# Rapidly growing non-tuberculosis mycobacterial (NTM) in sputum samples of Iraqi T.B. patients in TB reference lab. in Baghdad

**Hind. Hamed[1]*, Dhuha. S. Saleh[2]**
[1]Department of Anatomy, College of Medicine, University of Baghdad, Baghdad, Iraq.
[2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

**Abstract**
Non tuberculous mycobacteria (NTM) present in the environment, mainly in water and not transmitted from human to human. The lung is the most common target of NTM infections in human. The symptoms of NTM infection are: fever, weight loss and abdominal pain.
114 sputum samples were examined microscopically using Ziehl-Nelsen stain, all were positive. They cultured on Lowenstein-Jensen media.
The results of this study indicate presence of 3 (2.6%) of rapidly growing mycobacteria, phenotypically they resemble T.B. bacilli. They were diagnosed by biochemical tests, they were positive for catalase and pyruvate, negative for niacin, nitrate reduction test, sensitive for Thiophene-2-carboxyl acid hydrozide test& resistant for Para nitro Benzoic acid test. Drug susceptibility test showed that two of these isolates were resistant to isoniazid and rifampicin (MDR/multidrug resistance) while one isolate sensitive to all drugs. This study is a part of a subject aiming to estimate MDR-TB in Iraqi patient.
**Keywords:** NTM, MDR.

# المتفطرات سريعة النمو في قشع المرضى العراقيين المصابين بالسل الرئوي في المختبر المرجعي للتدرن في بغداد

**هند حامد شاكر *[1] و ضحى سعد صالح [2]**
[1]قسم التشريح، كلية الطب، جامعة بغداد، بغداد، العراق، [2]قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

**الخلاصة:**
المتفطرات سريعة النمو موجوده في البيئه وخصوصا في الماء ولا تنتقل من شخص الى اخر. المتفطرات سريعه النمو تصيب الرئتين واعراض الاصابه: الحمى، نقص في الوزن والم في البطن.
نتائج الفحص المجهري المباشر باستخدام صبغة زيل نيلسن كانت ايجابيه في 114 نموذجا من القشع ، تم زرع جميع العينات الموجبه لفحص القشع المباشر على الوسط الزرعي الصلب لوفنشتاين جنسن ، وبينت النتائج وجود 3 عزلات وبنسبة 2.6% من المتفطرات سريعة النمو. اخضعت العزلات الثلاث للفحوصات البايوكيميائية حيث اظهرت النتائج انها موجبه لفحص الكاتالايز والبايروفيت ، سالبه لفحص النياسين واختزال النترات ، حساسة لفحص TCH ومقاومه لفحص PNB. تم اختبار حساسية هذه العزلات لمضادات الخط الدوائي الاول واظهرت النتائج ان 2 من هذه العزلات كانت متعددة المقاومة الدوائية (MDR) من خلال مقاومتها للدوائين الايزونيازيد والرفامبسين بينما كانت العزلة الثالثة حساسة لجميع الأدوية. هذه الدراسة جزء من موضوع يهدف الى تقدير المقاومه الدوائيه للمرضى العراقيين المصابين بالتدرن الرئوي .

*Email: hindhamed75@yahoo.com

## Introduction

Non tuberculosis mycobacterial (NTM) infections cause morbidity worldwide [1]. Currently more than 125 different types of NTM exist in the environment. Those implicated in human disease can produce nonspecific symptoms [2], with clinical manifestations ranging from no symptoms or signs to destructive or even fatal disease. When symptoms and signs occur, they are often indistinguishable clinically and radiographically from those caused by Mycobacterium tuberculosis [3]. However, the pattern of resistance and treatment outcomes for NTM disease can be significantly different from TB, resulting in different implications for public health [4].

Though widely recognized for causing symptomatic disease in developing countries, the role of NTM in pulmonary or systemic disease in developing countries is not well described. This is largely attributable to lack of diagnostic facilities for culturing and identification of mycobacteria. Since TB is usually endemic and life threatening in these areas, patients are treated for TB [18].

There is no specific information regarding the role of NTM in causing infection or disease. Patients are treated based on sputum smear exams using standard first line TB therapy depending on clinical criteria in conjunction with World Health Organization (WHO) guidelines [5]. As NTM are often resistant to first-line anti-TB medication, presumably many of these cases would be considered treatment failures, and subsequently treated for multidrug resistant disease (MDR-TB defined as resistance to both isoniazed and rifampicin, the two most important first-line drugs) [6]. Because these bacteria considered as an important therapeutic problem. The present study aims to evaluate this percentage in a sample of Iraqi T.B. patients.

## Patients and method:

This study was done at chest and respiratory diseases center/TB reference lab NRL in Baghdad. Sputum samples were collected from each patient the first one was taken from patient where his/her arrival just reached the institute; second one was collected at next morning before breakfast; and the third one was collected after breakfast. All samples collected in sterile clean screw capped plastic cups were submitted for lab tests. All sputum samples were directly examined by (ZN stain) Ziehl-Nelsen stain technique and that was done according to Brooks and Brown [7, 8].

