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MOLECULAR IDENTIFICATION OF MYCOBACTERIUM ISOLATED FROM BIRDS IN IRAQ

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Abstract

110 samples from birds species (42- chickens, 15-pigeons, 25-pet birds, 11-turkeys, 1- ostrich, 10-ducks, 6-parrots) samples of birds was included liver, spleen, intestine and rectal swab, fifteenths isolates (13.6%) out of 110 birds samples were identified as *mycobacterium spp*; all of isolates were belong to mycobacterium based on their ability to grow on LJ medium, cultural characteristics, acid-fast stain and biochemical tests. The (11.9 %) (5/42) from Chickens, (30%) (3/10) from Duck, (20%)(3/15) from Pigeons (12%) (3/25) from Pet birds, (16.6%)(1/6) from Parrot Mycobacterium isolates were identified 5/15 (33.3%) isolate was belong to *M. tuberculosis, 3*/ 15 (20%) isolate was belong to *M. intracellulare,* group of these isolates *were* 1/15 (6.6%) isolate was belong to *M. avium, group of isolates* were reported 3/15(20%)to belong *M. spp,* followed by *M. tuberculosis Complex* 1/15 (6.6%) other spp Include *M. abscessus* 1/15 (6.6%), *M. fortiutum* 1/15 (6.6% ten out of 15 (66.66%) isolate were recover from rectal swap followed by liver 2(13.3%), isolate 1 (6.6) from spleen, while 2 (13.3 %) from Intestine, from rectal swap *M. tuberculosis* isolated 2 /10 (20%), *M. intracellular* 2 /10 (20%). *M. tuberculosis complex* 1/10 (10%) *M. avium*, 1/10 (10%), *M. ssp* 2/10 (20%), *M. abscessus* 1/10 (10%) and *M. fortuitum* 1/10 (10%), from intestine *M. tuberculosis* 2/2 (100%) and other isolates from liver as *M. tuberculosis* 12(50%) and *M.spp* 1/2(50%). From spleen *M. intracellulare* 1/1 (25%) These results were the first study isolated this spp of mycobacteria in birds in Iraq

Keywords: *M. tuberculosis, M. intracellulare, M. avium, M. tuberculosis, Complex M. abscessus , M. fortiutum.*

Introduction

Mycobacteria are aerobic, non-motile, acid-fast bacteria There are more than 150 species of mycobacteria, which can be classified based on, growth rate, growth substrate and pigmentation, Mycobacteriosis is granulomatous infectious disease that affects liver, spleen, intestine, and bone marrow, lungs, ovary, oviduct and testes (Fulton and Thoen .2003).All bird species can be infected, but classic tubercles rarely develop in Passeriformes, Anseriformes Columbiformes, Psittaciformes, (Bougiouklis *et al.*, 2005; Abbas, 2008; Gavrilina *et al.*, 2019). Mycobacteria in birds, principally *Mycobacterium avium*, *M. intracellulare*. (Grange *et al.*, 1990, Tortoli, 2006; Zhu *et al.*, 2019), avian tuberculosis caused by *M. avium* (Dorrestein, 1997, Dvorska *et al.*, 2007; Shitaye *et al.*, 2008; Kriz *et al.*, 2010). *Mycobacterium fortuitum* is the most pathogenic nontubercolus mycobacteria occasionally cultured from lesions in avian species (Hoop *et al.*,1996, Brown-Elliott *et al.*, 2002). Infection of birds with *M. tuberculosis* in birds. Has been documented in exotic carnivores and psittacinebirds is of critical importance to wildlife veterinarians, public health officials and zoos. (Thoen *et al.*, 1977; Kearns, 2003 Jasim *et al.*, 2010). For rapid identification of mycobacteria 16S rRNA gene is the best method (Issa & Salman, 2017). This study aimed to identify mycobacterium in birds.

