

# Microscopic and Molecular Diagnosis of Mycobacterium Tuberculosis in Erbil city-Iraq

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## Abstract

This study was carried out in consultation clinic of the Chest and Respiratory Diseases Center Erbil city-Iraq from 1<sup>st</sup> January to 31 December 2018 .

The results explained that from total of 1986 consecutive suspects with TB disease in Erbil city 103(%5.2) were pulmonary tuberculosis (PTB) infection depended on microscopic examination of sputum specimens with AFB stain and sputum culture on Lowenstein Jensen Media While histopathology screening (Biopsy- In cooperation with histopathology's physician ) indicate that from 1883 (%94.8) negative cases 8(%0.4) was extrapulmonary tuberculosis (EPTB) infection.

According to nationality, most cases were Iraqi from Erbil city 52 (%50.5) while the number of non-national patients Syrian were 13(%68.4) and others were from different nationalities 6(%31.6), while Iraqi emigrant patients were 32(%31.1). According to age group most cases were at age group 25-34 and 15-24 years were 27(%26.2) and 22(%21.3) respectively and only 2(% 1.9) patients were more than 65 years old .Most of patients were male 55(%53.4) and 48 (%46.6) were females.

Detection of (TB) by GeneXpertMTB/RIF assay for contributing to the rapid diagnosis of the disease and their resistance to drug rifampin (RIF) indicated that all 103(%100) samples were positive to GeneXpertMTB the target DNA detected by real time PCR which is needed to start treatment quickly, while in 11(%10.6) samples the Rifampicin resistance DNA was detected which is need treatment with second-line agents. Conclusion: The results of current study indicate that diagnosis and detection of TB infection by microscope, culture and rapid diagnosis by PCR have the same accuracy.

## Introduction

Tuberculosis (TB) is an air-borne infectious disease caused by *Mycobacterium tuberculosis* (*M. Tuberculosis*) that typically attacks the lungs (Pulmonary) and can affect different parts of the body (Extrapulmonary) [1]. It is an aerobic, non-motile bacillus. Because of waxy coating on the cell surface, it is neither gram- positive nor – negative, but its impermeable to gram staining [2]. Air droplet containing bacilli is the main method to transmit to a healthy individuals by inhalation .They also may be produced by sputum induction, aerosol treatments, aerosolization during bronchoscopy, and through manipulation of lesions or processing of tissue or secretions in the hospital and laboratory. It is usually curable if properly treated and may be fatal if not treated [3].

Each year, more than 8 million new cases of TB occur, and approximately 3 million cases die from the disease , 95% of these cases occur in, developing countries [4]. A study revealed estimation the burden of TB on Iraq that 14,800 -15,300 people being infected by TB each year in Iraq [5].

In cooperation with the World Health Organization and Health Cluster partners, International Organization for Migration (IOM) Emergency Health Program has been supporting the Iraqi Ministry of Health's National

Tuberculosis Program (NTP) since 2014. Between 2014 and 2017, more than 300 TB cases were detected by IOM at NTP health facilities among internally displaced persons and Syrian refugees in the Kurdistan region and Kirkuk [6].

Therefore the aim of this study attempted to screening and diagnosis TB disease by using many methods to detecting new cases in Erbil city- Iraq in 2018.

## Materials and Methods

**1. Sample collection:** A total of 1986 consecutive suspected with TB disease referral from the whole districts of Primary Health Care Centers in Erbil Governorate from January to December 2018 which attended a consultation clinic of the Chest and Respiratory Diseases Center in Erbil. Diagnosis (In cooperation with Specialist physician) was based on clinical evaluation of patients.

## 2. Sputum collection and processing

All TB suspects attending care and treatment clinics were counseled about the study asked to give sputum samples for smear microscopy and culture. The patients were advised to rinse their mouth twice with water before producing the specimen and this helped to remove food and any contaminating bacteria in the mouth. They were instructed to take two breaths, coughed vigorously and expectorated the material in into the sterile 50ml cap screw-capped bottle. This process allowed sputum to be produced from deep in the lungs. The TB suspects were asked to hold the sputum container close to the lips and spit into it gently after a productive cough. Three sputum specimens during two days were collected from TB in order to increase the susceptibility of detection Mycobacterium TB infection.

