



## The First Molecular Identification of *M. fortuitum*, *M. porcinum* and *M. cosmeticum* from Pulmonary Patients with Negative Tuberculosis in Iraq

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### ABSTRACT

A total of 105 fresh sputum samples were collected from patients with negative acid-fast for pulmonary tuberculosis, which was obtained from the Chest and Respiratory Diseases Center in Baghdad city. The samples were decontaminated, concentrated and cultured on Lowenstein (LJ) medium, incubated at 28-30°C and observed the growth after 3 days-4 weeks. The results indicated Non-tuberculous mycobacteria (NTM) were isolated 4/105 (3.8%) from the patient, and all of these isolates belong to RGM (rapidly growing mycobacterium) based on their ability to grow on LJ medium, cultural characteristics, acid-fast stain, and biochemical tests. At molecular identification, *M. fortuitum* 50% (2/4) was isolated from a male who was smoking for a long time, *M. porcinum* 25% (1/4) was isolated from diabetic females and the last isolate was *M. cosmeticum* 25% (1/4). All the patients were elderly patients aged 41-75 years; this is the first study of isolating these species from pulmonary infections in Baghdad city.

**Keywords:** *M. porcinum*, *M. cosmeticum*, *M. fortuitum*, NTM, Pulmonary infection

### INTRODUCTION

Non-tuberculous mycobacteria (NTM) (also called environmental, atypical or mycobacteria other than Tuberculosis (TB) are mycobacterial species which are non-members of the *M. tuberculosis* complex and *M. leprae*. NTMs are free-living organisms in a variety of environmental reservoirs (natural, soil, and water, aerosols, protozoans, humans, and animals). Luckily, NTM is less virulent than the *M. tuberculosis* complex, but most NTM is a major cause of infection in both immunocompromised and immunocompetent persons, animals and birds [1,2]. Over 170 NTM species were identified [3]. Although the prevalence of TB has declined globally especially in the developed world, there is an increased incidence of humans NTM infections in last year's and will increase more in the future as a result of the growing numbers of immunosuppressant and modernity and the development of diagnostic techniques [3,4]. The source of human NTM infections occurs due to the environmental exposure, causing many infections features which depend on the location of the infection and the immune status of the individuals [5]. Non-tuberculosis mycobacteria pulmonary disease (NTM-PD) represents about 80%-90% of NTM-associated diseases, which is increasing and is considered as an emerging public health. NTM-PD occurs in humans with either an immunosuppressed or abnormal lungs structure, such as in bronchiectasis, cystic fibrosis, or Chronic obstructive pulmonary disease (COPD) [6-10].

Despite the rare transmission of NTM infection, the transmission directly from human to human was reported [6,11-15]. The most pathogenic NTM species are *M. avium*, *M. intracellulare*, *M. kansasii*, *M. xenopi*, and *M. abscessus* [16]. And the most common NTM-PD pathogens are *M. avium*, *M. intracellulare*, *M. chelonae*, *M. abscessus*, and *M. fortuitum*, the prevalence of these species vary with geographical area [17,18]. The diagnosis of NTM at the species level requires a long time by using conventional biochemical tests methods leading to non-significant identification [19]. So, use of 16S rRNA gene analysis is the gold standard method for significant and rapid identification of NTM for the correct epidemiological control study and treatments [20]. The aim of this study was to identify the NTM negative tuberculosis patients in the Baghdad city.

## MATERIALS AND METHODS

### Sputum Samples Collection

A total of 105 sputum samples were collected from November 2017 to April 2018, patients negative acid-fast for pulmonary tuberculosis was obtained from Chest and Respiratory Diseases Center in Baghdad City. About 5-10 ml of sputum was collected in a sterile container and transported to the laboratory in a cool box.

