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Isolation of *Mycobacterium tuberculosis* and testing their susceptibility to antimicrobial agents by using Bactec 960.

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Abstract:

This study was done from 3/7/2011 to 1/1/2012 , different clinical samples were collected from (1235) patients that coming to the Specialized center for chest and respiratory diseases / Baghdad to investigate *Mycobacterium tuberculosis* and study their resistant to first and second line antimicrobial agents by using Bactec 960.

The results obtained from direct smear examination with Ziehl – Neelsen stain in (294) patients that indicated these patients are Infected with tuberculosis .These samples has been cultured on solid medium Lowenstein Jensen media (L.J.) and liquid medium (middle brook 7H8 broth base) in the Mycobacteria Growth Indicator Tubes (MGIT) using a device (BACTEC MGIT 960 System).Results of (275) samples that represented (22.27%) from total samples were showed a significant real growth that belong to bacteria *Mycobacterium tuberculosis*.

From (275) isolates tested to 1st line anti TB (streptomycin (SM), isoniazid (INH), rifampin (RMP) and ethambutol (EMB)), (196) isolates were resistant to the first line antimicrobial agents that used in the treatment of tuberculosis by using Bactec 960. (125) isolates (63.77%) showed resistance to multiple antimicrobial drugs (MDR-TB).

Furthermore, the sensitivity test was done on thirty isolates that characterized by multiple drug resistance criteria (MDR-TB) on second line antimicrobial agents to investigate Extensively Drug-Resistant Tuberculosis(XDR-TB) between these isolates. The results showed that the best antibiotics in their impact on the isolates (MDR-TB) is (PAS) P-aminosalicylic acid as the percentage of resistance was (3.3%).

Key Word: *Mycobacterium tuberculosis*, Bactec 960 , MDR-TB.

الخلاصة:

جمعت 1235 عينة سريرية مختلفة من المرضى الوافدين الى معهد الامراض الصدرية والتنفسية / بغداد تراوحت اعمارهم بين (4 أشهر الى 70 سنة) من كلا الجنسين للفترة من 3-7-2011 لغاية 1-1-2012 لعزل بكتريا *Mycobacterium tuberculosis* ودراسة حساسيتها لمضادات التدرن الخط الاول والثاني بأستعمال تقنية Bactec 960.

أظهرت نتائج الفحص المباشر للمسحات المصبوغة بصبغة زيل – نيلسون ان (294) مريضاً يعاني من مرض التدرن، زرعت العينات على الوسط الصلب Lowenstein Jensen media (L.J) والوسط الزرعي السائل Modified middlebrook 7 H8 broth base في انابيب خاصة Mycobacteria Growth indicator Tube (MGIT) باستعمال منظومة (BACTEC MGIT 960 System) وقد اظهرت النتائج ان (275) عينة (22.27 %) من المجموع الكلي للعينات نمواً حقيقيا وتبين انها تعود لبكتريا*Mycobacterium tuberculosis* .

أختبرت حساسية هذه العزلات لمضادات التدرن الخط الاول (الايزونيازيد والستربتومايسين والاثيمبتول والريفامبسين) باستعمال تقنية الباكتيك 960، وأظهرت النتائج ان (196) عزلة من اصل (275) كانت مقاومة لمضادات التدرن. من جانب اخر كانت (125) عزلة من اصل (196) بنسبة (63.77 %) مقاومة متعددة للمضادات (MDR-TB).

اختبرت حساسية (30) عزلة ذات مقاومة متعددة للمضادات تجاه مضادات الخط الثاني (السايكلوسيرين والاثينومايد والسبروفلوكساسين والاميكاسين والكانامايسين والريفابيوتين وكلاريثرومايسين والفانكومايسين) لدراسة المقاومة الواسعة (Extensively Drug-Resistant Tuberculosis) (DR-TB) بين العزلات المحلية لبكتريا التدرن .
أظهرت النتائج ان افضل المضادات في تاثيرها هو p-Aminosalicylic acid كانت نسبة المقاومة لها واطئة (3.3%).