At the laboratory, the standard AFB direct smear microscopy using ZN staining was done on the initial sputum to confirm TB diagnosis of suspected patients.

### 2.1 Microscopic examination of sputum specimens with AFB procedure:

Smear was prepared on clean, unused glass slide labeled with laboratory number.

1. There was only one smear per slide and a swab stick or loop was used to collect representative portion of the sample for smearing.
2. After drying, fixation was done by gentle heating.
3. The entire slides were flooded with ZN 1% carbolfuchsin solution. Each slide was heated slowly by Bunsen burner until steam arises. Steaming was maintained for 5 minutes by using intermittent heat.
4. After 15 min. each slide was rinsed individually in a gentle flow of water until all free stain was washed away. Excess rinsed water was drained off by tilting the slide.
5. The slide was poured with H<sub>2</sub>SO<sub>4</sub> 20% (for remove carbolfuchsin) and let it 2 min.
6. After 2 min. wash the slide thoroughly with water and excess water drained off from the slide.
7. The slide was poured with 0.1% methylene blue for 60 seconds.
8. The slide was washed with water and excess drained off by left standing to allow air dry.
9. Sputum smears were examined for AFB after staining under oil immersion
10. The degree of ZN smear positivity was quantified as in table (1):

**Table (1): Degree of positivity in ZN stain smear [7].**

Number of AFB/field	Results
No AFB/300 field	Zero (-Ve)
1-9 AFB/ 100 field	the exact number of AFBs was indicated
10-99 AFB/100field	+1
1-10 AFB/1 field (at least 50 field)	+2
> 10 AFB/1 field (at least 20 field)	+3

### Sputum Culture and Identification

Sputum samples were processed and only one tube at a time was uncapped for addition of solutions. Buffer solution was prepared as individual aliquots in single use tubes and opened five minutes after centrifugation at 3000xG for 15 minutes. After centrifugation, it was cultured on slopes of Lowenstein Jensen (LJ) media.

1. The Lowenstein Jensen Media was inoculated with specimen after decontamination and neutralization, according to test procedures recommended by the National TB Control Program [7].
2. The medium was incubated at 35-37°C. Protect from light. Tubes media should be incubated for 72 hours with loosened caps to be sure the evaporation of the liquid from the culture and the closure of the bottle caps in order to prevent dehydration of media and also to examine the contaminated contaminants found.
3. The media was examined within 5 to 7 days, and weekly thereafter for up to eight weeks.
4. The plates were examined under light for the appearance of macroscopic growth.
5. The tubes were examined under light and magnifying mirror for macroscopic growth. Record and describe colony morphology on the first day growth is observed *M. tuberculosis* on LJ medium after 6 weeks of cultivation, typically nonpigmented, rough surface, dried colonies.
6. To make sure that the colony of *M. tuberculosis*; part of the colony is taken by needle planting and melted in a drop of saline solution on the slide, leave the specimen on the slide to dry and then stabilize by gentle heating and stained by ZN staining technique and examine for presence of AFB.

The amounts of bacterial growth of bacterial colonies are recorded as in table (2):

**Table (2) The amounts of bacterial growth according to bacterial colonies**

<b>Number of colonies</b>	<b>results</b>
<b>No colonies</b>	Negative
<b>&lt; 50 colonies</b>	Actual count
<b>100-50 colonies</b>	+1
<b>200-100</b>	+2
<b>500-200 colonies</b>	+3 (Almost Confluent)
<b>Over 500 colonies</b>	+4 (Confluent)

+ : Positive cultures for *M. tuberculosis* confirm the diagnosis of TB disease. [7].

