibacillary in specimens, and in addition, Extra-pulmonary TB is difficult to diagnose as compared to pulmonary TB, such as pleural effusions, lymphadenitis, meningitis and others, almost all of these sites require an invasive procedure to obtain specimen for diagnosis. As a result, the disease most often remains undiagnosed and untreated specimens. So, a rapid, sensitive, and specific diagnosis is needed owing to limitations of traditional microbiological methods (paucibacillary nature of specimens) and extensive differential diagnosis [39]. Such as the Gene Xpert assay, the most efficient and useful technique to diagnosis the extra-pulmonary specimens, as in this study detected TB bacilli with highest

positive result (90.91%), and the negative result was (9.09%). Because as previously mentioned this technique capable to detect even one bacilli whether viable or not, this was on the same line with [29], who get high sensitivity (96.7%) of Gene Xpert for extra-pulmonary specimens. Twenty extra-pulmonary specimens were mainly caused by the species *M.tuberculosis*, as this technique specific to diagnosis MTB. But the two negative isolates may belonged to other species of *M.tuberculosis* complex. The reliability of Z-N staining and culture on solid and liquid media for extra-pulmonary specimens as shown in this study were lower and less reliable than it in pulmonary specimens. The main drawback is the low product of TB bacilli in extra-pulmonary.

#### Biochemical typing tests:

Regarding the biochemical tests, most isolates from human disease belong to the species *M. tuberculosis*. However, depending on geographical and epidemiological circumstances, it may be necessary to differentiate species within the *M. tuberculosis* complex.

In the present study six biochemical typing tests were used to identify of *M.tuberculosis* complex from mycobacteria other than tuberculosis, including niacin accumulation, nitrate reduction, and heat-labile catalase (68°C) reactions, Para-nitro benzoic acid (PNB) inhibition test, Thiophene-carboxylic acid hydrazid sensitivity, and growth on stone-brink medium. Sixty-six of diagnosed pulmonary isolates were identified with these biochemical typing tests as shown in Table (5) which revealed the majority of pulmonary isolates belonged to *M.tuberculosis* at total percentage (90.90%).

Sixty six isolates (100%) were positive for niacin production, although all mycobacteria produce niacin, comparative studies have shown that, *M. tuberculosis* accumulated the largest amount of nicotinic acid (niacin) and its detection is useful for its definitive diagnosis. Niacin negative *M.tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests. The niacin test should not be used alone to identify *M. tuberculosis* because several other species *M.simiae*, *M.chelonae*, *M.canettii* gave positive results to this test but *M.bovis* gave negative results, while *M.africanum* gave variable results. This fact alone emphasizes the importance of performing the supportive test of nitrate reduction to confirm the identify of *M.tuberculosis* [33].

Positive results for nitrate reduction also were given by (100%); without negative results. This test is particularly useful for differentiating *M. tuberculosis*, which gives a positive reaction, from *M. bovis*, which is negative [45].

According to growth on L-J media in presence of Para-nitrobenzoic acid, out of sixty-six isolates, sixty-five isolates were inhibited (98.48%) which belong to *M.tuberculosis*, while growth percentage were (1.52%), as growth of MTB is inhibited by Para-nitrobenzoic acid (PNB), whereas, (Non-tubercular mycobacterium) NTM are resistant. So this test contributes to the differentiation of species of the *M. tuberculosis* complex. Para-nitro benzoic acid inhibits the growth of *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* [38].

Thiophene-2-carboxylic acid hydrazide (TCH) is useful in the differentiation of MTBC when performed together with other tests [12].

According to TCH there were (98.48%) resistant isolates and (1.52%) were susceptible. (TCH) Permitted the differentiation of *M. tuberculosis* that grows

in the presence of the compound as opposed to other species such as *M. bovis*. Some species of NTM are positive for this test. This test is useful to distinguish *M. tuberculosis*, which grows in the presence of this compound, from other members of the *M. tuberculosis* complex. "*M. canettii*" and most non-tuberculous mycobacterial species are also positive to this test (26, 45). Among sixty-six isolates, two isolates (3.03%) gave positive results to the heat stable Catalase test at PH7/68°C and sixty-four were negative (96.97%). Catalase is an antioxidant enzyme responsible for eliminating molecules of hydrogen peroxide from the cells that are produced during respiration. The reaction results in the release of water and free oxygen (20). The 68°C catalase is a heat-tolerance test measuring the catalase activity at high temperature. Characteristically, *M. tuberculosis* gives negative results, as do other species in the *M. tuberculosis* complex [4].

An important step to diagnose *M. bovis* is culturing the sputum specimens on the specific medium (stone brink medium), this method is used for detection of samples with low mycobacterial load [13]. Thirty-four isolates (51.52%) were identified as *M. bovis* and *M.tuberculosis* on the basis growth on stone-brink medium, and thirty-two isolates (48.48%) showed negative results. *M. bovis* and other species of the *M.tuberculosis* complex (*M. microti* and *M. africanum*) are unable to use glycerol, Thus, these organisms will often fail to grow on Löwenstein-Jensen medium, [20].

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