### Culturing sputum:

All sputum samples were cultured on conventional LJ media (Lowenstein-Jensen media) according to [9, 10]. The negative specimens were discarded after 8 weeks incubation and positive samples were reported. The results of culture were reported depending on IUATLD, as shown in table 1.

**Table 1-** result of culture on Lowenstein Jensen media as considered by International Union Against Tuberculosis and Lung Disease [11].

| **Reading** | **Report** |
|---|---|
| No growth | Negative |
| 1-9 colonies | Positive number of colonies |
| 20-100 colonies | + |
| 100-200 colonies | + + |
| 200-500 almost confluent growth | + + + |
| >500 colonies confluent growth | + + + + |
| Contamination | Contamination |

### Biochemical test:

Mycobacterial species were identified depending on some biochemical tests:

**Niacin test:** 1.5 distilled water was added to actively growing culture (4 weeks old/50-100 colonies), the surface of growth was scraped and stabs were made through the slops. The slops were stand horizontally for 30 minutes, at room temperature, 0.5 ml of the suspension was transferred to sterile tube, 0.5 ml of cyanogens bromide and 0.5 ml of the 4% aniline solution were added to the mixture. Yellow color was appeared within 10 minutes and that indicate that these colonies were *M. tuberculosis*, and the colorless was indicated that the colonies were NTM [12].

**Nitrate reduction test:** A loop full of actively growing culture (4 weeks old), was re-emulsified in 2 ml of nitrate substrate ($NaNo_3$ 0.1%), gently the suspension was shaken and incubated at 37°C in water bath for 3 hours, one drop of HCl was added to the mixture, followed by 2 drops of 0.2% sulfanilamide and 2 drops of 0.1% of N-naphthalene diamine, the mixture was examined immediately for the appearance of pale pink color which indicate presence of NTM [12].

**catalase test:** 1 ml freshly prepared Tween 80 hydrogen peroxide reagent was added to the L.J bottle inoculums. After 5 minutes, foam was

measured by a ruler and result reported, high catalase > 45mm of foam, low catalase < 45mm of foam [13].

**Thiophene-2-carboxyl acid hydrozide test (TCH):** 0.2 ml of mycobacterial suspension (dilution $10^{-3}$) was added to slop contain 2 µg/ml TCH and then incubated at 37°C for 4 weeks, NTM was sensitive to TCH, if there is no growth on media with TCH [14].

**Para nitro Benzoic acid test (PNB):** 0.2 ml of mycobacterial suspension (dilution $10^{-3}$) was added to slop contain 2 µg/ml PNB and then incubated at 37°C for 4 weeks. NTM was resistant to PNB, if there is growth on media with PNB [9].

**Drug susceptibility test (DST):**

Drug susceptibility tested by proportional method. After preparation of L.J media and before coagulates, the media were dispensed in 4 volumetric flasks (200 ml in each), and mixed with different antibiotics (streptomycin, isoniazid, rifampicin and ethambutol), the mixture were dispensed in screw-cap bottle and the coagulated performed at 85°C for 45 minutes. The bottles were in slant position in the oven to get slops. Mycobacterial suspension was prepared by added a loop full of actively growing culture (2-4 weeks) to 1ml of distilled water. The suspension was adjusted in turbidity to 0.5 McFarland standard visually. Then, five serial dilution were prepared ($10^{-1}$-$10^{-5}$). The dilution $10^{-2}$, $10^{-4}$ were discarded. L.J media with and without antibiotics were inoculated with different dilution ($10^{-1}$, $10^{-3}$, $10^{-5}$). The mixture were incubated at 37°C for 3-4 weeks [15] and [16].

**Statistical analysis:**

The chi-square test was used for the statistical analysis. A p-value less than 0.05 was considered significant as shown in figure 1 and table 2.

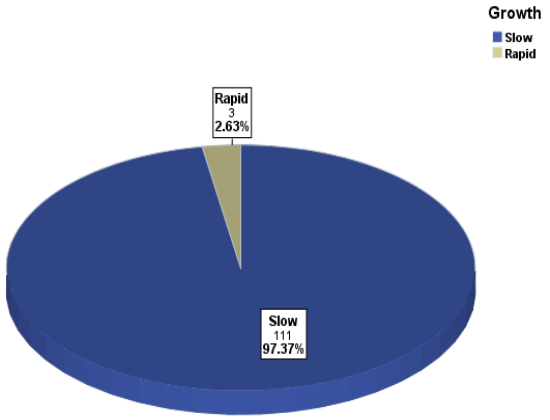


**Figure 1-** Distribution of study sample according to type of growth. P value < 0.001

**Table 2-** distribution of study sample according to growth.

| Growth | N (111) | 100.0% | P value |
|---|---|---|---|
| Slow | 111 | 97.4 | < 0.001 |
| Rapid | 3 | 2.6 | |

## Results

**Microscopic examination:**

This study was done at chest and respiratory diseases center/TB reference lab NRL in Baghdad during the period 1st of March 2012 to 1st of September 2012, 114 samples were examined under oil immersion lenses (power 100x) of light microscope, the presence of clumping rode shape bacilli with red color in blue background with mucous material and epithelial cells, positive results.

