Isolation and Molecular Identification of Nontuberculous *Mycobacterium* from Different Species of Fish in Karbala Province, Iraq

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**A B S T R A C T**

This study was designed for isolation and molecular identification of Nontuberculous *Mycobacterium* (NTM) from fish during the period between October and December 2017 from Karbla province-Iraq. This study included 200 fresh fish samples from four different species including *Spondyliosoma cantharus, Liza abu, Carassius carassius* and *Cyprinus carpio*. Three samples of each fish were taken including gills, muscles and all internal organs. The samples were processed by decontamination, concentration of 4% sodium hydroxide, and 0.1 ml of sediment was streaking on Löwenstein Johnson (LJ) media; then the bacterial cultures were incubated at 28-30 °C for 3 days up to 4 weeks and suspected colonies were stained with acid fast stain to confirm the presence of *Mycobacterium*. Further identification, biochemical tests were carried out to confirm the diagnosis of isolates, PCR was done using 16s RNA gene for all isolates, *hsp65* gene was used in unidentified NTM spp and to confirm the others. Results revealed that out of 200 fish samples, 19 isolates 9.5% were identified as NTM belonged to Rapid Growth *Mycobacterium* (RGM). of the total isolates, 18.26% was investigated from *Liza abu* (Kishni, Abu khraiza). NTM (RGM) isolates on spp level identified six spp of these isolates. *M. porcinum* was 26.32% which was followed by *M. fortuitum* of 21.05%, others included *M. neworleansense* and *M. mucogenicum* 10.5% of each, *M. cosmeticum* and *M. paliens* 5.26% of each. The distribution of NTM spp in the fish organs, nine out of 47.37% NTM isolate were recovered from gills followed by muscles 36.84 %, while 15.79% from internal organs. These results were the first study concerning isolation of these spp of NTM from fish in Iraq, and some spp are not reported in other studies. This study concluded that the fish is an importance source or reservoir for NTM, especially the pathogenic spp.

**Keywords**: NTM, fish, *Liza abu*, RGM, Iraq

**INTRODUCTION**

Nontuberculous mycobacteria (NTM) infections (*mycobacteriosis*) affect many aquatic or marine animals (fish, tropical aquarium fish, amphibians, and reptiles) causing chronic diseases, and are considered as a throughout the body by the circulatory or lymphatic system (1). The average of the incubation period of it is major cause of morbidity and mortality in free-living fish; and infections may lead to production and economic losses. Different clinical signs of mycobacteriosis in fish may be existed depending on the main sites of the infection and its severity or no clinical signs may associate, after invading the mycobacterial organisms into the body and spreading about 3 months and the form of the disease is either acute or chronic. In acute form, the infection is characterized by
heavy growth of the organism causing death of animals within 16 days (2-4). The chronic form, which is most commonly, is characterized by developing of granuloma in internal organs and animals may survive for maximum of 4 to 8 weeks. Signs may be seen with this disease including exophthalmos (bulging eyes), cachexia, pigmentation in fish body, ulcerative dermal necrosis, skeletal changes, swollen and distended abdomen, ulcers and eroded fins and tail rot, heavy mucus coating on the body surface, loss of scales, loss of appetite, weight loss, non-healing open ulcers, skeletal deformities such as curvature spinal or stunting defect pale gills lethargic. They may detach from other fishes and aggregate in corner of the holding facility (5). Nodular lesions on skin, miliary tubercles occurs in the liver, spleen and kidney which is histologically corresponding to granuloma; in addition to the fact that an affected fish may die, the mortality may reach up to 50% with no clinical signs (5, 6).

Some NTM pathogens that infect fish are highly virulent and are pathogenic to human; so, it may be considered as a zoonotic in a public health concern and its spread from fish, birds, and animals to human causing epidemics in nature (7-13). Conventional biochemical tests are not a significant method for NTM identification at the species level in addition, it waste of time (14). The sequencing of the 16S rRNA gene is universally the gold standard method, especially, for significant and rapid identification of NTM for the correct epidemiological control study and treatments, sequencing of the hsp65 gene as the second best alternatives (15-17). This study aimed to isolate and identification of NTM spp. from fish.