### Cultivation of Sputum

After digestion and decontamination according to Petroff's method [21], 0.1 ml of sediments was inoculated to the Lowenstein (LJ) medium and blood agar was incubated at 28-30°C and observed the growth after 3 days-4 weeks [21]. Ziehl-Neelsen stain was done before and after inoculation to the LJ medium. The isolates were identified by the growth characterization which includes pigmentation test, growth on MacConkey agar without crystal violet, catalase test (3 types), nitrate reductions, Simmons citrate utilizing test, NaCl tolerance, urease test, tween hydrolysis test, arylsulfatase test (3 and 14 days) and by molecular identification.

### Molecular Identification of NTM

The genomic DNA of the bacteria was extracted according to the protocol of Wizard Genomic DNA Purification Kit, Promega. Conventional PCR was performed to identify the NTM species on 16S rRNA using 27F and 1492R primers (27F AGAGTTTGATCTTGGCTCAG) and (1492R TACGGTTACCTTGTTACGACTT). PCR reaction mixture was adjusted to a total volume of 25µl, which included: 12.5 µl of PCR premix, 1µl of each forward and reversed primers, 2µl of DNA, nuclease-free water 8.5µl and 2µl of the template. The amplification mixture was transferred to thermocycler and the program was started for amplification as shown in Table 1.

**Table 1 PCR program for detection of 16s rRNA**

Step	Temperature (°C)	Time (m/sec)	No. of cycles
Initial denaturation	95	05:00	1
Denaturation	95	00:30	35
Annealing	60	00:45	
Extention	72	01:00	
Final extention	72	0.7000000	1
Hold	10	10.000000	

After PCR amplification, 1% agarose gel electrophoresis was used to confirm the presence of amplification. PCR products were then sent for the Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation-Korea. The results were analyzed and the similarity was achieved using Basic Local Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) and SepsisTest, EzTaxon.