Materials and Methods

Samples Collection

A total of (110) birds were chickens, duck, pigeon, pet birds, turkey, parrot, ostrich (rectalswab, spleen, liver, intestine) were collected in Baghdad collected from (December 2018 to April 2019) governorate. The samples from birds were aseptically, each part was cut into small particles and transmitted to sterile mortar contain sterile (0.85%) normal saline and grounded, then the supernatant transfers into sterile tube (Pfyffer, 2015). Each sample were digested and decontaminated, sterile 4% NaOH in equal amount of was added. Then incubated at 37°/ 15 min with shaking. Then centrifuged at for 15 min at 3,000 rpm and The deposit was neutralized by 2N HCL. 0.1ml. of deposit was inoculated by 0.1ml. of deposit and incubated at 28-30°C, (Petroff, 1915; Cruickshank *et al.*, 1975 and observed the growth after 3day- 8weeks. At the same time from the sediments of each sample, smears were prepared and stained with ziehl-Neelsen. TBc ID test: take 100 µl of liquid culture (suspension) add directly to the TBc ID test. The end result after 15 min. The presence of two color line positive for *M. tuberculosis* complex. however, if given one color line was considered as negative for *M. tuberculosis* complex (Martin *et al.*, 2011)

Also cultured on blood agar as sub culturing at 28-30°C for 7 days. Diagnosis was based on the characteristics of bacterial colonies, rate of growth, acid fast stain, growth on MacConkey agar without crystal violet, chromogenes production, catalase test, nitrate reductions, NaCl tolerance, urease test, and by molecular identification (Kent & George, 1985)

Molecular Identification of Mycobacteria

According to the WizardGenomic DNA Purification DNA was isolated from bacterial growth, Promega. PCR Kit, protocol was performed for identify Mycobacteria spp.

on (MYCGEN-F 5`-AGA GTT TGATCC TGG CTC AG-3`,MYCGEN-R 5`-TGC ACACAG GCC ACA AGG GA-3`,MYCAV-R 5`-ACCAGA AGA CAT GCG TCT TG-3`

MYCINT 5`-CCT TTA GGC GCA TGT CTT TA-3`), 1ml of culture for 2min at 13000 rpm then solution added to pellet and vortex. Incubated at 37 ºC in water bath for 30min.

After incubation samples centrifuge at 13000 rpm for 2min.. From Lysis Solution, 600µl. added and mixing were Incubate at 80°C, for 5 min, then lysis cooled., 3µl of RNase Solution. Mixed, incubated for 15 mins at 37°C., 200µl of Precipitation Protein add to cell lysate. Vortexing incubated at (-30) centrifuge for 5 min at 13,000 rpm. Diluted DNA transfer to tube with 600µl isopropanol. Mixing then centrifuge as in "Pellet Cells". 70% ethanol, 600µl were added centrifuge at13,000 rpm for 2 min aspirate Ethanol and air-dried the pellet. rehydrated in 100µl of Solution for 1 hour at 65°C.

Table 1 : PCR program for detection of mycobacteria

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

agarose gel used to detect the presence of amplification PCR.

Solutions

DNA ladder marker 1 X TAE buffer, Ethidium bromide loading dye (10mg / ml).

Agarose preparation

100 ml of 1X TAE was taken. and1. 1 gm of agarose added to the buffer. Then heated to boiling until dissolved. Then add 1µl of Ethidium Bromide to the agarose. to get mixed The agarose was stirred ,The cooled at 50-60C°.The solution poured into the gel tray and sealed the edges with cellophane tapes and solidify for 30 min. The comb removed. The tray was filled with1X TAE-electrophoresis buffer 3-5 mm over the surface of the gel.

DNA loading

5µl of PCR products was loaded to well. at 100v/50mAmp for 75min Electrical power was turned on DNA moves from Cathode to plusAnode poles. The Ethidium bromide stained the bands in gel by using Gel imaging

Results

The results of bacterial isolation revealed that the mycobacteria were 15 isolates out of (110) birds. The colonies of the isolated appeared within (3 days-8weeks). acid-fast rods presence in Ziehl-Neelsen (ZN) The isolates grown at 28-30,37°C,. Colonies on LJ were typical nonpigmented (Figure 1). On acid-fast stain long rods or short, branching were observed (Figure 2)on the MacConkey agar pink color colonies (Figure 4). all isolates give negative except sample (F)positive from TBc ID test. (Figure 3).