### 3. Tuberculin Skin Test

A tuberculin skin test is done. Purified protein derivative (PPD) (Tuberculin Mammalian), used as TB antigens in a tuberculin skin test

### 4. Identification of *M. tuberculosis* complex strains by GeneXpertMTB/RIF assay.

#### Sample collection

After the patients rinsed their mouth twice with water., inhaled deeply and coughed vigorously, one ml of sputum per sample were collected into the sterile screw-capped specimen collection container. the lid on the collection device was screwed. Sample can be stored at a maximum of 35C° for less than 3 days and at 4C° for 4-10 days.

#### Prepare sample

- The lid of each sputum collection container unscrewed, directly in the collection container 2 volumes of sample reagent to 1 volume of sample were added .
- The lid replaced and shaken vigorously by vortex.
- After 10 min of incubation at room temperature , the specimen was shaken vigorously .

- After 5 min. of incubation, sample was perfectly fluid before being processed, with no visible clumps of sputum.

### Prepare cartridge

The cartridge was labeled with the sample ID, the cartridge lid was opened, at least 2 ml from the collection container transfer with the plastic pipette to the cartridge then the cartridge lid was closed.

The DNA was extracted in biological safety cabinet. The primers (forwards 5') and (reverse 5') amplify a 123 bp region of the IS6110 was used. The DNA (2.5micro/l) of *M. tuberculosis* was amplified in a 2.5micro/l reaction containing 2.5Mm MgCL<sub>2</sub>, 200µM nucleoside triphosphates, 25pmoles of each primer and 1 unit of Taq polymerase in 1X PCR Buffer II 50mM KCL, 10mM Tris-HCL pH 8.3). The cycling parameters was held 95C° for 4 min, followed by a 30 cycle PCR comprised of denaturation at 94C° for 30s, 68C° for 30s, and 72C° for 30s and 72C° for 10 minutes.

### Results and Discussion

The current results stress the importance of frequent reviewing of frequency, severity, and outcome of TB infection. The positive sputum smears for AFB examinations and culture on slopes of (LJ) media was shown in Figure (1). From 1986 cases, the number and percentage of who were suspected to have tuberculosis after showing clinical manifestations and radiological changes for tuberculosis were submitted for conventional laboratory diagnosis and were positive for at least one of the diagnostic criteria were 103(5.2). While the number and percentage of negative case were 1883 (94.8) as showed in Figure (2).

Figure (3) explained that all of these patients 103 (100%) were pulmonary tuberculosis (PTB) who were diagnosed by Zihel-Nelsen staining technique, culturing. While histopathology (Biopsy) indicate that from 1883 negative cases 8(0.4) was have extrapulmonary tuberculosis (EPTB).

Other study determine the incidence of tuberculosis in Babylon province-Iraq, in which sixty TB cases were diagnosed distributed into 40 pulmonary TB and 20 extrapulmonary TB [8]. World Health Organization (WHO) estimates of tuberculosis incidence by country in 2015 the Estimated rate in Iraq 43 per 100,000 population [9].

The result in table (3) indicated the number of total respiratory symptomatic individual registered, self referred was 92(89.3) While Public was 6(5.8%) Private positive sample was 5(4.9). Community, Prisons and Military percentage was 0(0%).

The number and percentage of TB registered patients according to nationality, as it shows the most case were national 52 (50.5) while the number of non-national patients was 19 (18.4) and emigrant patients was 32(31.1) (Figure,4).

The number and percentage of national TB patients according to age group most case was at age group 15-24, 16 (30.8) then 25-34 reached 10(19.2) and 7(13.5) for age group 35-44, while age group 1-4 and 5-14 and 45-54 and 55-64 and more than 65 years number and percentage was 3(5.8), 4(7.7), 5(9.6), 5(9.6) and 2(3.8) respectively (Table,4).

The number and percentage of emigrant TB patients according to group most case was at age group 25-34 12 (37.6) then 35-44 and 55-64 each of them reached 6(18.7) (Table,5).

The number and percentage of non-national TB patients according to age group most case was at age group 35-44 was 12 (63.2) then 25-34 was 5(26.3) and 45-55 was 2(10.5) (Table,6).

Table (7) represented the number and percentage of TB patients according to gender, as it shows the most of national and emigrant patients were females 22(42.3), 17(53.1) respectively while most number of non-national patients was male 18(94.7) and generally male cases was 55(53.4) as compared with females 48 (46.6).

