Bacteriological and Molecular studies on *Mycoplasma* infection in turkey from Egypt.

Hassan W.H.[1*], Abou-shama U.H.[2], Dardeer M.A.[3], Zain A. Z.[4]
[1]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.
[2]Department of Microbiology, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt.
[3] Mycoplasma Department, Animal Health Research Institute, Dokki, Giza, Egypt.
[4] Dean of the Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt.

[*] Corresponding author E-mail: dr_walidh@yahoo.com

Abstract

In this study the prevalence of *Mycoplasma* species was studied in diseased turkey flocks showed respiratory symptoms, sinusitis, lameness as well as decrease in egg production in Al Minia and Sohag Governorates by both culturing and serological methods. Six field isolates were tested by PCR and compared with the standard *M. gallisepticum* reference (R) strain. All the examined field isolates were identified as *M. gallisepticum*. RAPD-PCR was used as technique to differentiate between the strains of the same *Mycoplasma* species. DNA profiles of five *M. gallisepticum* field isolates were compared with that of *M. gallisepticum* reference strains (F and R) using fan primer. The banding patterns of that isolates were highly similar to those of *M. gallisepticum* reference strains. The *in vitro* evaluation of antimicrobial activities using the minimum inhibitory concentrations (MICs) test against the isolated *Mycoplasmas* proved that the tilmicosin and tylosin possessed the lowest MICs compared with other six antimicrobials, so it is recommended to be used for *in-vivo* treatment and also they are effective in eradication programmes of field *Mycoplasma* infection in poultry. In conclusion, *M. gallisepticum* is the predominant cause of turkey respiratory manifestations. RAPD-PCR technique detects the genetic diversity in the natural populations among field isolates. Tilmicosin and tylosin had the lowest MICs than other antimicrobials used in this study against avian *M. gallisepticum*.

Key words: Mycoplasma, RAPD, MIC, turkey

Introduction

Mycoplasma, belonging to the class *Mollicutes*, is a small free living highly fastidious and slow growing microorganism, (Nicolas and Ayling, 2003). *Mycoplasma* infections engender enormous economic losses to broiler, layer and breeder flocks because of downgrading of carcasses, reduced feed conversion efficiency and egg production, hatchability losses and increased costs of medication, egg treatment and control programs (Stipkovits and Kempf, 1996; Ley and Yoder, 1997).

The most important *Mycoplasmas* of avian species are *Mycoplasma gallisepticum (MG)*, *Mycoplasma synoviae (MS)*, *M. iowae (MI)* and *M. meleagridis (MM)* (Fan *et al.*, 1995a). Both *M. gallisepticum and M. synoviae* are the etiological agents of severe respiratory disease in both chickens and turkeys. *M. iowae* and *M. meleagridis* affect turkeys only (Fan *et al.*, 1995a). In addition to these pathogenic *Mycoplasmas*, others infect birds and may interfere with the rapid diagnosis.

Three main approaches are used for the diagnosis of avian mycoplasmosis: organism isolation, biochemical identification, serology, polymerase chain reaction (PCR) (Ewing *et al.* 1996).

Isolation of pathogenic avian *Mycoplasma* organisms is difficult as they are relatively fastidious organisms that grow slowly, sand may require up to 3 weeks prior to detectable growth. Therefore, serological assays such as the rapid serum plate agglutination (SPA) and haemagglutination inhibition (HI) tests have been used routinely. The SPA is a rapid, relatively inexpensive, and sensitive test, hence, it has been widely used for sero-diagnostic monitoring of poultry flocks (Kleven *et al*., 1998). Problems of low sensitivity, cross reactions, and non-specific reaction were encountered with rapid serum plate agglutination and (HI) tests (Ewing *et al*., 1996).

Recently, molecular biological techniques such as PCR and Randomly Amplified Polymorphic DNA (RAPD) or the Arbitrarily Primed PCR (AP-PCR) have been applied for detection and identification of *Mycoplasmas*.

Mycoplasmal mgc2 gene sequences have been determined to detect vaccinal strains and the challenge R strain. The mgc2 PCR has a faster turnaround time than other PCR methods (LP and gapA), therefore, among these PCR methods evaluated, mgc2 PCR is the method of choice (Garcia *et al*., 2005).