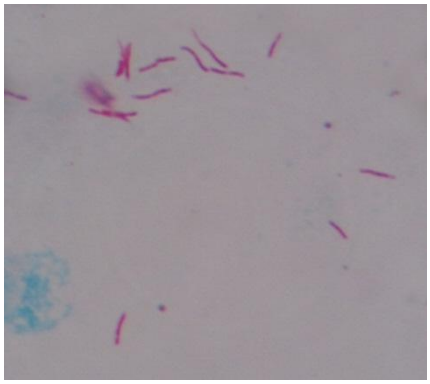

**Figure 2-** clumping rod shape bacilli in ZN stain (x100).

Phenotypically there were no significant differences between *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria as shown in figure 2.

**Culturing results:**

Culturing results indicate presence of rapidly growing, pigmented, small and convex colonies on three L J medium (2.6%) as shown in figure 3, while the results of 111 sputum sample appears as non-pigmented, large coliflower colonies. Culturing period was 8 weeks (slowly growing) as shown in figure 4.

Culturing time, colony shape and pigmentation were the most important criteria for differentiation between classical T.B. bacilli & the non –tuberculosis bacilli.

**Figure 3-** small, convex, and pigmented NTM colonies growth on LJ medium (Lowenstein-Jensen medium).

**Figure 4-** large, coliflower, and not pigmented *Mycobacterium tuberculosis* colonies growth on LJ medium (Lowenstein-Jensen medium).

**Biochemical tests:**

Application of biochemical test indicate that the three isolates of rapidly growing bacteria were positive for catalase and pyruvate, negative for niacin, nitrate reduction test. Sensitive for TCH and resistant for PNB, while the other 111 isolates of slowly growing bacteria were negative for catalase and pyruvate, positive for niacin, nitrate reduction test. Resistant for TCH and sensitive for PNB as shown in table 3.

**Table 3-** Identification of M.T.B cases by biochemical methods.

| | **Biochemical tests** | | | | | |
|---|---|---|---|---|---|---|
| | **Niacin production** | **Nitrate reduction** | **Catalase test** | **TCH (Thiophen-2-carboxylic acid hydrazid)** | **PNB (Para nitro benzoic acid)** | **Pyruvate medium** |
| **MTB[1]** | + (97.3%) | + (97.3%) | - (97.3%) | R (97.3%) | S (97.3%) | - (97.3%) |
| **NTM[2]** | - (2.6%) | - (2.6%) | + (2.6%) | S (2.6%) | R (2.6%) | + (2.6%) |

**[1]MTB = Mycobacterium tuberculosis**
**[2] NTM= Non-tuberculosis mycobacteria**

**Drug susceptibility test (DST):**

Drug susceptibility test showed that two of isolates were MDR according to their resistancy to isoniazid and rifampicin while the third isolate was sensitive for all therapeutic drugs.

**Discussion:**

According to the colonial morphology in LJ media, 3 isolates (2.6%) were NTM with convex, small pigmented rapidly growing colonies, and the results of biochemical test were showed that these isolates were Non-tuberculosis mycobacteria and all results were in agreement with studies on the same bacteria [16,17].

Drug susceptibility tests indicated that 2 isolates were MDR according to the resistance to isoniazid and rifampicin, while the third was sensitive to all drugs, these results were in agreement with other studies [18].

According to registering data from national reference laboratory in Iraq (NRL) there was 15 NTM cases from 1800 patients (0.8%) within the same period of the present study.

The International Union Against Tuberculosis and Lung Diseases (IUATLD) reviewed data from some countries and found the percentage of NTM in these countries: Denmark (5.3%), France (6.5%), Germany (12.2%), Italy (2.5%),

Turkey (33.9%) [19], Iran (14.5%) and North America (2%) [20].

NTM is a recognized cause of mycobacterial infection worldwide [21, 22]. Currently, very little data exists on the NTM species distribution in pulmonary specimens. Yet, it is well known that important differences in species distribution exist between countries and regions [23, 24].

In developing countries, the presumption is that most pulmonary symptoms resembling mycobacterial disease are caused by *M. tuberculosis*. This is largely because of the lack of appropriate diagnostics in resource-limited environments as well as the endemic nature of *M. tuberculosis* in these areas [25, 26].

NTM in this study was isolated from three tuberculosis patients. In a similar study it was found that patients infected with *Mycobacterium tuberculosis* were in some instances co-infected with NTM. NTM are found more easily in previously damaged or diseased tissue and that could explain why they were isolated from patients with pre existing TB and this support the results of the present study where the three cases were of chronic type [18].

In our study, 2.6% of clinically T.B. infection cases could be attributed to NTM. This suggests the need to consider NTM disease in patients who fail first line and re-treatment regimens. It is also highlights the need to study NTM infections in TB endemic areas and to evaluate the impact of these infections on TB disease. Multidrug resistance may erroneously be suspected in these patients. Careful and repeated bacteriological examination as well as clear communication between physician and microbiologist is crucial.

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