The results of study focused on patients' characteristics of age, gender and type of tuberculosis in Baghdad, Iraq for the period of 2012-2016, depict that patients of age group of (15-24) year old had experienced detected tuberculosis cases and concluded that female patients had experienced more detected cases of tuberculosis than male ones. [10]. A study demonstrated, that an extremely high rate of acquisition, of TB infection in childhood and adolescence results in very high TB prevalence rates in young adults who are most at risk, of acquiring HIV, infection [11]. Also in Malawi most of the TB cases was male at age group 35-44 and 25-34 [12].

In India, the Revised National Tuberculosis Control Programme (RNTCP) detects nearly three times more male than female TB patients [13]. In the same place [14] found excess of male TB case. In Southwestern the number and percentage of TB was 258 (66.5%) males and 175 (44.8%) female [15]. The higher rates of notification for male may be due to biological function like sex hormones, genetic background, difference in exposure due to behavioral risk factors (smoking, occupation, dietary differences, indoor air pollution), Socio-cultural components (stigma, income, awareness, differential access to health care and health care seeking behavior) [16].

A study describe the epidemiology of TB in immigrants by using national surveillance data in Finland. During 1995-2013, 7030 (84.7%) native and 1199 (14.4%) immigrant cases were identified. The proportion of immigrant cases increased from 5.8% in 1995 to 32.1% in 2013, consistent with increasing immigrant population (2.1-5.6%) and decreasing incidence of TB in the native population (from 12.1 to 3.5/100 000) [17].

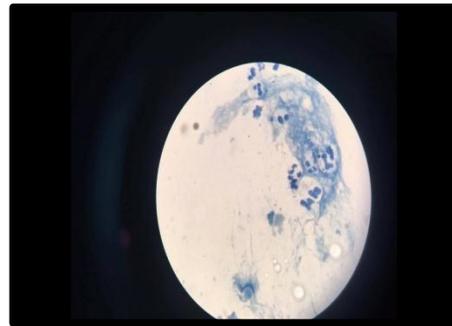
A total of 9,412 new tuberculosis (TB) cases were reported in the United States in 2014, with an incidence rate of 3.0 cases per 100,000 persons, a decrease of 2.2% from 2013, while the proportion of cases among foreign-born persons has increased. In 2013, the percentage of TB cases among those born outside the country was 64.6%. [18]. Approximately 450,000 persons are admitted to the United States on an immigrant visa each year, and 50,000–70,000 as refugees. Applicants for either an immigrant visa or refugee status are required to undergo a medical examination overseas before being allowed to travel to the United States [19]. In our study we suggested that medical examination (chest radiographs , sputum smear and cultures) to all allowed to enter our city in order to separate TB cases from healthy and decreasing transmission and at same time the most recent data are available rigorous diagnostic and treatment programs can be implemented in areas with TB incidence .

The number and percentage of TB cases which were test by GeneXpertMTB/RIF assay ,a new test that is detect tuberculosis (TB) control by contributing to the rapid diagnosis of the disease and their resistance to drug rifampin (RIF). All 103(%100) cases was positive to GeneXpertMTB the target DNA detected by real time PCR which is need to start treatment quickly , while in 11(%10.6) cases the Rifampicin resistance DNA was detected Resistance to Rifampicin or other first-line drugs usually indicates the need of second-line agents (Table,8) .

Table (9) demonstrated identification of *M. tuberculosis* by tuberculin skin test .From 1986 suspected case all of them was negative if the area of skin where received the PPD injection not swollen or is only slightly swollen 48 to 72 hours after the injection, the test results was negative. A tuberculin skin test is done to find people, who have TB, including those who have been in close contact with someone known to have TB.