The RAPD method has been used to study heterogencity in closely related organisms (Fan *et al.*, 1995b). This method detects differences in the DNA sequences at sites in the genome that are defined by the used primer. The number and the length reveal sequence variation of amplified products, this may be phylogenetically conserved. The RAPD method is advantageous for strains or isolates identification (Geary *et al.*, 1994).

Only few vaccines are available for the control of avian mycoplasmosis. Test and slaughter is the most effective method for controlling mycoplasmosis however, the emergency of multiage complexes renders it impractical to be adopted (Levisohn and Kleven, 2000). Consequently, the control of *M. gallisepticum* infection in broiler breeder by chemotherapy is the most practical way to minimize economical losses. On the other hand, resistance has developed to a number of antimicrobials, therefore evaluation of some antibiotics to detect the most effective group for *M. gallisepticum* prophylaxis and treatment purposes using minimum inhibitory concentration technique (MIC) is critical (Valks' and Burch, 2002).

The present work was planed to identify *Mycoplasma* species isolated from turkey by PCR and to evaluate the ability of RAPD-PCR for identification and differentiation between pathogenic and vaccinal strains among field isolates of *M. gallisepticum*. In addition, evaluation of some antibiotics to detect the most effective group for prophylaxis and treatment purposes for *M. gallisepticum* infection using MIC technique was achieved.

2. Materials and Methods

2. 1. Samples:

A total of 975 samples including swabs from sinuses, tracheas and tissues from lungs, tracheas, inflamed joints and reproductive organs as well as 550 serum samples were collected from diseased turkey flocks showed respiratory symptoms, sinusitis, lameness as well as decrease in egg production in Al Minia and Sohag Governorates. The samples were stored freezed at -4 ^{o}C and transmitted in ice bags to the laboratory for identification.

2.2. Isolation and identification of *Mycoplasmas*:

Liquid and solid media were used for the isolation and propagation of *Mycoplasma* according to Frey *et al.* (1968). Genus determination and biochemical characterization were carried out as described by Erno and Stipkovits (1973). Serological identification was conducted using growth inhibition test as described by Clyde (1964). Locally prepared rabbit antisera against *M.*

gallisepticum, *M. gallinarum* and *M. pullorum* were used.

2. 3. Polymerase chain reaction (PCR) and Random amplified polymorphic DNA (RAPD):

2.3.1. DNA extraction and purification (Fan *et al.*, 1995b**):**

Mycoplasma reference strains and *Mycoplasma* cultures were grown in Frey's broth, 5 ml of a 24 hour broth cultures were centrifuged for 10 minutes at 12000 r.p.m. The pellet was washed twice in 1ml of phosphate buffered saline (PBS) pH 7.2 and suspended in 50 µl PBS. The cell suspension was heated directly at 100°C for 10 min. in a heat block to break the cell membranes, then cooled on ice for 5 minutes. Finally, the cell suspension was centrifuged for 5 min. and the supernate containing DNA was collected and stored at -20°C until use for PCR.

2.3.2. Specific PCR primers: Two primers were prepared according to Ferguson *et al.* (2005):

Forward primer Mgc2 1F was (5'-GCTTG TGTTCT CGGGTG CTA-3').The sequence of Reverse primer Mgc2 1R was (5'-CGGTGGAAAACCACCAGCTCTTG-3'). The primers were prepared by Sigma Company, Germany.

2.3.3. RAPD-PCR primers (Sigma, Germany)**:** The single primer was synthesized as described by Fan *et al.*, (1995b). Amplification of the sequence of this primer was as follows: 5'- AGG CAG CAG GTA GGG AAT-3.

3.4. Polymerase chain reaction (PCR) procedure for *M. gallisepticum* (Ferguson *et al.*, 2005)**:** The reaction mixture (total volume of 50 µl) were 5 µl of 10 X reaction buffer (Applied Biosystem, USA), containing 50 ng of template DNA, 1ml of 10 mM dNTP mix (Sigma, Germany), 1.5 ml of 25 mM $MgCl_2$ and 1.0 ml of the primer. Then 2U of DNA *Taq* polymerase (Applied Biosystem, USA), was added and the mixture was completed by ultra-pure distilled water to 50 µl.