Introduction:

Mycobacterium tuberculosis (*M. tuberculosis*) is one of the worlds' most successful and sophisticated pathogens. It is estimated that over 2 billion people today harbour latent *M. tuberculosis* infection without any clinical symptoms. Since most new cases of active tuberculosis (TB) arise from this (growing) number of latently infected individuals [1].

Mycobacterium tuberculosis is infecting one third of the global population. Currently, tuberculosis management and control is potentially devastating threat worldwide due to emergence of drug resistant strains. The modern, standard short-course therapy for TB recommended by the World Health Organization is based on a four-drug regimen that relies on direct observation of patient compliance to ensure effective treatment [2]. Globally, prevalence of tuberculosis (TB) has reached epidemic levels with ever increasing proportion of Multi-Drug Resistant (MDR) and more recently, extensively drug resistant tuberculosis (XDR TB) [3].

MDR TB cases threaten the effectiveness of chemotherapy for both treatment and control of TB and require the use of second-line drugs that are more expensive, toxic, and less effective than first-line anti-TB drugs[4]. Extensively drug-resistant (XDR) tuberculosis is defined as disease caused by *Mycobacterium tuberculosis* with resistance to at least isoniazid and rifampicin, any fluoroquinolone, and at least one of three injectable second-line drugs (amikacin, capreomycin, or kanamycin) [5].

Extensively drug-resistant (XDR) tuberculosis has received substantial attention in recent years, both from the general public and scientific communities [6]. Rapid methods enabling accurate susceptibility testing of first-line and second-line drugs are critical for the early diagnosis of MDR TB and extensively drug-resistant tuberculosis and the initiation of effective regimens. Various drug susceptibility testing (DST) methods that use solid media, including the agar proportion method (AP) and other methods, have the drawback of prolonged turnaround times (TATs). The World Health Organization and the U.S. Centers for Disease Control and Prevention have recommended the use of liquid culture systems for the diagnosis of tuberculosis and DST to improve TATs [7].

In the quest for new non-radiometric, culture-based strategies which allow both rapid detection of acid-fast bacilli and testing of susceptibility to antimicrobial agents, new liquid medium-based systems, such as the MB/BacT , ESP Culture System II, MB Redox, and the Mycobacteria Growth Indicator Tube 960, have become available. They all aim not only at recovering mycobacteria from clinical specimens but also at generating antimicrobial susceptibility testing (AST) data with a

shorter turnaround time than that observed with the current "gold standard," the agar proportion method [8]. The aims of this study were to isolation of *Mycobacterium tuberculosis* and testing their susceptibility to antimicrobial agents by mycobacterium growth indicator tube using Bactec 960.

Materials and Methods:

Sample collection:

Different clinical samples were collected from (1235) patients that coming to the Specialized center for chest and respiratory diseases / Baghdad to investigate *Mycobacterium tuberculosis*. The samples were from sputum (n=685), bronchial washing (n=196), biopsy (n=5), CSF (n=7), pleural fluid (n=138), Gastric lavage(n=71), Urine (n=71), laryngeal swabs(n=15), pus(n=9), ascitic fluid(n= 28) and others (n= 10).

Sample processing and inoculation:

Samples were homogenized and decontaminated with NALC-4% NaOH, 2.9% citrate (N acetyl L cysteine-sodium hydroxide citrate) method. Equal volume of NALC-NaOH citrate solution was added to the sample. After mixing and incubation at room temperature for 15 min, specimens were concentrated at 3000 g for 15 min. Supernatant was decanted and pellet was suspended in 2 mL (pH-6.8) of Phosphate Buffer Saline (PBS). A suspension of 500 µL and 100 µL was used to inoculate MGIT 960 tubes and Lowenstein Jensen (LJ) slants, respectively [8 , 9].