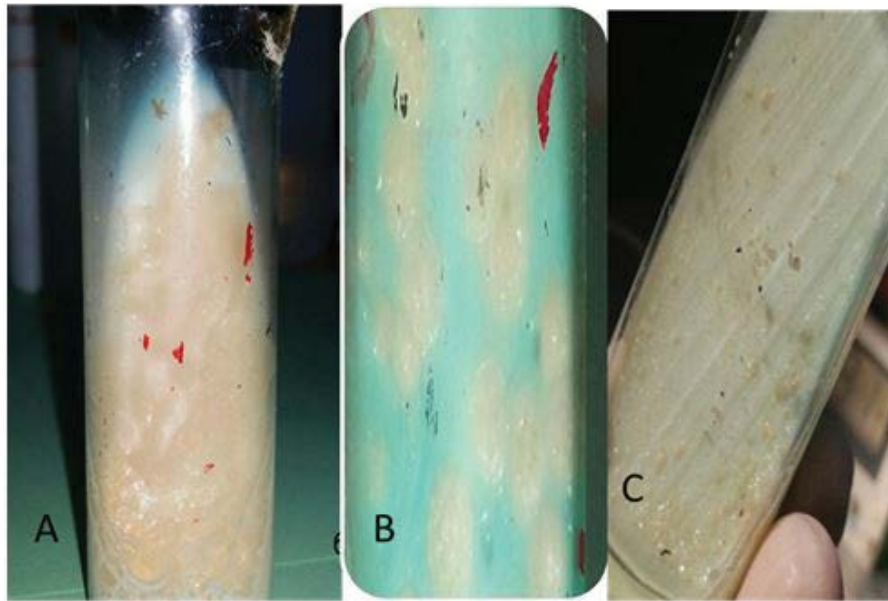
## RESULTS

### Cultural Characteristics and Biochemical Tests of the Isolates

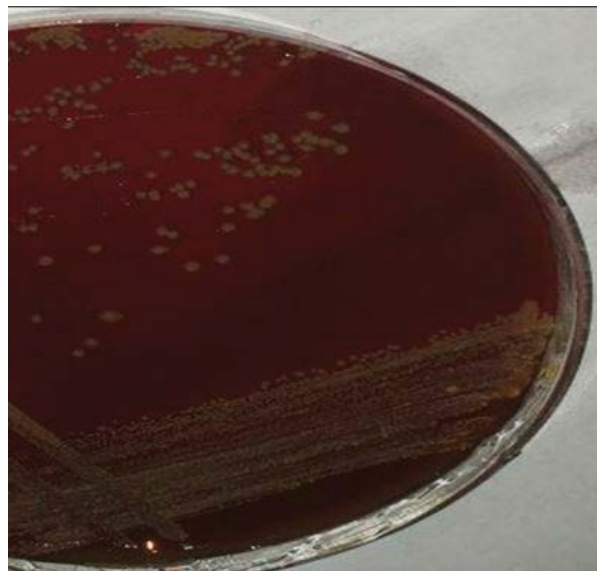
The isolates were grown at 28-30°C, two isolates were grown within 7-10 days and others were growing within 5-7 days, while in subculturing growth was within 3-4 days, except two isolate in which growth was within 5-6 days. Colonies on LJ were smooth white and creamy lobulated and/or white rough in long incubation tend to become pale yellow in subculturing, in which one isolate was scotochromogens (yellow-orange) (Figure 1). On blood agar medium it converted white color colonies to beige color (Figure 2). Three of the isolates didn't produce pigmentation in dark as well as when exposed to light.

On acid-fast stain short or long rods, no cord formation, no cross-barring, non-capsulated and non-spore forming,

some cell aggregates structured branching were observed (Figure 3). The growth on the MacConkey agar without crystal violet was observed as pink color colonies (lactose ferment) (Figure 4). Biochemical tests revealed that these isolates were negative for Simmons citrate agar, positive for urease, as well as nitrate reduction, catalase test at room temperature and at 68°C, semiquantitative catalase <45 mm was observed (Table 2). NTM were isolated 4/105 (3.8%) from a patient from TB center, and they belong to RGM based on their ability to grow on LJ medium, cultural characteristics, acid-fast stain, and biochemical tests.



**Figure 1** NTM on LJ isolated from human: A: *M. cosmeticum*, yellow to orange colonies color. B: *M. porcinum* white rough lobulated colonies. C: *M. fortuitum* white, round colonies.



**Figure 2** Showing growth of non-tuberculous mycobacteria on blood agar

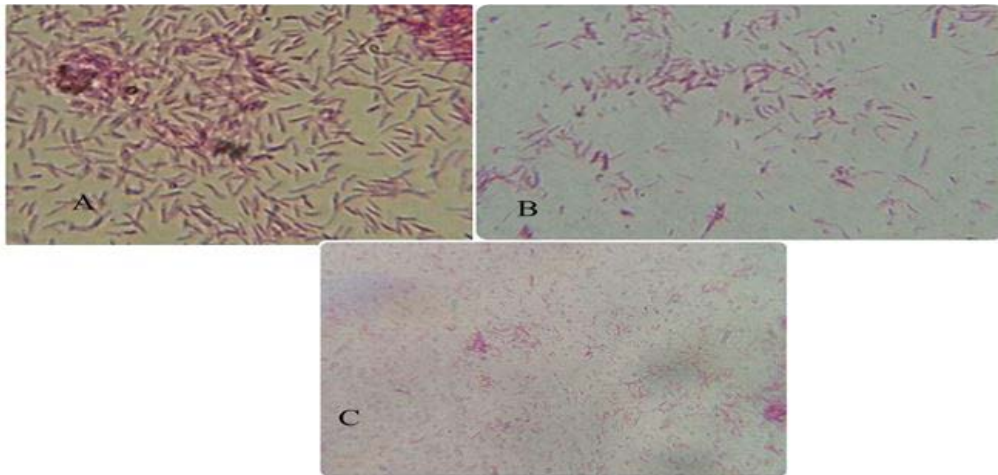


Figure 3 Showing acid-fast bacilli: A: *M. cosmeticum*, B: *M. porcinum*, C: *M. fortuitum*