Using thermal cycler machine and *Mycoplasma gallisepticum* thermal cycler program; the amplification consisted of an: initial step at 94°C for 3 min., followed by 40 cycles as follows: 94°C/20 sec., annealing, 55°C/40 sec.and extension 72°C/1 min. This was followed by a final elongation step at 72°C for 5 min. Aliquots of amplified products (10 µl) were electrophoresed through 1% agarose gel and DNA was visualized by Ultraviolet transilluminator after ethidium bromide staining then photographed.

3.5.Random amplified polymorphic DNA (RAPD) procedure (Fan *et al.*, 1995b**):** The reaction mixture (total volume of 50 µl) were 5 µl of 10 X reaction buffer (Applied Biosystem, UK), containing 50 ng of template DNA, 1µl of 10 mM dNTP mix (Sigma), 1.5 µl of 25 mM $MgCl_2$ and 1.0 µl of the primer. Then 2U of DNA *Taq* polymerase (Applied Biosystem, UK), was added and the mixture was completed by ultra-pure distilled water to 50 µl. PCR was performed on a PTC-100 Programmable Thermal Controller (M. J. Research Inc,). The reaction conditions were as follows: 3 cycle of 15 sec. at 94°C (denaturation), 2 min. at 28°C (annealing), 3 min. at 74°C (extension) and then for 35 cycles of 15 sec. at 94°C, 2 minutes at 45°C, and 3 min. at 74°C. 10 µl aliquots of amplified DNAs were electrophoresed in 2% agarose gel in TBE containing 0.5% ethidium bromide at 100V. Five micrograms of 100bp DNA ladder (Pharmacia) were also run in each gel as a standard for size determination of DNA fragments. The DNA was visualized using UV transilluminator, and photographed.

Visualization was done in transiluminator (Spectroline, Model 312A, 312 nm Ultraviolet, USA) and photographs were taken by UV camera (Polariod DS 34 direct screen instant camera, England).

4. Determination of Minimal Inhibitory Concentration (M.I.C.) by Micro-broth method.

Tests were performed in duplicate as described by Senterfit (1983). The antimicrobials were tested in serial twofold dilution at concentrations ranging from 10 to 0.0048 µg/ml and tests were repeated if the back-titration of CCU, which was carried out alongside, did not fall in the required range of 10^3 to 10^4/0.2ml.

3. Results

The primary isolation of *Mycoplasma* spp. from 975 collected samples from diseased turkey flocks yielded 234 isolates (24%) from Al Minia and Sohag Governorates. The highest recovery rate of *Mycoplasma* (36%) was from sinus swabs in Sohag followed by lung in Sohag (34%), tracheal swabs in Al Minia (33%), sinus swabs (32%) in Al Minia, trachea in Sohag (24%), tracheal swabs in Sohag (20%), trachea in Al Minia (8%), lung in Al Minia (6%), inflamed joint in Al Minia (4%), reproductive organ in Sohag (4%) and from inflamed joint in Sohag (2%), reproductive organ in Al Minia (2%). The results revealed the presence of two distinct biochemical groups of isolates from diseased turkey flocks. *Mycoplasma* species were biochemically identified as described by Sabry (1968) by using glucose fermentation test, arginine deamination and the formation of film and spot test into two groups.

Group **I** (*M. gallisepticum*) 153 isolates were obtained (54 and 45 from sinus swabs, 22 and 9 from tracheal swabs, 2 and 11 from Lung tissue, 2 and 4 from tracheal tissue, 1 and 1 from inflamed joint, 1 and 1 from reproductive organs tissue of the examined turkeys in Al Minia and Sohag Governorates respectively). Group **II** *(M. gallinarum*) 81 isolates were obtained (26 and 27 from sinus swabs, 11 and 6 from tracheal swabs, 1 and 6 from lung tissues, 0 and 2 from tracheal tissues, 1 and 0 from inflamed joints and finally 0 and 1 from reproductive organs tissue of the examined turkeys in Al Minia and Sohag Governorates, respectively).