Biochemical identification:

The LJ slants were observed weekly till 8 weeks of growth. Growth was monitored for colony morphology, pigmentation and stained by Ziehl Neelsen (ZN) method to date it for presence of acid fast bacilli (AFB). The LJ positive for mycobacterium was subjected to conventional biochemical differentiation by niacin, nitrate and semi-quantitative catalase tests [10].

Drug susceptibility testing

Antimicrobial susceptibility testing for first and second line was done by agar dilution method using LJ medium in the Proportion method and by using Bactec 960. First line antimicrobial agents: streptomycin (SM), isoniazid (INH), rifampin (RMP), ethambutol (EMB). second line antimicrobial agents: (CS) Cycloserine, (ETH) Ethionamide; (CP) Ciprofloxacine; (AM) Amikacin; (KM) Kanamycin; (RFB) Rifabutin; (PAS) P-Aminosalicylic Acid; (CTH) Clarithromycin; (V) Vancomycin.

Stock solutions of antibiotics were prepared and sterilized by passing through a filter (0.22 µm). The stock solutions were stored at 4 ºC .The antibiotic solution of the required concentration was added to LJ medium and set in the slopes and inspissated at 80ºC[11,12]. The growth of the *Mycobacterium tuberculosis* was scraped from fresh LJ slants and suspended in 5 ml of distilled water. It was homogenised with glass beads by vortex and turbidity was adjusted to Macfarland standard 1 with distilled water. Three drops of this suspension were added to the drug containing bottles. The bottles were incubated at 37 ºC and were aerated twice a week for 3 weeks. Control strain used was *Mycobacterium tuberculosis* H37Rv (ATCC27294).

BACTEC MGIT 960 system.

Bactec MGIT 960 drug susceptibility testing supplement, 100 µl of the drug stock solution, and 0.5 ml of the suspension containing *M. tuberculosis* were added to an MGIT. The GC did not contain any drugs. Drug susceptibility testing sets were entered into the Bactec MGIT 960 instrument and continuously monitored until a susceptible or resistant result was obtained. The drug

susceptibility testing set results were reported by the instrument [8].

Results and Discussion:

The results obtained from direct Smear examination with Ziehl–Neelsen Stain in (294) patients that indicated these patients are Infected with tuberculosis .These samples has been cultured on solid medium Lowenstein Jensen media (L.J.) and liquid medium (middle brook 7H8 broth base) in the Mycobacteria Growth Indicator Tubes (MGIT) using a device (BACTEC MGIT 960 System).

A results of (275) samples that represented (22.27%) from total samples were showing a significant real growth that belong to *Mycobacterium tuberculosis* through doing biochemical tests (nitrate reductase) and (niacin-catalase examination), and largest proportion of bacterial growth was from sputum samples that had reached (32.8%) (Table-1). The underdeveloped world is still suffering from TB, as shown by the following statistics. The incidence of TB ranges from less than 10 per 100,000 in North America to 100 to 300 per 100,000 in Asia and Western Russia to over 300 per 100,000 in Southern and Central Africa. There is one death from TB every 15 s (over two million per year), and eight million people develop TB every year. Without treatment, up to 60% of people with the disease will die. Essentially all these cases are in the Third World [13].

Table-1 : Distribution of samples and the percent of the positive samples for *Mycobacterium tuberculosis.*

Sample	N0. of sample	Positive sample on LJ	%
Sputum	685	225	32.8
Pleural fluid	138	11	7.9
Bronchial washing	196	26	13.2
Urine	71	1	1.4
Gastric lavage	71	7	9.8
Laryngeal swabs	15	1	6.6
Pus	9	1	11.1
Ascistic fluid	28	2	7.1
Biopsy	5	1	20
CSF	7	0	0
Others	10	0	0
Total	**1235**	**275**	**100**

