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ISOLATION AND IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* AND STUDY OF MULTIDRUG RESISTANCE (MDR-TB)

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ABSTRACT

The current study aimed to monitor and control the multidrug resistance of *Mycobacterium tuberculosis* bacteria isolates and to detect its prevalence. The study was conducted at the Institute of Chest & Respiratory Diseases / Baghdad. Different clinical samples were collected from 500 patients to detect the presence of *Mycobacterium tuberculosis* bacteria by using conventional methods. The direct microscopic examination of Zeihl-Neelsen stained smears showed that 64 (12.8%) out of 500 of the samples were positive for the bacteria. All samples were cultured on Lownstein Jensen (L.J) medium, and 90(18%) of them showed true growth of tuberculosis bacteria. Microscopic examination and culture on Lownstein Jensen medium of sputum sample showed the highest positive percentage (15.9% and 23.1%) respectively, and the results of the biochemical and differential tests showed that all the isolates belonged to *M. tuberculosis* bacteria. No significant differences were found in the incidence of tuberculosis between males and females (51% and 49%) respectively, while significant differences ($P<0.01$) were shown in the incidence of the disease among different age groups 23% in the age group (21-30) years followed by 22% in the age groups (31-40) and (41-50). The sensitivity of all tuberculosis isolates (90 samples) were tested against the first-line anti-tuberculosis drugs (streptomycin, rifampicin, isoniazid and ethambutol) by using the proportion methods, which showed that 53.35% (38 out of 90 isolates) were resistant to those drugs, and 30% (27% isolates) were resistant to one of them, while 12% (11 isolates) were resistant to more than one of these drugs, whereas 11.1% (10 isolates) showed TB multi-drug resistance characteristics (MDR-

TB). The highest resistance of the isolates was to isoniazid (38.9%) followed by (25.6%) to rifampicin followed by (17.8%) to streptomycin, while the lowest resistance (11.1%) was to ethambutol with highly significant differences at (P <0.01). The current study showed a real problem was found in the resistance of TB bacilli to antibiotics, because of the high percentage of isolates of these bacteria which resisted the first line anti-tuberculosis drugs. The study also found that Isoniazid and Rifampicin had the highest resistance, which leads to a real risk to the development of MDR-TB isolates in Iraq.

KEYWORD: *Mycobacterium tuberculosis,* isolation, identification, multidrug resista.

INTRODUCTION

Mycobacterium tuberculosis is the main cause of tuberculosis, and it is one of the most infectious diseases that caused a large number of deaths in different communities at different times.[1] *M. tuberculous* bacteria are bacilli with[2-4] microliter length and (0.2-0.5) microliter width, non-motile, obligate aerobic, mesophilic, growing at $37C^0$.[2,3] They are non-capsulated and non-spore forming bacteria, however, recent studies suggest that *M.marinum* and *M. bovis* may possibly be able to form spores.[4]

The most common form of tuberculosis is pulmonary TB. It can cause extra pulmonary TB i.e outside the respiratory system. Because the spread of the disease through air occurs only among patients with pulmonary tuberculosis, so the diagnosis of bacteria from pulmonary samples is very important to control the spread of the disease.[5] Tuberculosis is still one of the most devastating infectious diseases globally. It is thought that about one-third of the world's population is infected with TB bacilli, and it is estimated that the rate of transmission and new infections occur at a rate of one person per second.[6] According to the World Health Organization (WHO), tuberculosis recorded the second highest mortality rate among all infectious diseases worldwide, and it remains a health burden worldwide despite the progress and development of the diagnosis and treatment protocols of this disease. It is estimated that approximately 10.4 million people are infected with tuberculosis and about 1.8 million people died of the disease in 2015.[7] Iraq is among the eight countries in the Middle East region that recorded the highest incidence of tuberculosis. According to the recent estimates issued by the ministry of health, it has been shown that the infection rate was 45 per 100,000 of the population, although Iraq has a reference laboratory located in Baghdad at the specialized chest and respiratory disease center, and 124 TB sub-laboratories, as well as a program of follow-up of TB patients under treatment.[8]