**MATERIALS AND METHODS**

**Sample Collection**

A total of two hundred fresh fish belonged to four fish species were collected from various fish selling markets at Karbala province, Iraq from October to December 2017 from Krabla city. Fish species included were Spondyliosoma canthus (n=35), Liza abu (n=104), Carassius carassius (n=46), and Cyprinus carpio (n=15). Fish were autopsied in a biosafety cabinet; an individual set of sterile instruments was used for each animal, 70% ethanol was applied as antisepsis to the body of the fish. Three samples were taken from each fish included all internal organs (O), Gills (G), and Muscles (M) were removed aseptically, and each part was cut into small pieces then transmitted to sterile porcelain mortar containing sterile normal saline (0.85%) have been mashed, then the supernatant transferred into sterile container (18).

**Cultivation of Samples and Identification of NTM**

After digestion and decontamination of samples according to (19-21) methods, a loop full of sediment was inoculated to the LJ medium (Hi media, India) and blood agar (Hi media, India) then incubated at 28-30 °C, and the growth observed within 3 days-4 weeks. Ziehl-Neelsen stain (Syrbio, Swiss) was done before and after culturing the samples; and the isolates were identified depending on colonies features, pigmentation production, ability of growth on MacConkey agar without crystal violet (Hi media, India), in addition to, biochemical tests which included catalase production test (room temperature, semiquantitative catalase), Nitrate reductions, Simmons citrate utilizing, NaCl tolerance, urea hydrolysis (urease) and arylsulfatase (at three and fourteen days).

Polymerase chain reaction (PCR) was performed after genomic DNA of the bacteria was extracted; and conventional PCR was done to detect two genes in clinical isolates of NTM including: universal primers 16S rRNA gene 1400pb for all isolates by using Forward primer- 27AGAGTTGTGATCCTGGCTCAG, reverse primer - 1942 TACGTTACCTGTGTTACGT and hsp65 gene 249pb was used for unidentified Mycobacterium spp.; then to confirm the others, Forward primer-GCCAAGAGACCCAGYAGCGT and Reverse primer – GGTGATGAGCCCTCGTTGC were used.

The PCR reaction mixture contained 25 µL volume as follows: 12.5 µL of PCR premix, 1 µL of each forwarded and reversed primers, nuclease free water 8.5 µL and 2 µL of the template. Then the mixture was amplified using thermocycler [Master cycler-nexus, Eppendorf, Hamburg. Company???] and the program’s conditions were as follows: initial denaturation at 95 °C for 5 min of one cycle; followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 45 sec, and extension at 72 °C for 1 min; final extension at 72 °C for 7 min and hold at 10 °C for 10 min (one cycle) (22). While the PCR program for detection of hsp65 was done according to Akbari et al. (23); initial denaturation at 94 °C for 2 min of one cycle; denaturation at 94 °C for 30 sec, annealing at 62 °C for 30 sec, and extension at 72 °C for 1 min (40 cycle each); final extension at 72 °C for 6 min and hold at 10 °C for 10 min (one cycle). After that, 1% agarose gel electrophoresis was applied to confirm the amplification.

The PCR products were sent to Macrogen Corporation in Korea for Sanger sequencing using an ABI3730XL automated DNA sequencer. The obtained sequences for 16S rRNA gene were analyzed using SepsTest software and compared with known sequences in the GenBank (http://www. ncbi.nlm.nih.gov), EzTaxon-e, the obtained sequences for hsp65 were compared with those present in GenBank, and hsp65-BLAST database (hsp65-BLAST).