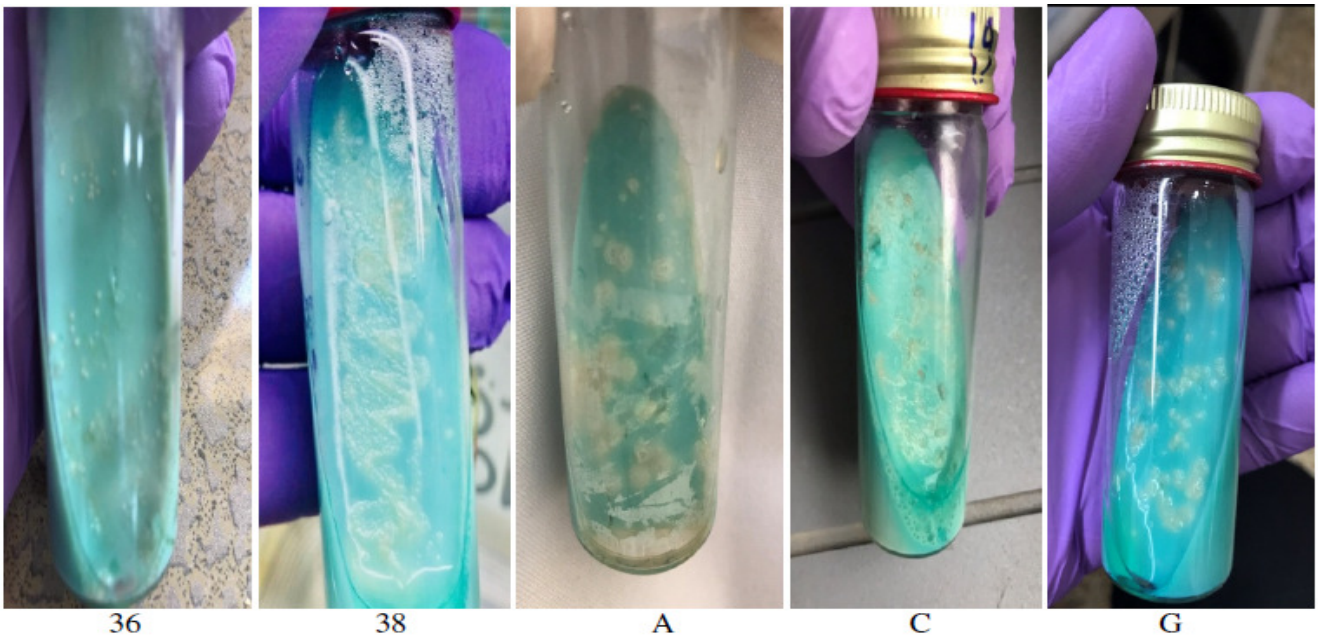


Fig. 1 : MTC & NTM on L J isolated from Birds : 36: *M.tuberculosis* after 25-34 days of cultivation, 37°C form typical non pigmented, rough, dry.38: M.spp after 14-20 days of cultivation, 37°C form typical non pigmented. A: *M. avium* after 27-30 days of cultivation, 37°C typical nonpigmented. C: *M. intraclluler* white nonchromogenic rapidly growing after 10-12days of cultivation. G: *M. fortuitum*. after 8-10 days of cultivation, 35°C form typical nonpigmented white, round, rough.