**Figure (1) TB colonies cultured**



**AFB smear positive on LJ agar**

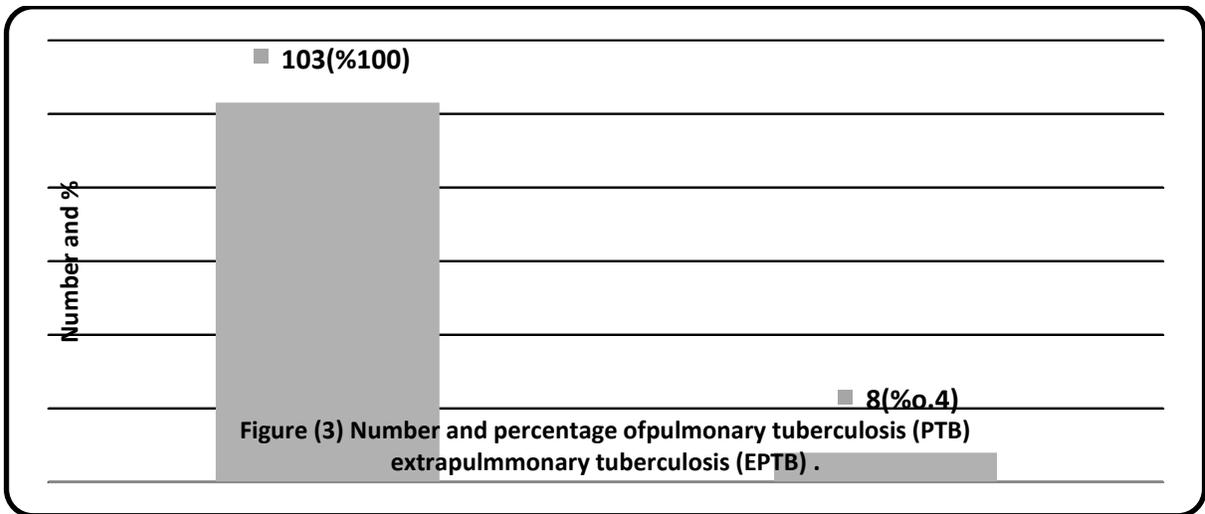
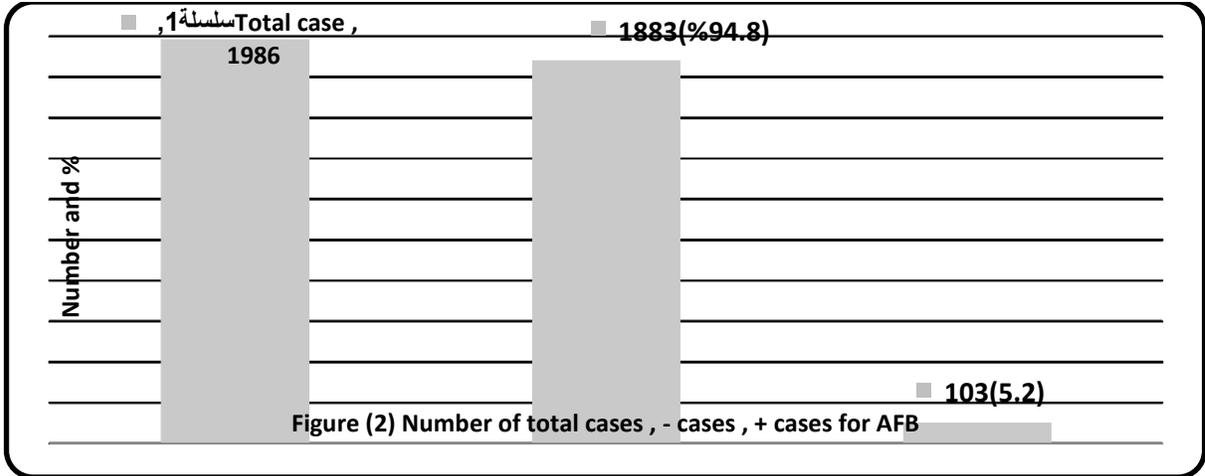
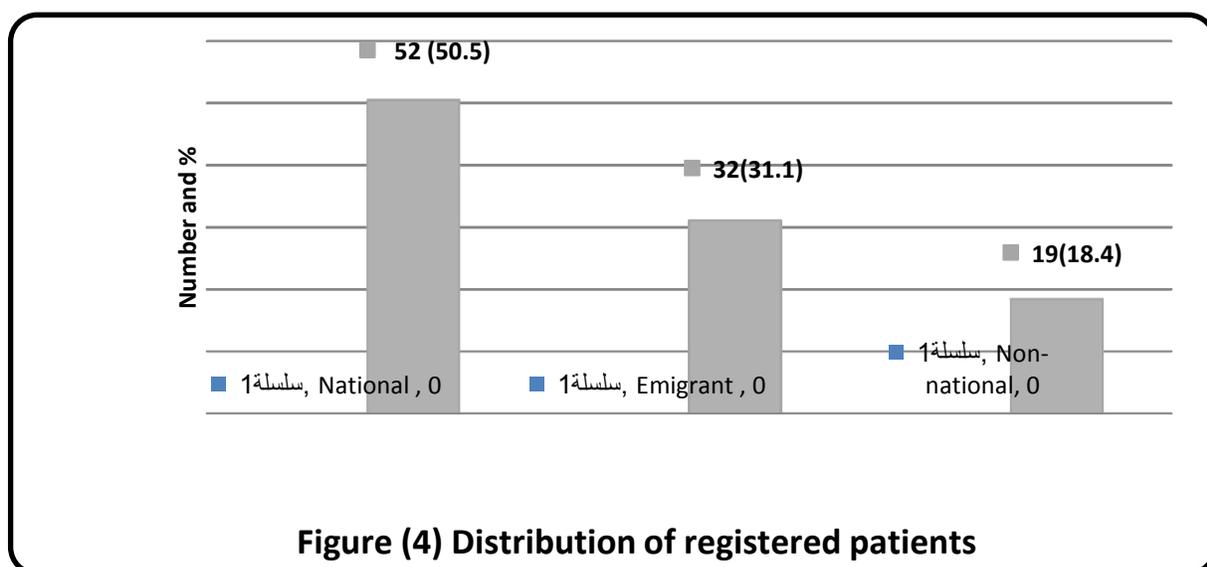