**RESULTS AND DISCUSSION**

Nineteen isolates (9.5%) out of 200 fish samples were identified as NTM; all of the isolates belonged to Rapid Growth Mycobacterium (RGM) based on the ability of...
isolates to grow on LJ medium, acid-fast stain, colony characteristics and biochemical tests. The over all of isolates 18.26 % (19/104) were investigated only from Liza abu (Kishni, Abu khraiza) which were isolated in December. Seven spp. of NTM on spp. level were identified. M. fortuitum group was the most predominant spp. among fish isolates 15/19 (78.95%). M. porcinum reported 5/19 (26.32%) followed by M. fortuitum 4/19 (21.05%), other spp. included M. neworleansense and M. mucogenicum 2/19 (10.5%), M. cosmeticum and M. pallens 1/19 (5.26%); in addition, three isolates were unidentified on spp. level including (M. septicum, M. peregrinum, M. porcinum, M. neworleansense, M. boenickei, and M. septicum, M. boenickei, M. peregrinum). This result agrees with Abbas (24) who isolated Mycobacterium 64%, 54 % both of them were belong to RGM from rectal swabs of different fish species; 17 (68%) was from Liza abu in Baghdad province. The above high isolates in comparison to the present result may be attributed to that the samples were from rectum (fecal) which are often harbored and more contaminated with microorganism such as Mycobacterium and depending only on cultural characteristics compared with present study which was more accurately in diagnosis. The results were also in agreement with a study in Nineveh province when it was found that M. fortuitum was the most common isolates (10%), then M. marinum 3.4% from different fish species belongs to Cyphindae (25). In a survey in NTM of fish from pet shop fish, the common pathogens isolated were M. marinum and M. fortuitum (M. fortuitum group) (26). Other study NTM found it was about 11.9% in freshwater fish tissue and fish products and the NTM spp. were included M. porcinum, M. peregrinum, M. fortuitumand, M. phocaicum, and M. mucogenicum (27). As well as M. porcinum and M. septicum, M. peregrinum were identified from infected goldfish, Guppy, and exotic fish species (13).

The distribution of NTM spp. in fish organs, nine out of 19 (47.37%) NTM isolates were discovered from gills followed by muscles 7 (36.84 %) while 3 (15.79%) was from internal organs. M. porcinum was frequently isolated from gills and muscles (66.66%) of each. M. fortuitum (75%) was isolated from muscles. M. neworleansense was only isolated from gills and only two M. mucogenicum was isolated from muscles and internal organs, while other spp. had only one isolate of each organ. M. porcinum, M. neworleansense, M. boenickei, and M. septicum, M. peregrinum were isolated from gills and M. septicum, M. boenickei, M. peregrinum from internal organs (Table 1).

This to some extend came in line with a report of NTM were obtained from 1.7% fish; The species belonged to nine species using sequence analysis of the 16S rRNA gene, which was isolated more frequently from the skin and gills than from muscles or internal organs (28). This is the first time to isolate M. neworleansense, M. palls, M. cosmeticum and M. boenickei in Iraqi fish. The above spp. habitat in the Iraq environment, including water and the spp. were isolated from gills which might be colonized or transited in the gills although all NTM spp. were isolated in the present study showed to cause infection to man leading to variable clinical signs except for M. palls (29-32).

Das et al. (11) isolated the NTM from fish associated with granulomatous infection and the most of these pathogens were isolated from skin, gills and other tissues that confirmed by biochemical tests. the above suggested that the infection sound to be affected by the weather temperature, considering that low temperature is favorited for NTM pathogens. The above may agree with other studies in which same NTM spp. were isolated from fish and man in addition to other studies reffering to the possibility of getting infection from fish to man as the fish and its products may condiser as an important source of mycobacterial infections. Furthermore, (Kishni, Abu khraiza) reported the harboring of NTM may suggest that fish spp. is most important sources of NTM infections in human (11, 33, 34).

Table 1. Numbers and percentages of nontuberculous mycobacteria (NTM) spp. isolated from fish internal organs, Gills, and Muscles