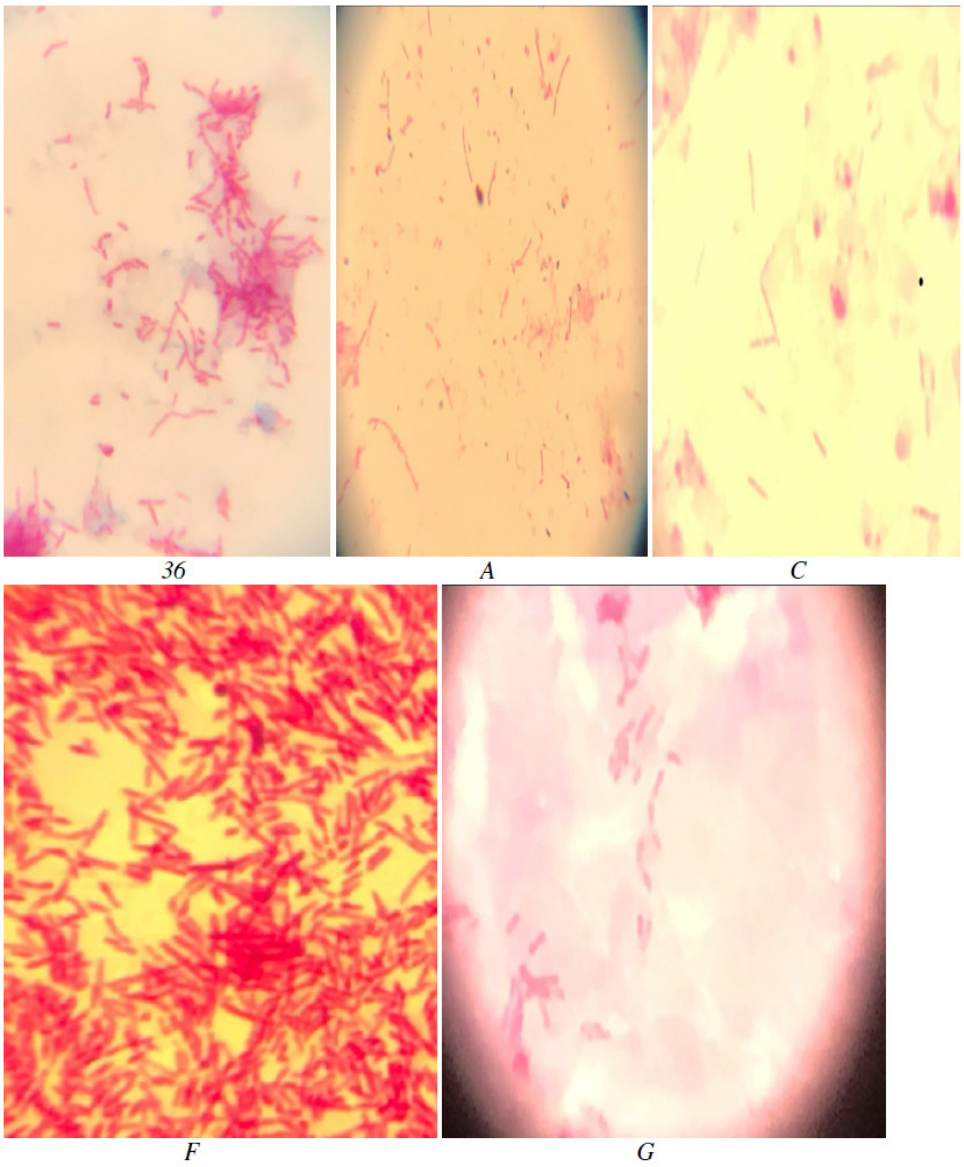
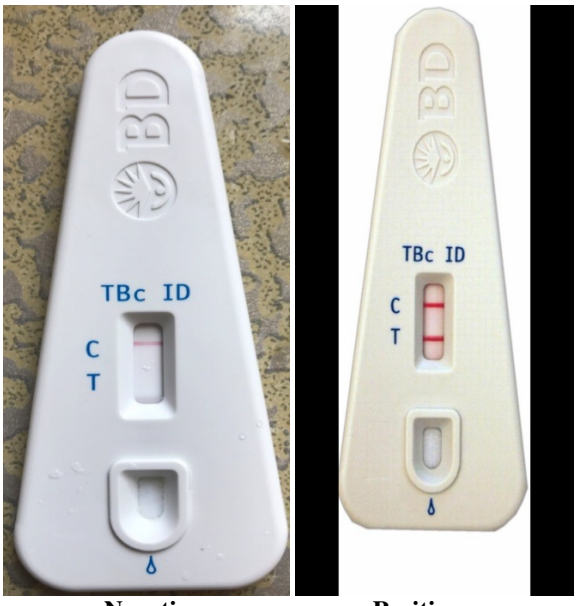