Table (3) Total number of respiratory symptomatic individual Registered and Number and percentage of positive sputum.

Referred by	Number of respiratory symptomatic individual registered	Number and percentage of sputum positive result
Self	1911	92(89.3%)
community	14	0(%)
Public	40	6(5.8)
Private	21	5(4.9)
Prisons	0	0(%)
Military	0	0(%)
Total	1986	103

**Table (4 ) Number and percentage of national TB patients according to age group**

Age groups	Number	%
1-4	3	5.8
5-14	4	7.7
15-24	16	30.8
25-34	10	19.2
35-44	7	13.5
45-54	5	9.6
55-64	5	9.6
65 and more	2	3.8
<b>Total</b>	<b>52</b>	<b>100</b>



**Table (5) Number and percentage of emigrant TB patients according to age group**

Age groups	Number	%
1-4	0	0
5-14	0	0
15-24	6	18.7
25-34	12	37.6
35-44	6	18.7
45-54	2	6.3
55-64	6	18.7
65 and more	0	0
<b>Total</b>	<b>32</b>	<b>100</b>

**Table (6) Number and percentage of non- national TB patients according to age group**

Age Group	N	%
25-34	5	26.3
35-44	12	63.2
45-55	2	10.5
<b>Total</b>	<b>19</b>	<b>100</b>

**Table (7) Number and percentage of TB patients according to gender**

Distribution of registered national patients according to gender		
Gender	Number	%
Male	22	42.3
Female	30	57.7
<b>Total</b>	<b>52</b>	<b>100</b>
Distribution of registered emigrant patients according to gender		
Gender	Number	%
Male	15	46.9
Female	17	53.1
<b>Total</b>	<b>32</b>	<b>100</b>
Distribution of registered non- national patients according to gender		
Gender	Number	%
Male	18	94.7
Female	1	5.3
<b>Total</b>	<b>19</b>	<b>100</b>