The incidence of different *Mycoplasma* spp. in diseased turkey flocks from Al Minia and Sohag Governorates using GI test which revealed that 140 isolates were *M. gallisepticum* belonging to biogroup I (80 isolates from Al Minia Governorate and 60 isolates from sohag Governorate with incidence of 66.1% and 53.1%, respectively), 71 isolates were antigenically related to *M. gallinarum* belonging to biogroup II (31 isolates from Al Minia Governorate and 40 isolates from sohag Governorate with incidence of 25.6% and 35.4%, respectively) and 23 were antigenically related to un-typed *Mycoplasma* belonging to biogroup III (10 isolates from Al Minia Governorate and 13 isolates from sohag Governorate with incidence of 8.3% and 11.5%, respectively). With a total incidence 59.9%, 30.3% and 9.8% for *M. gallisepticum*, *M. gallinarum* and untyped *Mycoplasma*, respectively.

It was noticed that, the serological identification of *Mycoplsama* spp. using SPA for 550 serum samples collected from diseased turkeys flocks in Al Minia and Sohag Governorates gave the following results: From Al Minia Governorate, 260 out of 350 were positive for *M. gallisepticum* with a percentage of 74.28 %, while 90 out of 350 were positive for *M. synoviae* with the percentage of 25.71%. From Sohag Governorate isolates 174 out of 200 were positive for *M. gallisepticum* with a percentage of 87%, while 26 out of 200 were positive for *M. synoviae* with the percentage of 13%.

As shown in Table (1), Fig. (2) the amplification with Fan 2 primer resulted in a characteristic bands for each of the strains under examination, the banding patterns of five field isolates studied with RAPD were highly similar to those of *M. gallisepticum* reference (F and R) strains.

The DNA banding patterns of the five field isolates showed that all tested isolates shared in five common bands at 190, 283, 375, 503 and 720 bp. A high similarity occurred between field isolate one, three and four.

In present work, the M.I.C. for different *M. gallisepticum* isolates isolated from diseased turkey flocks in Al Minia and Sohag Governorates were examined. Table (2) illustrates comparison of eight antimicrobials against two different *M. gallisepticum* field isolates from turkey flocks in Al Minia Governorate.

Table (2) revealed that Florfenicol was not effective for both field isolates; it has higher minimum inhibitory concentration (1.25 µg /ml for isolate "1" and 2.5µg/ml for isolate "2"). While Enrofloxacin was not effective for field isolate (2) with high minimum inhibitory concentration (5 µg/ml). The other five antibiotics were effective and had low MIC.

Spiramycin, Tilmicosin, Tylosin and Tiamulin had the lowest MICs (0.0048 µg/ml) for the field isolate "1". While Tilmicosin had the lowest MICs (0.0048 µg/ml) followed by Spiramycin, Tylosin and Tiamulin (0.0098µg/ml for these antibiotics) for the field isolate "2".

Table (3) showed the comparisons of eight antimicrobials against two different *M. gallisepticum* field isolates from diseased turkeys in Sohag Governorate. Florfenicol and Enrofloxacin were not effective for both field isolates (1 & 2), they had higher minimum inhibitory concentration (Florfenicol had MICs 2.5 mg/ml for isolate "1" and 1.25 mg/ml for isolate "2" and Enrofloxacin had MICs 10 mg/ml for isolate "1" and 5 mg/ml for isolate "2".

Tilmicosin had the lowest MICs (0.0048µg/ml) for both field isolates, followed by Doxycycline for both field isolates and tylosin for field isolate "2" which had MICs 0.0195 µg/ml.

Table (1): **RAPD- PCR analysis of *M. gallisepticum* reference strains and field isolates from diseased turkey flocks using Fan primer:**

No of bands	Reference *MG**(F)	Reference *MG* (R)	Field isolate (1) from Al Minia	Field isolate (2) from Al Minia	Field isolate (3) from Al Minia	Field isolate (4) from Sohag	Field isolate (5) from Sohag
1.			1200.97		1200.43	1200.65	
2.	1100.85	1100.02					
3.			860.05	860.77			
4.			720.45	720.39	720.06	720.33	720.58
5.	605.85	605.71		650.42	650.55	650.64	
6.	503.73	503.39	503.57	503.08	503.66	503.03	503.69
7.	410.90					410.71	410.88
8.	380.07	380.04	375.98	375.78	375.69	375.58	375.69
9.		283.94	283.32	283.15	283.57	283.89	283.40
10.	260.55	260.94	260.77		260.52		
11.			190.40	190.83		190.78	190.55
12.					90.98		