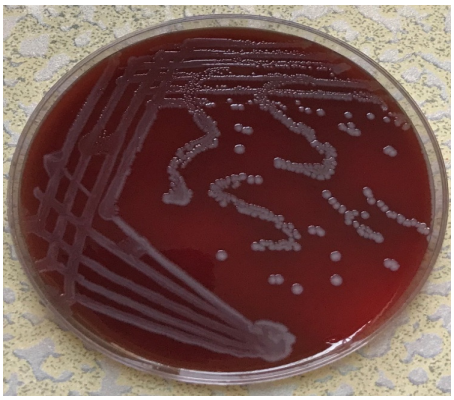
Fig. 2 : *Acid fast bacilli : 36: M. tuberculosis, A: M. avium, C: M. intracllular, F: M.tuberculosis complex, G: M. fortuitum..*



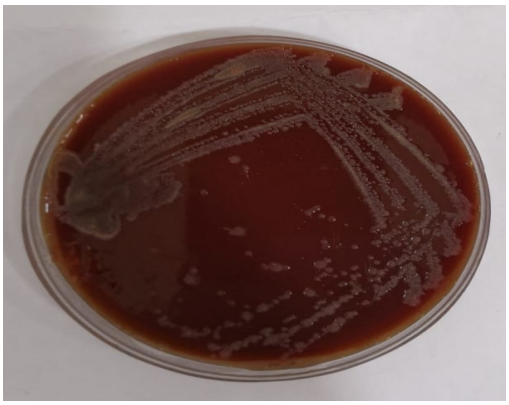
Negative **Positive**

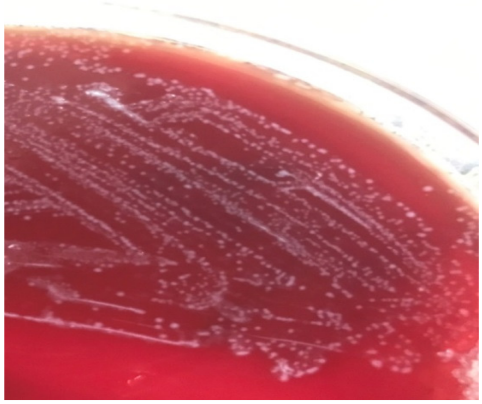
Fig. 3 : TBc ID test

the immunochromatographic assay (BD MGIT TBc ID): All samples of birds give negative except sample F(take from feces parrot)give positive result from test (*M. tuberclosis* complex).



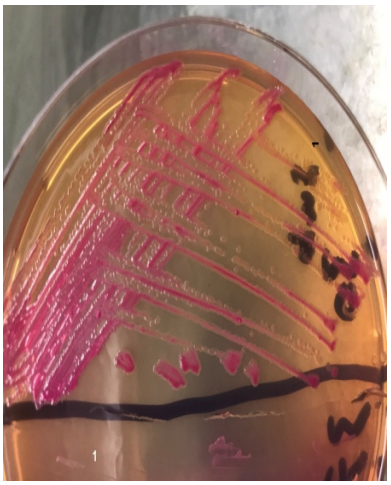
A

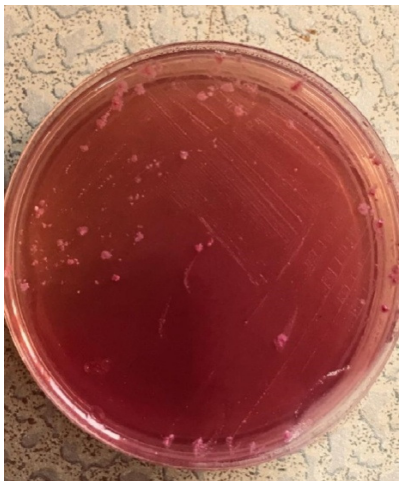




G *36*

Fig. 4 : On biood agar isolated from birds A: *M. avium*. G: *M. fortuitum*. 36: *M. tuberculosis*.