The use of effective anti-tuberculosis drugs such as streptomycin in 1941, isoniazid in 1952 and rifampicin in 1970 gave hope for treating the disease, with a 75% reduction in infection rate. However, the course of events changed unexpectedly in 1993 due to the epidemic of human immunodeficiency virus (HIV) when the WHO declared a global health emergency and decreased focus on tuberculosis-resistance programs, leading to the emergence of drug resistant tuberculosis bacteria, which appeared to be a major threat to the disease control. The World Health Organization (WHO) reported that more than half a million of new MDR-TB cases occurred during the year 2009.[9] There are a number of effective drugs against these bacteria, the most important of which according to the descriptions of WHO are: Rifampicin, Isoniazid, Streptomycin, Ethmbutol and Pyrazinamide. Anti-drug resistant tuberculosis is caused by strains of *M. tuberculosis* that resist at least two antibiotics including Isoniazid and Rifampicin, which are the most effective drugs in the treatment of tuberculosis.[10] According to WHO estimates for the year 2014, the resistance to rifampicin was as high as (7.6%) and to isoniazid exceeded (5.9%), therefore it needed monitoring and continuous follow-up. Molecular studies showed that the development of TB resistance to these antibiotics is mainly due to the mutation in inhA, katG and rpoB genes.[11]

Microscopic detection is the main diagnostic tool in poor countries of the world, where sputum is examined either by using light microscope with Ziehl-Neelsen stain or by using fluorescent microscope with Auramine-rhodamine stain.[12] When using the Ziehl-Neelsen stain, presence of 10,000 bacilli / milliliter is needed to report a positive result. *M. tuberculosis* bacilli appear in red color. Staining must be done for three successive times, and if the result is positive, it means that the patient is infected with active tuberculosis and he is contagious.[13,14] Bacterial culture is an international standard to diagnose tuberculosis. The sensitivity of this diagnosis is much greater than the microscopic examination, where it requires 10 *M. tuberculosis* bacilli per one millimeter to show a positive result. In addition to the sensitivity of this test, it can distinguish the morphologic characteristics and identify the genera of the bacteria as well as performing the sensitivity test to drugs. One of the conventional media used for T.B culturing is the Lowenstein Jensen medium (LJ), which is based on eggs, and the middle brooks medium, which is based on agar.[15]

This study aimed to investigate the prevalence of MDR-TB isolates by isolating and diagnosing *M. tuberculosis* from sputum and other samples and to detect the first line drug resistant TB bacteria by conventional methods.

METHODS

1. Sample culturing and bacterial diagnosis

A total of 500 sputum samples were collected from patients who visited the center of chest and respiratory diseases in Baghdad. Patient's age ranged from 1 to 96 years of both sexes for the period from 1/9/2016 to 1/1/2017. The samples were examined directly by Ziehl_neelsen staining method, and then positive and negative samples were cultured directly on the Lowenstein Jensen (L.J) agar medium according to the Petroff decontamination method pursuant to the laboratory manual.[16] The isolates were identified based on the morphologic and biochemical characteristics.[17]

2. Sensitivity test of bacterial isolates to first line anti-TB drugs

The proportion method for sensitivity was used according to the laboratory manual[18] as follows.

Preparation of the anti-bacterial stock solution.

- **Isoniazid:** (5) ml of the solution was taken by dissolving (10) mg of the antibacterial agent in (10) ml of sterile DW, and (45) of sterile DW was added. (5) ml of the latter solution was added to (45) ml of sterile DW and after filtration, (5) ml was transferred into (245) ml of Lowenstein Jensen (L.J) agar, until its final concentration reached (0.2) microgram/ml.
- **Rifampicin:** (21) mg of the antibacterial agent was dissolved in (5) ml of Ethylene glycol in a water bath at $70C^0$. After dissolving, (15) ml of sterile DW was added to it and then filtered. After that, (5) ml of the filtrate solution (which was filtered by 0.22 micrometer diameter filter paper) was transferred into (200) ml of Lowenstein Jensen (L.J) agar, until its final concentration reached (40) microgram/ml.
- Streptomycin: (2) ml of a solution prepared by adding (59) mg of the antibacterial agent to (5) ml of sterile DW, then added to (8) ml of sterile DW. (5) ml of the latter solution was added to (245) ml of Lowenstein Jensen (L.J) agar, until its final concentration reached (4) microgram/ml.