*MG: *M. gallisepticum*

Table (2): (MICs μg/ml) for the eight antimicrobials against two different *M. gallisepticum* field isolates isolated from diseased turkey flocks in Al Minia Governorate

No	Antibiotics	Isolate 1	Isolate 2
1	Doxycycline	0.0098 μg/ml	0.0195μg/ml
2	Florfenicol	1.25 μg/ml	2.5μg/ml
3	Enrofloxacin	0.315 μg/ml	5μg/ml
4	Lincospectin	0.039 μg/ml	0.156μg/ml
5	Spiramycin	0.0048 μg/ml	0.0098μg/ml
6	Tilmicosin	0.0048 μg/ml	0.0048μg/ml
7	Tylosin	0.0048 μg/ml	0.0098μg/ml
8	Tiamulin	0.0048 μg/ml	0.0098μg/ml

Table (3): (MICs μg/ml) for the eight antimicrobials against two different *M. gallisepticum* field isolates isolated from diseased turkey flocks in Sohag Governorate

No	Antibiotics	Isolate 1	Isolate 2
1	Doxycycline	0.0195 μg/ml	0.0195 μg/ml
2	Florfenicol	2.5 μg/ml	1.25 μg/ml
3	Enrofloxacin	10 μg/ml	5 μg/ml
4	Lincomycin/spectinomycin	0.315 μg/ml	0.078 μg/ml
5	Spiramycin	0.156 μg/ml	0.039 μg/ml
6	Tilmicosin	0.0048 μg/ml	0.0048 μg/ml
7	Tylosin	0.039 μg/ml	0.0195 μg/ml
8	Tiamulin	0.156 μg/ml	0.078 μg/ml

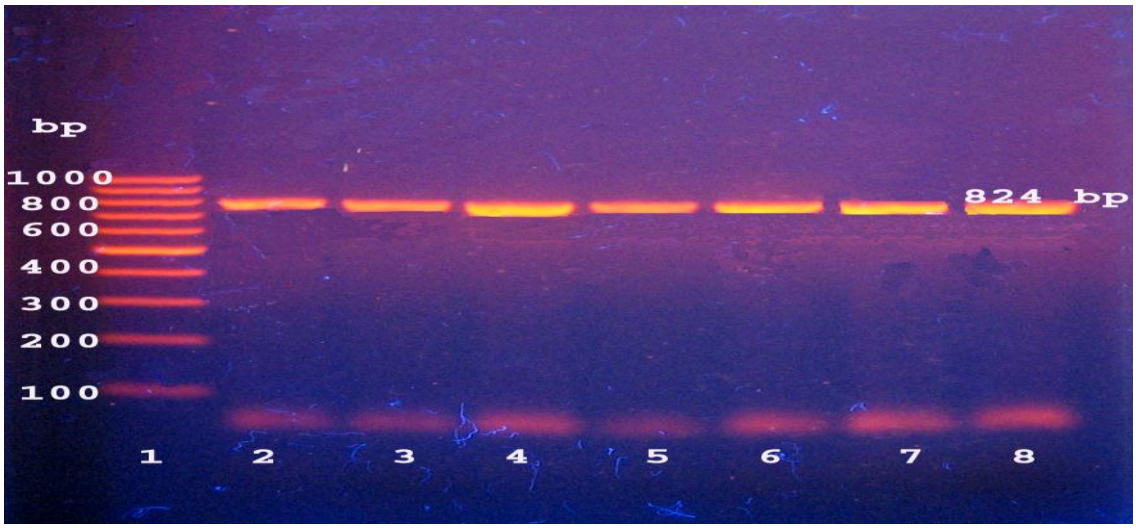

Fig. (1): The PCR products of specific DNA fragment (824 bp) of the *mgc2* gene of *M. gallisepticum* (Ferguson *et al.*, 2005).

Lane 1: 100 Base – pair Ladder
Lane 2: ***M. gallisepticum*** reference strain (R)
Lane 3-5: ***M. gallisepticum*** field from Al Minia Governorate.
Lane 6-8: ***M. gallisepticum*** field from Sohag Governorate.