Isolation of *Mycobacterium tuberculosis* from biological samples is essential in drug resistance survey and initiating treatment for cases suspected to developed drug resistance. In cases like treatment after default, failure and relapse, it is important for clinicians to know the status of drug resistance to previous treatment before the initiation of alternative anti-TB regimens[9].The performance of the fully automated Bactec MGIT 960 (M960) system and agar proportion method for the testing of *Mycobacterium tuberculosis* susceptibility to streptomycin (SM), isoniazid (INH), rifampin (RMP), ethambutol (EMB), and pyrazinamide (PZA) was evaluated with 275 clinical isolates. From (275) isolates tested to 1st line anti TB , we found (196) isolates

were resistant to the first line antimicrobial agents that used in the treatment of tuberculosis by using Bactec 960. One hundred twenty five (125) isolates (63.77%) showed resistance to multiple antimicrobial drugs (MDR-TB), results also showed a highest proportion of resistance to antimicrobial (Isoniazid)(52.94%) ,while the lowest resistance was to antimicrobial (Ethambutol) (3.92%) (Table2).

Resistance to antituberculosis drugs has been noted since the drugs were first introduced, and occasionally outbreaks of drug-resistant tuberculosis have been reported worldwide Isoniazid and Rifampicin are the key stone drugs in the management of TB. While resistance to either isonicotinic acid hydrazide (INH) or rifampicin may be managed with other first line drugs, MDR-TB demands treatment with second line drugs that have limited sterilizing capacity and are more toxic [14]. Isoniazed resistance is the most common form of antituberculosis drug resistance encountered, whether in isolation or in combination with other drugs, INH is a prodrug that is activated by the catalase peroxidase enzyme encoded by the *kat*G gene to generate a range of highly reactive species which then attack multiple targets in *M. tuberculosis* [15]. A multidrug-resistant *M. tuberculosis* strain is currently defined as one that is resistant to at least isoniazid (INH) and rifampin (RMP) or more antituberculosis drugs. The timely and systematic monitoring of the susceptibility of *M. tuberculosis* isolates to front-line drugs is essential for (i) rapid detection of drug-resistant strains, (ii) effective treatment of patients, and (iii) prompt and adequate public health measures to prevent or reduce the spreading of drug-resistant TB. [16].

Table-2: Susceptibility of the 275 isolates of *M. tuberculosis* to First line antimicrobial agents.

Antimicrobial agents	Resistant %
Streptomycin (SM)	13.73
Isoniazid (INH)	52.94
Rifampin (RMP)	29.41
Ethambutol (EMB)	3.92

Furthermore, we were done the sensitivity test on thirty isolates that characterized by multiple drug resistance criteria (MDR-TB) on second line antimicrobial agents to investigate Extensively Drug-Resistant Tuberculosis (XDR-TB) between these isolates. The results showed that the best antibiotics in their impact on the isolates (MDR-TB) is (PAS) P-Aminosalicylic acid as the percentage of resistance (3.3%) (Table 3).

A study done in Iran reported 12 (10.9%) XDR strains from 113 multi-drug resistant tuberculosis strains [17]. In Hong Kong, nine out of the 75 multi-drug resistant tuberculosis strains (12%) had extensive drug resistance with simultaneous resistance to ethionamide, amikacin, ofloxacin and

cycloserine[18]. From India, 5 XDR-TB cases were recognized from 68 multi-drug resistant tuberculosis strains [19].

Drug-susceptibility testing is still widely used as a tool for the selection of effective regimens to successfully treat tuberculosis patients (particularly multidrug-resistant tuberculosis), as well as for evaluation of programme efficiency and development of strategies to cope with the problem of drug-resistant tuberculosis. However, to provide reliable results, the drug-susceptibility testing method under evaluation for routine use must be carefully calibrated with representative clinical isolates of *Mycobacterium tuberculosis* strains.

Table-3: Susceptibility of the 30 isolates of MDR *M. tuberculosis* to second line antimicrobial agents.

Antimicrobial agents	Resistant %
Cycloserine	10
Ethionamide	33.3
Kanamycin	10
Rifabutin	20
P-Aminosalicylic Acid	3.3
Clarithromycin	10
Ciprofloxacin	6.6
Amikacin	6.6
Vancomycin	10

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