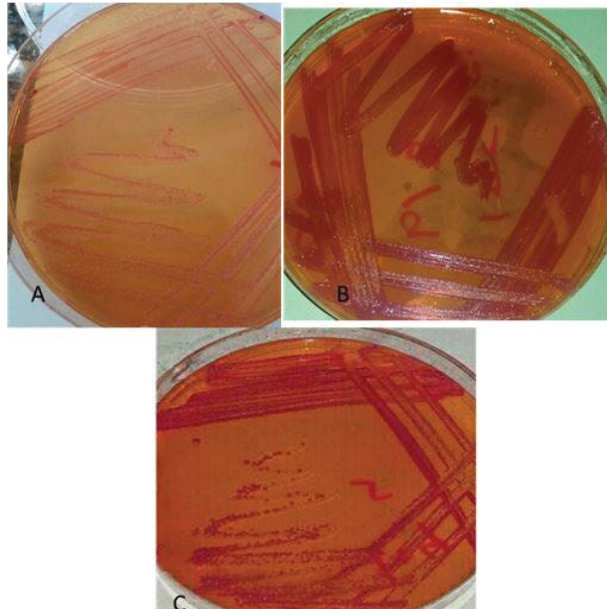


Figure 4 Showing A: *M. cosmeticum*, B: *M. porcinum*, C: *M. fortuitum* on MacConkey agar without crystal violet

Table 2 Grow on MacConkey agar without crystal violet and biochemical tests of isolated NTM species

*No of isolate/Spp	NaCl 5%	Growth time (days)	Growth on MacConkey agar	Subculture growth time	Mannitol sugar agar
<i>M. fortuitum</i>	+ve	5-7	+ve/pink	3-4	-ve
<i>M. fortuitum</i>	+ve	5-7	+ve/pink	3-4	+ve
<i>M. porcinum</i>	+ve	7-10	+ve/pink	3-4	-ve
<i>M. cosmeticum</i>	-ve	7-10	+ve/pale pink	5-6	-ve

\*Species identification by 16S rRNA gene analysis

**Molecular Identification**

Based on 16S rRNA gene analysis, similarity was achieved using Basic Local Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) and SepsisTest results which identified 4 species of GRM (NTM), 3 isolates belonged to *M. fortuitum* group which represents the highest percent (75%) including *M. fortuitum* 50% (2/4) similarity was 100% with *M. fortuitum* strain CT6, *M. porcinum* 25% (1/4) similarity was 100% with *M. porcinum* strain Y17 and *M. cosmeticum* 25% (1/4) similarity was 100% with *M. cosmeticum* strain 5102B.

According to age and gender, the results indicated that all isolates were isolated from aged patients age ranging from 41-75 years and both male and female. They had the same clinical signs of TB including a persistent cough, loss of appetite and weight loss. *M. fortuitum* was isolated from males; one of them was smoking for long period. *M. porcinum* was isolated from a diabetic female with no response to treatments (Table 3).

**Table 3 NTM species isolated according to age and gender**

No. of patient	Gender	Age (year)	Clinical signs	Notes	Species
1	Male	53	Persistent cough, loss of appetite and weight loss		<i>M. cosmeticum</i>
2	Male	75	Persistent cough, loss of appetite and weight loss	Smoking for a long time	<i>M. fortuitum</i>
3	Female	60	Persistent cough, loss of appetite and weight loss	Suffer from diabetes, don't a response to treatment	<i>M. porcinum</i>
4	Female	41	Persistent cough, loss of appetite and weight loss		<i>M. fortuitum</i>