**Table (8) The number and percentage of TB cases according to GeneXpert MTB with Rifampicin Resistance, Qualitative by Real Time PCR.**

Tests	Total samples	The Target DNA detected	Target DNA not detected
GeneXpertMTB	103	103(%100)	0(%0)
Rifampicin Resistance	103	11(%10.6)	92(%89.4)

**Table (9) The number and percentage of case according to Tuberculin Skin Test**

Other tests	Total negative case	Positive number and %	Negative Number and %
Identification of other TB cases by Tuberculin Skin Test	1875	0(%0)	1875(%100)

### Conclusion

The reduce of Tuberculosis Ventilation with fresh air is especially important, sterilizing fomites, using masks and ultraviolet irradiation of air in the room is desirable. Treating the patient with effective antituberculosis chemotherapy . Additionally, IOM is providing TB awareness campaigns, training more medical staff, providing support to TB screening facilities as well as early detection and diagnosis of TB cases in IDP camps and other emergency sites for Iraq's displaced.

### CONFLICT OF INTERESTS

**There are no conflicts of interest.**

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## التشخيص المجهرى والجزئى لعصيات السل في مدينة أربيل- العراق

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### الخلاصة

أجريت هذه الدراسة في المستشفى الخاص بالأمراض الصدرية والتنفسية في مدينة أربيل - العراق للفترة ما بين ١ كانون الثاني إلى ٣١ كانون الأول ٢٠١٨ م، بينت النتائج بأن من مجموع ١٩٨٦ حالة مشكوك فيها بمرض TB أحيلوا من مراكز الرعاية الصحية في مدينة أربيل بأن ١٠٣ (٥.٢%) حالة كانوا مصابين بسل الرئوي (PTB) اعتماداً على الفحص المجهرى للبلغم بواسطة صبغة AFS و الزرع على وسط L-J، في حين أشار فحص العينة histopathology (بتعاون طبيب histopathology) إلى أن من ١٨٨٣ (٩٤.٨%) حالات سلبية ٨ (٠.٤%) انتقل السل فيهم إلى خارج الرئتين (EPTB).

وطبقاً لجنسية المرضى تبين أن أكثرهم كانوا من سكان المدينة من العراقيين ٥٢ (٥٠.٥%)، بينما كان عدد المرضى الأجانب من السوريين ١٣ (٦٨.٤%)، والبقية كانوا ٦ (٣١.٦%) من جنسيات أخرى، وقد سجلت ٣٢ (٣١.١%) حالة في المهاجرين العراقيين (التازحين). أما حسب الفئة العمرية فقد تبين أن أغلب المصابين كانوا من الفئة العمرية ٢٥-٣٤ و ١٥-٢٤ سنة ٢٧ (٢٦.٢%) و ٢٢ (٢١.٣%) على التوالي ووجدت حالتان فقط (٣.٨%) بعمر أكثر من ٦٥ سنة. أما وفق جنس المرضى فإن أغلبهم كانوا ذكوراً ٥٥ (٥٣.٤%)، أما الإناث فـ ٤٨ (٤٦.٦%).

إن كشف (TB) بواسطة GeneXpertMTB / RIF يساهم في التشخيص السريع للمرض ومقاومة البكتريا للمضاد ريفاميسين، حيث أشارت النتائج إلى أن العينات كافة ١٠٣ (١٠٠%) كانت إيجابية ل GeneXpertMTB، وتم الكشف بواسطة PCR، وهذه الحالات تحتاج إلى المعالجة السريعة، وفي الوقت نفسه كانت ١١ (١٠.٦%) عينة مقاومة لمضاد ريفاميسين، وهم بحاجة إلى الجيل الثاني من العلاج. الاستنتاجات النتائج من دراستنا تشير بأن التشخيص وكشف عدوى TB بالمجهر، الزرع وتشخيص سريع بواسطة PCR لها نفس الدقة في نفس الوقت إختبار tuberculin skin لاشخاص الذين في التماس المباشر مع الشخص المصاب ب TB ضرورية

الكلمات الدالة: سل Mycobacterium، العراق، أربيل، رئوية، خارج رئوية.