3. Preparation of bacterial suspension

The bacterial suspension was obtained from the newly cultured bacteria after their growth on the solid agar (6-8 weeks after cultivation). The culture was diluted, then 10^{-1}, 10^{-3} and 10^{-5} cell / ml from the dilutions were cultured on culture medium containing rifampicin and on other culture media containing other antibacterial drugs prepared in the above paragraph.

Culture was also done on other media not containing antibacterial agents as a control. After incubation at $37C^0$ for 6-8 weeks, presence or absence of growth was observed as mentioned by.[19]

RESULTS

1.4 Isolation and diagnosis of *Mycobacterium tuberculosis*

The samples were examined by Ziehl-Neelsn stain and direct microscopic examination. The bacteria were identified according to their morphology under the light microscope at 100X magnification. They appeared as thin, straight or slightly curved bacilli with a red or pink color within a blue background as shown in figure (1). In this study, 64 samples (12.8%) showed positive results, and sputum samples recorded the highest positive result for the microscopic examination (15.9%), followed by the cerebrospinal fluid (14.2%), followed by the bronchial lavage (8.9%), and finally the pleural fluid sample which recorded (4.8%). No infection was recorded in ASF samples. Significant differences ($P<0.05$) were found between the samples with positive results.

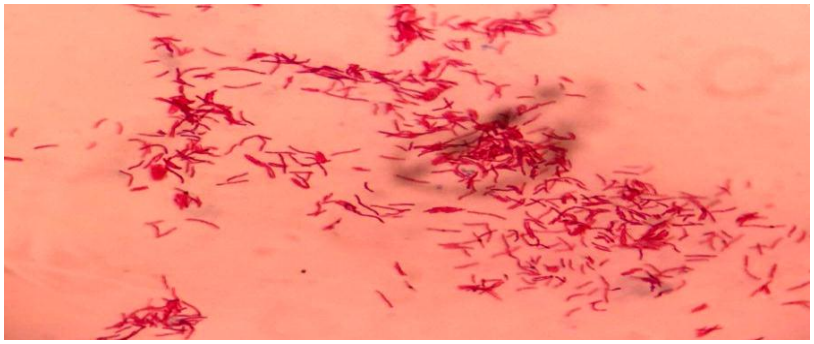

Figure (1): A acid – fast bacilli, Zeihl – Neelsen stain

All the (500) samples were cultured on the Lowenstein Jensen media (LJ). The culture results showed a true growth of 90 (18%) isolates. The T.B bacilli were diagnosed after growth on (LJ) media depending on colony color, shape and consistency. The colonies appeared as colorless to yellowish with rough surface and wrinkled colonies resembling cauliflower as shown in figure (2).

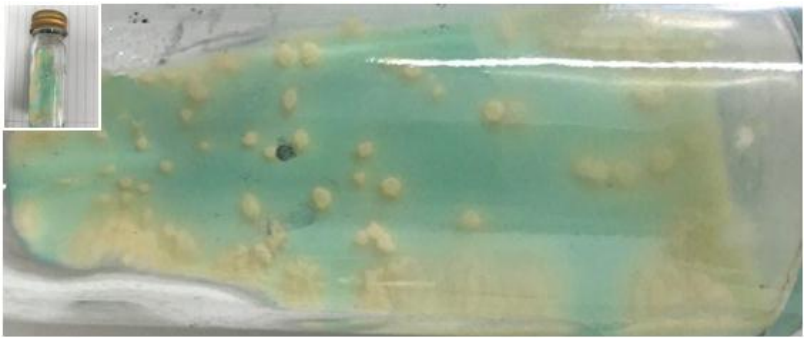

Figure (2): Shape colonies of *mycobacterium tuberculosis* on LJ media.