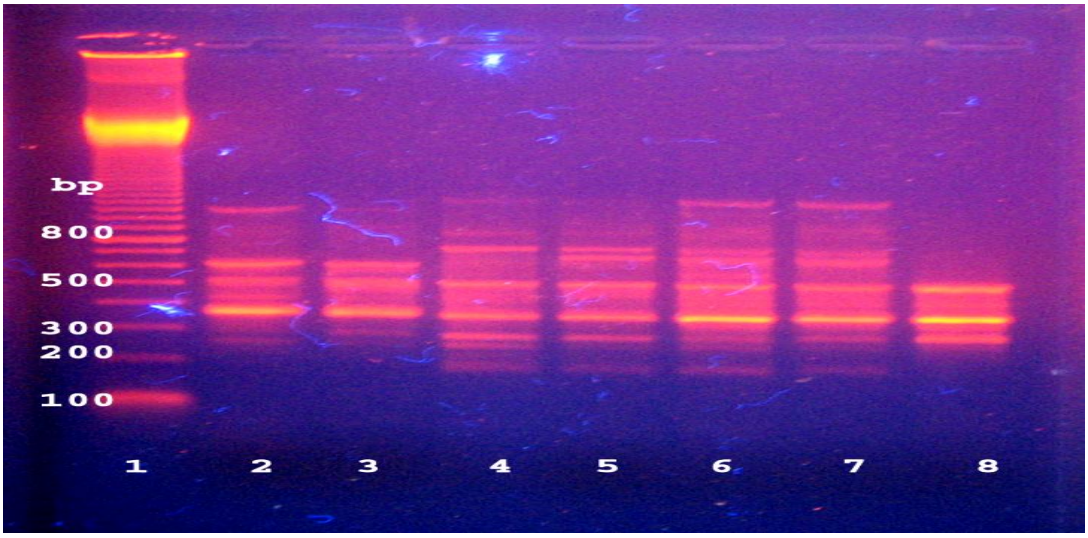

Fig. (2): RAPD- PCR analysis of *M. gallisepticum* reference strains and field isolates from Turkey flocks using Fan 2 primer:
Lane 1: 100 Base – pair Ladder
Lane 2: *M. gallisepticum* vaccinal strain (F)
Lane 3: *M. gallisepticum* reference strain (R)
Lane 4-6: *M. gallisepticum* field isolates from Al Minia Governorate.
Lane 7-8: *M. gallisepticum* field isolates from Sohag Governorate.

4. Discussion

The primary isolation of *Mycoplasma* spp result agrees with that of Abd El-Rahman (2007) who isolated *Mycoplasma* from diseased turkey flocks with an incidence of 24.5%. Lower percentages were recorded by El-Ebeedy *et al.* (1984) (3.4% of two-month to one year old turkeys, 21.9% of dead turkeys, 5.3% of infertile egg and 8.7% of dead-in-shell embryos), Jessup *et al.* (1983) (14.3% of wild-type turkeys and 8.3% of domestic turkey), Rott *et al.* (1989) (9.5% of turkey poults), Sharaf (2000) (8.57% of one day apparently normal examined turkey and 20% of apparently normal 45 day old turkey) and El-Seify (2004) (19.6%). On the other hand higher percentages were recorded by Zhang and Ji (1989) (26.7), Rott *et al.* (1989) (31% of semen samples of male turkeys), Mottles and Hing (1970) (37.7%), Timms (1967) (39.6%), Sharaf (2000) (54.35% of one day diseased turkeys and 51.42% of 45 day old diseased chickens).

The biochemical results revealed the presence of two distinct biochemical groups of isolates from diseased turkey flocks. *Mycoplasma* species were biochemically identified as described by Sabry (1968) by using glucose fermentation test, arginine deamination and the formation of film and spot test into two groups. These results coincide with those reported by Abd El-Rahman (2007) who classified the *Mycoplasma* organisms isolated from diseased turkey flocks into the same two biochemical groups.

The results of isolation, biochemical and serological identification indicated that *M. gallisepticum* is the predominant isolate. These results are parallel with those mentioned by Sokkar *et al.* (1986) who revealed that 24% of the examined samples were *M. gallisepticum* positive and Dardeer *et al.* (2006) who revealed that 63.49% of the examined samples were *M. gallisepticum* positive and Abd El-Rahman (2007) who revealed that 31.7%

of the examined samples were *M. gallisepticum* positive.