| Nontuberculous mycobacteria spp. | Internal organs | Gills | Muscles |
|---------------------------------|----------------|-------|--------|
|                                 | Number | %    | Number | %    | Number | %    |
| M. porcinum                     | 1      | 33.33| 2      | 66.66| 2      | 66.66|
| M. fortuitum                    | 1      |      | 1      | 25   | 3      | 75   |
| M. neworleansense               | 1      |      | 2      | 100  | -      | -    |
| M. boenickei                    | 1      |      | 1      | 100  | -      | -    |
| M. porcinum, M. neworleansense, M. boenickei | 1 | 50  | -     | -    | 1(50) | 50   |
| M. septicum, M. boenickei, M. peregrinum | 1 | 50  | -     | -    | 1(50) | 50   |
| M. septicum, M. peregrinum      | 1      |      | 1      | 100  | -      | -    |
| M. mucogenicum                  | 1      | 100  | -      | -    | -      | -    |
| M. palls                        | 1      |      | 1      | 100  | -      | -    |
| M. cosmeticum                   | 1      | 100  | -      | -    | -      | -    |
| Total                           | 3      | 15.79| 9      | 47.37| 7      | 36.84|
The cultural characteristics of the NTM isolates of multiplying colony types appeared on the media and the isolates grew at 28-30°C within 5-10 days as range in the first isolation. In sub culturing grew within 5-6 days. *M. fortuitum* group colonies had a smooth white, creamy some of them appeared as a rough, wrinkled, and irregular edge on LJ. In long incubation, it tended to be white to slightly beige, small in diameter and translucent on blood agar for 3 days at 35°C. *M. mucogenicum* isolates were mucoid. The colonies of *M. pallens* and *M. cosmeticum* isolates had an orange-yellow color, Scotochromogens and the other isolates had non-produced pigmentation neither in dark nor after exposing to light (Figures 1, and 2). All the isolates grew on MacConkey medium without crystal violet except *M. pallens*; and the reaction on this media were differentiated by lactose ferment (pink) and lactose non ferment (pale yellow).

In addition to the above, all the *M. fortuitum* group isolates had pink color while the two isolates *M. ucogenicum* had a pale yellow; and on acid fast stain, the isolates of NMT were short-long rods and no cord formation, noncapsulated and spore forming (Figures 3 and 4). Biochemical tests revealed these isolates were negative for Simmons’ citrate, positive for urease, Catalase test at room temperature and semiquantitative catalase < 45 mm. Other biochemical showed differences between isolates as it is shown in (Table 2). These results were similar to the phenotypic characteristics described previously (16, 35, 36). Identification of Mycobacteria is limited by using classical methods such as morphology, growth characteristics and biochemical tests which may give an inaccurate or limited diagnosis due to unreactive biochemical tests, new or novel Mycobacterium spp. discovered causing a confusing to identify at species level (38, 39). Moreover, some Mycobacterium spp. are phenotypically similar, causing suspicion in the diagnosis (40, 41).
Table 2. Biochemical tests result of the nontuberculous mycobacteria (NTM) spp

| No. | Species (No. of isolate) | Nitrate Reduction | NaCl 5% | Catalase test at 68 °C | Growth on MacConkey | Mannitol sugar agar |
|-----|-------------------------|------------------|--------|------------------------|---------------------|-------------------|
| 1   | *M. fortuitum* (4)      | +                | +      | 2 +ve, 2 +ve           | +ve, pink           | -                 |
| 2   | *M. porcinum* (5)       | +                | +      | +                      | +ve, pink           | -                 |
| 3   | *M. neworleansense* (2) | +                | +      | +                      | +ve, pink           | +                 |
| 4   | *M. boenickei* (1)      | -                | +      | +                      | +ve, pink           | -                 |
| 5   | *M. cosmeticum* (1)     | +                | +      | +                      | +ve, pink           | -                 |
| 6   | *M. mucogenicum* (2)    | -                | -      | -                      | +ve, pale, yellow   | -                 |
| 7   | *M. pallens* (1)        | -                | -      | -                      | -                   | -                 |