2.4 Relationship between age and tuberculosis

The age of patients in the current study ranged between (1- 96) years and was divided into age groups as shown in figure (3). The variance of the number of patients in each age group was seen with significant differences at (P <0.01). The highest percentage of patients (23.3%) was in the age group (21-30) years, followed by (22.2%) in the age groups (31-40) and (41-50) years, while the lowest percentage was (1.1%) was recorded in over than 85 years patients. No infection was reported in the (1-10) year's age group.

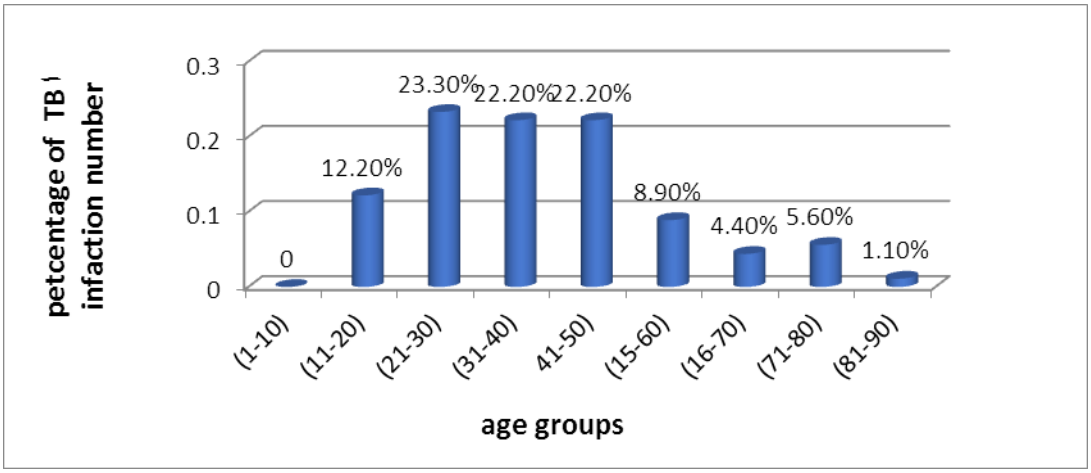

Figure (3) Represents the percentage of TB infection according to the age groups(P>0.0).

3.4 relationship between gender and tuberculosis

Results of the current study revealed no significant differences in the percentage of TB infection between both sexes. The percentage of infection in males was (51%0 in comparison with females (49%) as shown in figure (4).

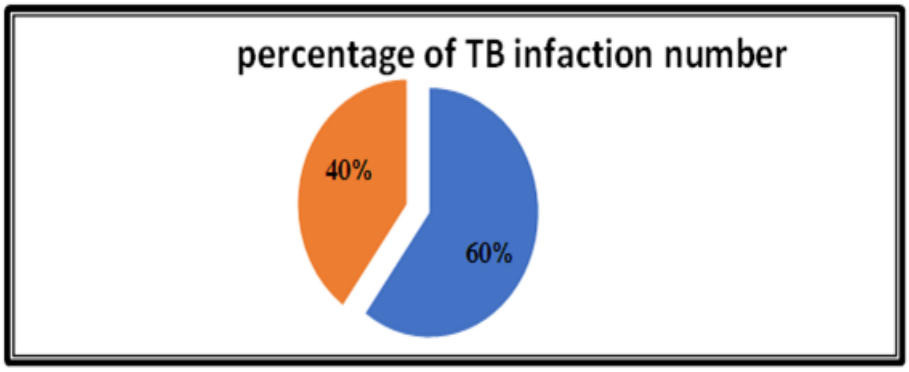

Figure (4) The percentage of TB infection according to gender.