In the present study, *M. gallisepticum* PCR assays targeted the *mgc2* gene which encodes cyt-adhesion protein of *M. gallisepticum* and also known to play a role in attachment process. As shown in Fig. (1) the six field isolates from diseased turkey flocks were tested and compared with *M. gallisepticum* reference R strains. The PCR amplification products were electrophoresed on 1% agarose gel strained with ethidium bromide which gave a characteristic fragment at 824 bp for all examined reference and field strains. Similar results were detected by Ferguson *et al.,* (2005) who mentioned that the use of *mgc2* forward and reverse primers could identify this gene in all the examined strains which gave fragment at 824 bp.

The amplification with Fan 2 primer resulted in a characteristic bands for each of the strains under examination, the banding patterns of five field isolates studied with RAPD were highly similar to those of *M. gallisepticum* reference (F and R) strains (Table 1 & Fig. 2). The DNA banding patterns of the five field isolates showed that all tested isolates shared in five common bands at 190, 283, 375, 503 and 720 bp. A high similarity occurred between field isolate one, three and four. Our results are in agreement of those obtained by Fan *et al.,* (1995b); Abd El-Gawad, (2005) and Abdel-Megied, (1994) who stated that field isolates were highly similar to those of *M. gallisepticum* reference strains.

In present work, the M.I.C. for different *M. gallisepticum* isolates isolated from diseased turkey flocks in Al Minia and Sohag Governorates were examined.

From Table (2 & 3) tiamulin had a high minimum inhibitory concentration in both isolates in Sohag Governorate in comparison to the tilmicosin and tylosin. This may due to previous exposure to tiamulin mediation before sampling this agreed with (Abd El-Ghany, 2009) who stated that it is recommended that testing the efficacy of the drugs in-vitro before application in-vivo to overcome the problem of drug resistance.

Our results showed that the tilmicosin and tylosin had the lowest MICs than other antimicrobials, so it is recommended to be used for in-vivo treatment and they are effective in eradication programs of field *MG* infection in poultry. These results agree with Abd El-Ghany (2009) and Eissa *et al.,* (2009b).

The tilmicosin had the lowest MICs followed by tylosin. Our results are in coincide with those obtained by Anonymous (1981); Fan *et al.,* (1995a); Cerda *et al. (*2002); Abd El-Ghany (2009) and Eissa *et al.,* (2009a&b).

In conclusion, it was found that *M. gallisepticum* is the predominant cause of turkey respiratory manifestations and infra-orbital sinusitis in Al Minia and Sohag Governorates.

Culture method for isolation of avian *Mycoplasma* is still remained the gold standard for definitive diagnosis although it is slow and time consuming.

In spite of shared antigens among *Mycoplasma* species, SPA test can be used as an initial screening test followed by another confirmatory test.

Specific PCR is a rapid, sensitive and accurate technique used for identification of *Mycoplasma* infection (characteristic fragment at 824 bp for *mgc2* cyto-adhesion gene). By this technique, we can avoid the false positive and false negative results encountered with serological techniques. PCR overcomes the waste time and low recovery rates associated with cultural methods.

RAPD-PCR technique is a more recent technique which can be used for rapid differentiation of different strains of *Mycoplasma* species. Also it is a reproducible method for comparing the *Mycoplasma* field isolates in epidemiological studies. The technique detects the genetic diversity in the natural

populations among field isolates. It could help in tracing the source of infection.

The results of RAPD-PCR suggested that the amplification with Fan 2 primer produced characteristic bands for each strain under examination, the banding patterns of five field isolates from diseased turkey flocks in Al Minia and Sohag Governorates were highly similar to those of *M. gallisepticum* reference strains.

Tilmicosin and tylosin had the lowest MICs than other antimicrobials used in this study against avian *M. gallisepticum* isolated from diseased turkey flocks in Al-Minia and Sohag Governorates, so it is recommended to be used for treatment and they are effective in eradication programs of field *M. gallisepticum* infection in turkey flocks.

It is recommended to test the *in vitro* efficacy of the drugs before application *in vivo* medication to overcome the problem of drug resistance and interference with the results of MICs test.

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