G *H*

Fig. 5 :NTM grow on macConkey ager without crystal violat from Birds isolates G: *M. fortuitum* on macConkey ager (pinke colonies), H: *M.abscessus.*.

Molecular Identification

in this study analysis of 16S rRNA gene and primers the similarity done in the National Center for Biotechnology Information (NCBI by using Basic Local Search Tool and SepsiTest results which identified 15 species of mycobacterium spp.

5/15(33.3%) isolate was belong to. *M. tuberculosis*, 3/ 15 (20%) isolate was belong to *M. intracellular*, group of these isolates were 1/15(6.6%) isolate was belong to. *M. avium*, group of isolates were reported 3/15(20%) to belong M.spp, followed by *M. tuberculosis* Complex 1/15(6.6%). 1/15(6.6%) isolate was belong to *M. abscessus*, other spp include. *M. fortiutum* 1/15(6.6%) Figure (6, 7, 8).

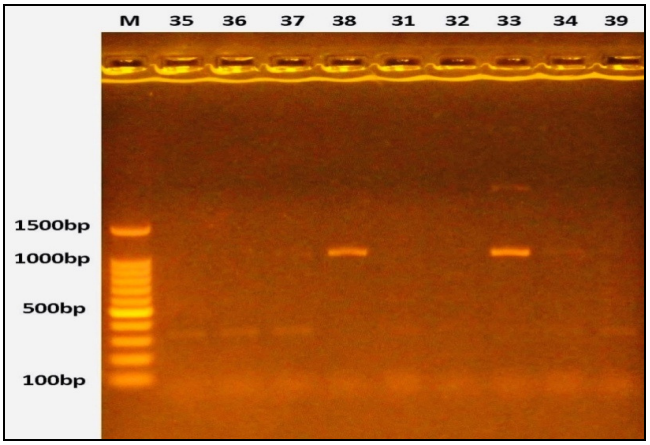


Fig. 6 : Electrophoretic separation of PCR products obtained by multiplex PCR of genomic DNAs of mycobacteria, isolated from feces and tissue samples of positive reactor chickens. The DNA for each lane is as follows:

lane 1, molecular-weight markers representing DNA fragments (Bio-Rad); lane 31, 35, 36, 37 and 39: *M. tuberculosis*, lane 33, 34 and 38: M. spp.

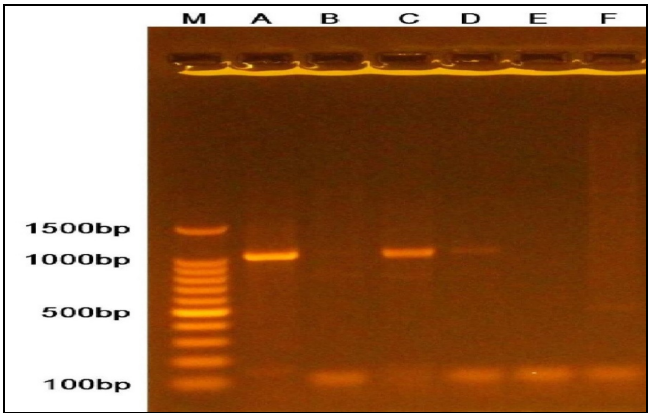


Fig. 7 : Electrophoresis separation of PCR products obtained by multiplex PCR of genomic DNAs of mycobacterium, isolated from feces and tissue samples of positive reactor chickens. The DNA for each lane is as follows:

lane 1, molecular-weight markers representing DNA fragments (Bio-Rad); lane **A**: *M. avium*, lane **B,C** and **D**: *M. intracellular*; lane **F:** *M. tuberculosis* complex.