The 16S rRNA gene was amplified from 19 fish isolates using conventional PCR, which was amplified a product size of approximately 1400 bp (Figure 5). In all cases, the sequence similarity reading for all four public databases ranged between 95.19-100%. SepsITest blast ranged between 97.2-100%, Genebank 96.63-100%, EzTaxon-e 95.19-10 and 0% Hsp-BLAST 100%. According to this database reading in this study, a similarity of 16S rRNA or hsp65 gene and 16S rRNA were reported and fish isolates were 7 isolates give similarity 100%: 3 isolates were *M. fortuitum*, 3 isolates were *M. porcinum*, 1 isolate was *M. pallens*, while 10 isolates give range of similarity between 95.19-99.9%: 1 isolate were *M. fortuitum*, nine isolates were each isolate was gave vary between two or three species (Table 3). This isolate can be differentiated using biochemical test on isolate even if biochemical test could not be differentiated on level spp. Finally, the other two isolates divided into one had only *M. spp.*, and one was unidentified by 16S rRNA. Therefore, hsp65 gene to detect 5 spp. was used and this gene gave 100% similarity to these isolates with exception to two isolates in which they varied between two or three spp. These isolates were not differentiated on spp. level as shown in Table 3 even by biochemical test. In study of Joao et al., (43) mycobacteria based on two highly conserved genes 16S rRNA and hsp65 were used to identify the gen and several public databases to perform the data analysis explaining the cause of using two genes, the 16S rRNA which is universally used for bacteria identification. Nevertheless, this has several limitations even so it is rare in some cases (*M. terrae* and *M. celatum*complex); and two copies of the 16S rRNA gene, with different sequences, could be found in the same mycobacteria (44). Williams et al. (45) used “16S rRNA sequence analysis for successful performance of 30 NTM isolates giving results of two isolates to fail of show 16S rRNA PCR product and in this study, the identification was taken as the closest match generated by GenBank blast search analysis, had similarities ranged from 96 to 100% homology over fragment sizes of 448 to 635 bp. They explained the above as there were no standards for the interpretation and analysis of sequence data in a diagnostic setting.

Therefore, all results should be interpreted on an individual basis in conjunction with the clinical information; and sequence analysis of 16S rRNA did not differentiate among *M. peregrinum*, *M. septicum* and *M. fortuitum*; *M. kansasii* and *M.gastri*; *M. mucogenicum* and *M. ratisbonense*; *M. chelonae*, *M. abscessus* group and *M. fuerth* and they suggested that the reason is according to what Han et al. (46) has referred to. The inability to distinguish between *M.*
kansasii and M. gastri using 16S rRNA gene sequence analysis has been noted previously, however, these species could be differentiated by culture characteristics. The study concluded that fish is a potential source of pathogenic NTM spp. that may increase the risk of public health. In addition to that, using sequence analysis for hsp65 gene with 16S rRNA primer and using multiple public databases are good methods for identifying NTM at the species’ level.

Table 3. Results of Sequences Analysis and Identification of nontuberculous mycobacteria (NTM)S of Fish isolates by four databases