### DISCUSSION

In the present study, *M. fortuitum*, *M. porcinum*, and *M. cosmeticum* were isolated from respiratory infections. There are no accurate or extensive studies on NTM in human in Iraq especially at the molecular identifications, we found 3.8% of NTM pulmonary infections which belong to RGM. This result agrees with Saleh, et al., who founded that NTM from sputum from TB patients was 114 (2.6%) in Baghdad city using Ziehl-Nelsen stain and cultural characteristics, and the 3 isolates belong to RGM [22]. Al-Mussawi, isolated biochemically 16/150 (10.6%) NTM from sputum samples from T.B suspected patients [23]. *M. chelonae* represents 4 (2.6%) in Basra, Iraq. Around 7 (4.6%) NTM species were identified among humans in patients with TB-like symptoms and isolated *M. fortuitum* (14.3%) [24]. Senanayake, et al., from 2004 to 2009 found 25% of NTM from pulmonary and extrapulmonary infections [25]. On another hand, Otchere, et al., isolated 43/1755 (2.5%) NTMs in human, Varghese, et al., found 5.39% NTMs pulmonary infections [26,27]. Although the incidence of NTM infections is increasing globally, the isolation rate of NTM is different according to a different area of the world [28], many factors contributed to these differences. So, it is hard to compare our results with other countries or even within the country published reports, due to several reasons: periods of study variation, type and numbers of samples (infected or uninfected) and feature of the people examined, also due to variations in dealing with samples, and isolation methods and variations in the prevalence of NTM [29].

NTMs and TB have the same conventional pulmonary infections and have similar clinical signs which lead to misdiagnosis as there is no sputum smear microscopic specificity [30-32]. NTMs are resistance to most first-line anti-TB drugs [6,33]. When NTMs are isolated from non-sterile sources such as bronchoalveolar lavage or sputum samples, especially with low colony numbers or where NTM are isolated from only one cultured specimen, it makes a firm diagnosis of NTM infections and is challenging to differentiate NTM infections from contamination, colonization or infection [34]. So, in the present study, the isolates were identified from a clinical patient with clinical signs resemble pulmonary TB, samples were kept away from environmental contamination. The patients were aged, and have a weak immune system, one had diabetes, and others were smoking since a long period, which means that the weak immune system takes a correct sample which is sputum, so the NTM isolates are the causative organisms in these patients.

*M. fortuitum* is one of the most common NTM-PD pathogens isolated from pulmonary infections in many reports, it isolated 7.92% in China and 1.2% in Zimbabwe [17,18,35,36].

In the past, *M. porcinum* has not been reported as a causative respiratory pathogen. Till Wallace, et al., found 52 cases of *M. porcinum*, only 4 isolates (8%) were identified from respiratory infection [37]. *M. porcinum* recovered 24 of 60 (40%) of GRM-NTM, 17 (70.83%) isolated from patients who had respiratory infections, in addition to others reports [35,38-41].

In medicine *M. cosmeticum*, is an opportunistic pathogen mostly causing cutaneous granulomatous lesions due to mesotherapy [42,43]. In the present study, the species is isolated from respiratory clinical specimens that agree with other studies, which is isolated from sputum specimens by Cooksey, et al., [44]. Reported 11.1% from 27 clinical NTM infections *M. cosmeticum* represent 2/3 (66.6%) of clinical patient sputum.

## CONCLUSION

Identification of mycobacteria are limited depending on morphology, growth characteristics and biochemical tests which may be given misidentification due to unreactive biochemical tests or new species discovered causing a confusion to the identification of the species level [45,46]. In addition, some mycobacteria species are similar phenotypically to each other causing doubt in diagnosis [47,48]. It is found that variable results of some of the biochemical tests attributed to using a large number of colonies and changes in the number of bacterial colonies that affect the biochemical reaction [49]. In the present study, variable results in one or two biochemical to the same species in different isolates have been found. Also in many studies, there are differences in some biochemical tests for the same species so in our study using DNA sequence analysis of the 16S rRNA gene region the gold standard was used for identifying the species level.

## DECLARATIONS

### Conflict of Interest

The authors have disclosed no conflict of interest, financial or otherwise.

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