4.4 Sensitivity of *M. tuberculosis* bacteria to first line antibacterial drugs

The sensitivity test was performed to (90) *M. tuberculosis* isolates against the first line antibiotics used for the treatment of tuberculosis including lsoniazid (I), Rifampicin (R), Ethambutol (E) and Streptomycin (S) by the Proportion method. These culture media contained the minimum inhibition concentration of anti-tuberculosis drug, as well as two control media which were free of antibiotics. The drug resistance of the tuberculosis bacteria was detected based on the phenotypic pattern of the bacterial colonies on the solid culture media.

The results of the sensitivity test showed that 48 out of 90 isolates (53.3%) had resistance to one or more antibiotics used in the current study, while 42 of 90 isolates (46.7%) were sensitive to all antibiotics. When the resistance property was distributed according the antibiotic type, it was found that resistance to lsoniazid resistance had the highest percentage 38.9% (35 out of 90 isolates), while resistance to Rifampicin was 25.6% and the resistance to Streptomycin was 17.8%, whereas the lowest resistance 11.1% (10 of 90 isolates) was recorded to Ethambutol antibiotic as shown in figure (5).

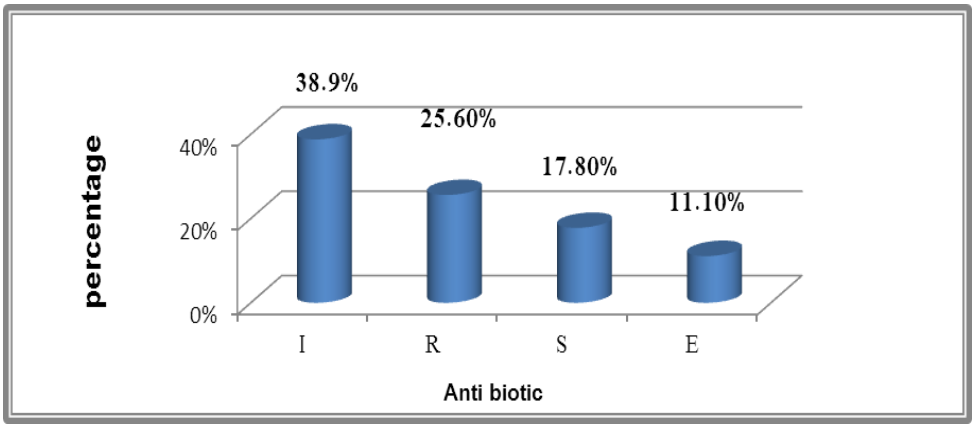

Figure (5): Percentage of number of antibiotics resistance to TB bacteria (P<0.01).

When the resistance property was studied according to the number of antibiotics, the isolates were distributed into: isolates which had resistance to one antimicrobial drug recorded 30% (27 of 90 isolates), isolates which were resistant to more than one antimicrobial drug 11of 90 (12,2%), while 10 isolates (11.1%) had multi- drug resistance with a highly significant difference at P<0.01) as seen in figure (6).

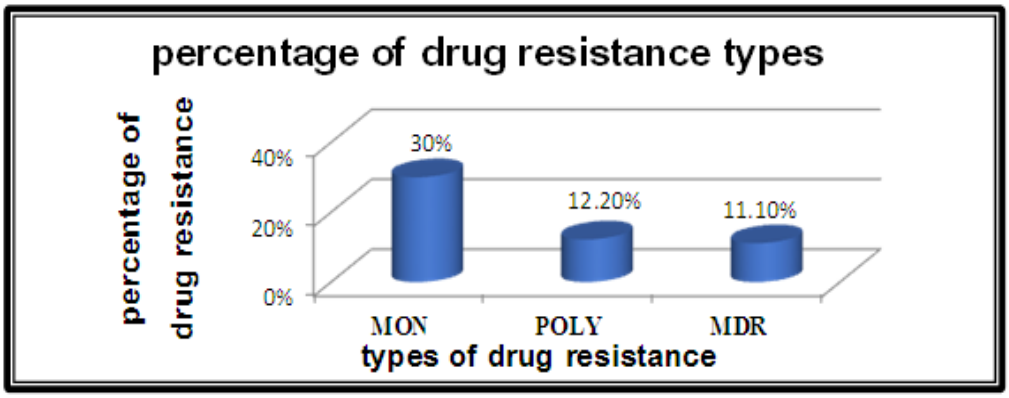

Figure (6): Percentage of multi-drug resistance to TB bacteria (P<0.01).