| Identification | 16S rRNA (similarity %) | hsp 65 gene sequencing (similarity %) | Differentiated by Biochemicals |
|----------------|-------------------------|-------------------------------------|-------------------------------|
|                | 1: sepsiTest blast 2: GenBank, 3: ExTaxon-e | 1: GenBank, 2: Hsp -BLAST |
| M. porcinum    | 100 99.9 99             | Not done Not done                   | M. porcinum                   |
| M. neworleansense | 100 100               | Not done Not done                   | M. porcinum                   |
| M. fortuitum   | 99.3 99.3 99.3         | Not done Not done                   | M. neworleansense             |
| M. boenickei   | 99.7 99.7 99.7         | Not done Not done                   | M. boenickei                  |
| M. fortuitum   | 100 100 99             | Not done Not done                   | M. boenickei                  |
| M. porcinum    | 99.7 99.7 99.8         | Not done Not done                   | Not done                      |
| M. boenickei   | 99.8 98.8 99.8         | Not done Not done                   | M. neworleansense             |
| M. porcinum    | 100 99.9 99             | Not done Not done                   | Not done                      |
| M. neworleansense | 100 100               | Not done Not done                   | M. porcinum                   |
| M. boenickei   | 100 100 100            | Not done Not done                   | M. neworleansense             |
| M. fortuitum   | 100 100 100            | Not done Not done                   | Not done                      |
| M. pallens     | 100 100 100            | Not done Not done                   | M. porcinum                   |
| M. porcinum    | 100 100 100            | Not done Not done                   | M. neworleansense             |
| M. boenickei   | 100 100 100            | Not done Not done                   | M. porcinum                   |
| M. neworleansense | 100 100           | Not done Not done                   | M. neworleansense             |
| M. boenickei   | 100 100 100            | Not done Not done                   | Not done                      |
| M. peregrinum  | 100 100 100            | Not done Not done                   | M. peregrinum                 |
| M. mucogenicum | 100 100 100            | Not done Not done                   | M. mucogenicum                |
| M. palens      | 100 100 100            | Not done Not done                   | M. mucogenicum                |
| M. septicum    | 100 100 100            | Not done Not done                   | Not done                      |
| M. peregrinum  | 100 100 100            | Not done Not done                   | M. septicum                   |
| M. boenickei   | 100 100 100            | Not done Not done                   | M. septicum                   |
| M. palens      | 100 100 100            | Not done Not done                   | M. septicum                   |
| M. porcinum    | 99.8 96 95.19          | Not done Not done                   | M. boenickei                  |
| M. mucogenicum | 99.6 99.47 99.86       | Not done Not done                   | M. mucogenicum                |
| M. scrofulaceum | 96 96 96              | Not done Not done                   | Not done                      |
| M. peregrinum  | 96 96 96              | Not done Not done                   | M. peregrinum                 |
| M. boenickei   | 96 96 96              | Not done Not done                   | Not done                      |
| M. septicum    | 96 96 96              | Not done Not done                   | M. septicum                   |
| M. peregrinum  | 96 96 96              | Not done Not done                   | M. septicum                   |
| M. boenickei   | 96 96 96              | Not done Not done                   | M. peregrinum                 |
| M. porcinum    | 96 96 96              | Not done Not done                   | M. peregrinum                 |
| M. neworleansense | 96 96           | Not done Not done                   | Not done                      |
| M. boenickei   | 96 96 96              | Not done Not done                   | M. neworleansense             |
| M. septicum    | 96 96 96              | Not done Not done                   | Not done                      |
| M. peregrinum  | 96 96 96              | Not done Not done                   | M. septicum                   |
| M. boenickei   | 96 96 96              | Not done Not done                   | Not done                      |
| M. porcinum    | 96 96 96              | Not done Not done                   | M. peregrinum                 |
| M. neworleansense | 96 96          | Not done Not done                   | M. neworleansense             |
| M. boenickei   | 96 96 96              | Not done Not done                   | Not done                      |

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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العزل والتشخيص الجزيئي للمتفطرات اللاسلية من مختلف أصناف الأسماك في محافظة كربلاء، العراق

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الخلاصة

صممت هذه الدراسة لعزل وتشخيص المتفطرات اللاسلية من الأسماك في محافظة لفترة خلال شهري أكتوبر وديسمبر 2017. تم جمع 200 سمكة من اربعة أنواع مختلفة من الأسماك حيث شمل هذا العد الأنواع Cyprinus carpio, Carassius carassius, Liza abu, Spondylotosoma cantharus, 15 سمكة من نوع Cyprinus carpio, 46 سمكة من نوع Liza abu, 104 سمكة من نوع Spondylotosoma cantharus. نقلت كل سمكة إلى مختبر الأسماك في كربلاء لإجراء اجراءات التصوير وعينات معالجة. تم حث السمكة في درجة حرارة تراوح بين 28-30 درجة مئوية لمدة 3 أيام - 4 أسابيع، صبغت المسماوات بصبغة acid fast لتشخيص الفطرات اللاسلية. تم تحليل السمكة من Point Palens exceptional DNA تكاثرها. هذه العدوى تأكد باختبارات كيميائية وتشخيص جين psp RNA في جميع السامك. تم اجراء جين 16sRNA Psp بالاضافة إلى استعمال جميع العينات في مختبر الأعضاء. و تم استخدام جميع العينات في مختبر الأعضاء من_point_Palens DNA. أظهرت النتائج أن 26.32% من M. porcinum متفطرات اللاسلية كانت تتأثر بالمتفطرات اللاسلية، بينما كانت 21.05% من M. fortuitum M. mucogenicum M. pseudotuberculosis M. novaearleanensis. المتفطرات اللاسلية كانت 47.37% من العينات تتأثر بالمتفطرات اللاسلية، بينما كانت 15.75% من العينات تتأثر بالمتفطرات اللاسلية. كشفت هذه الدراسة لأول مرة تأثيتساء للحماءات في الأسماك في العراق، ما يجعل هذه الدراسة مهمة لتحديد الفطرات اللاسلية وتحديد العينات للحماءات في الأسماك في العراق.