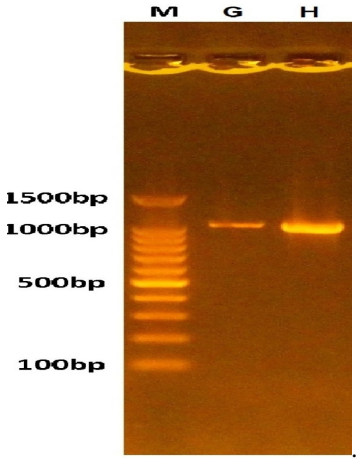


Fig. 8 : Results of the presence of 16s RNA gene of the birds MTC&NTM Isolate fractionated on 1% agarose gel electrophoresis stained with Eth.Br. Lane1:100bp DNA marker: G: *M. fortuitum*, H: *M. abscessus.*

Discussion

In the present study, The results indicated 5/15 (33.3%). *M. tuberculosis*, 3/ 15 (20%) *M. intracellular*, 1/15 (6.6%). *M.avium*, 3/15(20%) M.spp, 1/15 (6.6%) *M. tuberculosis* Complex, 1/15 (6.6%) *M.abscessus*, 1/15 (6.6%) *M. fortiutum* were isolated from bird's infections. This is the the first molecular identifications of mycobacterium spp in birds in Iraq .Previous studies reported in Iraq (baghdad province) Mycobacteria spp. isolated (10.3%). from (107) rectal swabs samples from pigeon and chickens by Waffa *et al.*, (2011) in Baghdad. While Abbas, 2016 isolated (35%)of mycobacteria from a pigeon chickens and pet birds,. Meanwhile, Previous studies reported the isolates belong to *Mycobacterium avium*, (Tell *et al.*, 2001; Dvorska *et al.*, 2003). study of Mayahi *et al.*, (2013) isolated 51 *Mycobacterium avium* subs. Avium from pigeons and molecular features of the isolated bacteria in Turkey avian mycobacteriosis were reported by Hasan *et al.*, (2016;Kul *et al.*, 2005; Terim *et al.*, 2010). In other study the presence of *Mycobacterium avium* in four birds from 130 psittacidaes, Godoy *et al*. (2009). Also in 38 water birds *Mycobacterium avium* subsp. avium was detected in faecal samples, tissue, in 18 (90.0%) birds. All 20 tissue isolates of and 8 MAA isolates in farm animals in zoological gardens, and their keeper (Dvorska *et al.*, 2007).Kaboudi et al(2017) isolated *Mycobacterium avium* (serotypes 1, 2 and 3) and Mycobacterium genavense in the case of avian tuberculosis. typical case in a chicken while Moravkova *et al.* (2011) isolated (*M. avium*) was (5.6%) in pheasants. such as *M. terrae M. fortuitum, M. diernhoferi, M. triviale, M. scrofulaceum, M. chelonae, M. flavescens, M. smegmatis, M. flavescens,* M. Stepień-Pyśniak *et al.*, (2016), also isolated *Mycobacterium avium* from the affected birds . From fecal samples of 60 other birds. Avian tuberculosis was detected in Iran and Greece (Bolfion *et al.*, 2010; Fragkiaelaki, 2005). In bangladish Reza *et al.* (2015) Three Mycobacterium spp. were isolated from dropping samples of poultry in Ethiopia Kindu1and Gashaw Getaneh 2 (2016), a total of seven (58.3%) chickens had gross lesions on On acid fast staining five (41.67%) In Poland Ledwon *et al.* (2008). The majority of Mycobacterium sp. identified was Mycobacterium fortuitum (38isolates). *Mycobacterium avium* was present in 16 samples. in Bangladesh Rahman *et al*. (2018) A fl ock of four-week old turkey chicks showed clinical signs such as fatigue, in-appetence, gasping and eventual death after having a very brief illnesses. Histopathologic findings were typical of avian tuberculosis, Laboratory confirmation was made based on lesions and using Ziehl-Neelsen (ZN) acid-fast stain. in China Zhu *et al.* (2018). In conclusion, birds act to contaminate their environment with Mycobacterium and a serious risk of bacterial spreading

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