DISCUSSION

The results of culturing on (L.J) medium showed that (27) isolates gave negative results for direct microscopic examination although they gave bacterial growth. This indicates that culturing of samples on the solid medium is more sensitive than direct microscopy to detect tuberculosis bacilli, where the sensitivity of direct microscopic examination ranged between 22-76%, and at least 5,000-10,000 bacillus / ml were required to diagnose the bacteria. However, microscopy is an important means for the detection of TB bacteria due to its rapid and easy preparation, low cost and simple procedure.[20]

The absence of infection in the age group (1-10) years may be attributed to BCG vaccination of children, while the high rates of infection in the current study, which were recorded in the age groups (21-30), (31-40)and (40-51) may be linked to several causes, including smoking and other factors that inhibit the immune system, such as certain diseases. This finding is consistent with other studies such as Al-Rubaie who found that the highest rate of TB infection was in people with mean age 35 years.[21] The results also agree with a study carried out by Al-Karimi in Baghdad, who recorded the highest rate of infection 36.6% in the age group (21-30) years.[22] Worldwide, studies indicated that tuberculosis epidemiology is age-varying according geographical regions. In developed countries with aging populations, the incidence of tuberculosis is highest in the older-aged population (more than 65 years), while

in developing countries it occurs in infants under 1 year of age, as their immune system is still immature and weak in addition to the weak immunization programs.[23] The absence of infection in the age group (1-10) years in Iraq is indicative of the effectiveness of the preventive vaccination program within the national program to control tuberculosis.

The results of this study showed that the rate of infection in females is close to the rate of infection in males, and this is an important result showing a change in the type of infection by sex, since these results do not correspond to previous studies conducted in Iraq, when Al-Jubouri indicated that the incidence of the disease in males is higher than females[24], and with Abood (2004) who reported (65.42%) rate of infection in males which is higher than females (34.58%).[25] These findings also didn't agree with other results recorded in other areas in the world such as Rosales et al in Honduras, who found that the infection rate in males is (66.5%).[26] Our results indicated that the incidence of the disease in Iraq is heading towards a type similar to that reported by the World Health Organization in its report in 2016, with a female infection rate higher than that of males. The increase in the sensitivity of female infection with tuberculosis in Iraq in recent years increased the contribution of women to society as they become more subject to social contact, making them more vulnerable to infection. The World Health Organization (WHO) reported that tuberculosis is one of the most important infectious and death-related diseases among women worldwide, with about 750,000 women dying from the disease and about 3 million women infected.[27]

Results of the present study also showed that the infection rate by single drug resistant bacteria was the highest, while infection rate by multi-resistant anti-bacterial drugs was the lowest. On the other side, refampicin resistance was the highest, followed by isoniazid, streptomycin and euthambutol respectively. These results agreed partially with a study conducted in Nepal[28], which reported that the infection rate of single drug resistant bacteria was the highest (19.2%) of 50 isolates, while the number of multi-drug resistant isolates formed the lowest rate (16.1%) of 50 isolates. The emergence of isoniazid and rifampicin-resistant TB bacteria poses a fatal threat to the patient's life as these isolates are described as multi-drug resistant according to the WHO statistics in 2015 when about 480.000 people were infected with (MDR-TB). Moreover, resistance to refampicin antibiotic has arisen to be the most effective drug mong 100.000 persons approximately who required to treatment by MDR globally. Treatment with multi drug resistant TB is currently successful only for 52% of patients, and this variation in the multi-drug resistant pattern is the reason why the WHO is

calling for the short-lasting treatment program strategy under direct supervision to prevent TB in both rich and poor countries, which is the only effective method to reduce mortality rate and to limit TB distribution worldwide to prevent the emergence of multi-drug resistant TB bacteria.[29]

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