j.Egypt.vet.med.Assoc 78, no 2. 219 – 232 (2018)

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *MYCOPLASMA GALLISEPTICUM* IN CHICKEN

By

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ABSTRACT

Mycoplasma gallisepticum (MG) is an important avian pathogen causes significant economic losses within the poultry industry. One of the options of the controlling MG infection is the isolation and identification of MG.215 samples collected from the lungs, tracheal biforcation, tracheal swabs, blood samples and frontal sinus aspirates were collected from broiler and layer flocks in Damietta governorate. Samples were cultivated on Frey's media giving 142 samples positive as (F. *Mycoplasmataceae)* as 66%. The (142) samples was used for digitonin sensitivity test and the results was (98) samples as digitonin positive (*Mycoplasmataceae*) (69%) and (44) samples was digitonin negative (*Acholeplasmataceae*) (31%).The (215) samples was characterized by PCR by using MG primer.

INTRODUCTION

Mycoplasma gallisepticum (MG) causes considerable economic losses to the poultry industry, especially in chickens and turkeys all over the world, associated with respiratory diseases, poor performance, and embryo mortality **(Eissa *et al.,* 2014).**

Mycoplasmas are the smallest known bacteria that are capable of replicating outside the cells. Occurs in humans and animals and has usual optimum growth temperature of 37ºC. Mycoplasmas are small prokaryotes, 300-800 nm in diameter. They lack in cell wall but are bounded by a triple layered plasma membrane **(Razin *et al.,* 1998 and Brown *et al.,* 2007)**. MG is the most pathogenic and economically significant bacterial respiratory pathogen of poultry. It is considered the most important of the pathogenic Mycoplasmas and Office international des Epizootics (OIE) has declared the disease caused by MG as notifiable **(OIE, 2004)**. It is a major problem in poultry industry worldwide and the infection is commonly known as chronic respiratory disease (CRD) of chickens and infectious sinusitis in turkeys **(Levisohn and Kleven, 2000)**.

Three main approaches are used for the diagnosis of avian mycoplasmosis, isolation, identification, detection of antibodies, and molecular detection of organism nucleic acid by polymerase chain reaction (PCR). Isolation and identification of pathogenic avian mycoplasmas organisms are difficult, slow growing, relatively fastidious organisms and might require up to 3 weeks for detectable growth. Therefore, serological assays such as the rapid serum plate agglutination test (SPA) and haemagglutination inhibition test (HI) tests have been used routinely. Because the SPA test is quick, relatively inexpensive, and sensitive, it has been widely used as an initial screening test for flock monitoring and serodiagnosis **(Kleven *et al.,* 1998)**. Problems of low sensitivity, cross reactions, and non- specific reaction were encountered with rapid SPA and HI tests **(Ewing *et al.,* 1996)**.

Molecular biology techniques such as PCR and Random amplified polymorphic DNA (RAPD) or the Arbitrarily PCR (AP-PCR) has been applied for detection and identification of Mycoplasmas **(Fan *et al.,* 1995).**

MATERIAL AND METHODS

Samples:

Two-hundred ninety-five samples were collected from chicken flocks at different ages all-over the seasons of the year. They included 98 broilers and 36 layers. The samples were collected from diseased chicken (Suffered from CRD and Complicated Chronic respiratory disease (CCRD) and dead chicken as shown in (Table 1).

Table (1): of collected samples from chicken flocks according to chicken status.

Types of chicken	Chicken status	Types of samples		No
Broiler (98)	Diseased chicken (60)	Blood		50
		Tracheal swab		60
		Organs	Lung	16
			Tracheal bifurcation	20
	Dead (38)	Organs	Lung	18
			Tracheal bifurcation	21
Layer (36)	Diseased chicken (25)	Blood		30
		Tracheal swab		25
		Frontal sinus aspirates		5
		Organs	Lung	16
			Tracheal bifurcation	22
	Dead (11)	Organs	Lung	4
			Tracheal bifurcation	8

Culture methods (Sabry and Ahmed, 1975):

A. Direct method:

Swabs or tissue homogenate were spread on Frey's agar media and incubated at 37°C with 5-10 % Co_2 and humidity for 10-14 days and examined under dissecting microscope for characteristic fried egg appearance of *Mycoplasma* colonies.

B. Indirect method:

Samples were inoculated into *Mycoplasma* broth then after 2-3 days of incubation the cultures were plated onto Frey's agar media. At the same time, it were transferred to broth for 3 days then it were spread on agar followed by Incubation at 37°C with 5-10 Co_2 for 2 weeks. Then

it was examined under stereomicroscope every 2-3 days for characteristic fried egg appearance of *Mycoplasma* colonies. If *Mycoplasma* colonies appear on the agar plate, agar blocks of *Mycoplasma* colonies were taken in broth medium and incubated at 37°C for 2-3 days then subjected to purification and further identification.

Purification of *Mycoplasma* cultures (Alder, 1954):

The broth cultures in which glucose were fermented (as indicated by colour change of phenol red from red to orange or slightly yellow) were filtered through 0.45 µm Millipore filter and the filtrate was plated on Frey's agar plates. After 3-5 days of incubation at 37°C in 10% CO_2 humidified atmosphere, single colonies were picked up and transferred into separate tubes with 1 ml Frey's broth medium for further subculture. The process is known as cloning of cultures. All cultures were spread on solid-medium without penicillin and thallium acetate to exclude L-form of bacteria.

Biochemical characterization:

A) Digitonin sensitivity test **(Freundt, 1973).**

Purified suspected Mycoplasma culture was examined by digitonin sensitivity test. Filter paper discs containing 0.02 ml of a 1.5% ethanol solution of digitonin was placed on Frey's plates inoculated by the running drop technique with 0.025 ml of cultures. The plates were incubated at 37 °C in moist carbon dioxide incubator for 3 days then examined for the development of inhibition zones around the discs. Mycoplasmas are digitonin sensitive, while Acholeplasmas are digitonin resistant.

B) Glucose fermentation test **(ERNQ and Stipkovits, 1972).**

Two ml of glucose media were added to 1 ml of suspected Mycoplasma cultures, and then incubated for 3 days at 37 °C in candle jar. No change in color indicates negative reaction while change in color to orange or yellow indicated positive reaction.

C) Arginin deamination test **(ERNQ and Stipkovits, 1972).**

Two ml of arginine media were added to 1 ml of suspected Mycoplasma cultures then incubated for 3 days at 37 °C in candle jar. No change in color indicates negative reaction while change in color to dark red to violet indicated positive reaction.

D) Arginine hydrolysis test **(Frenske and Kenny, 1976).**

Only 0.1 ml of viable Mycoplasma culture was inoculated into 0.9 ml. of the tested medium and incubated at 37 °C aerobically for 7 days along with uninoculated control tube. A dark red

or reddish violet color change indicated a positive test and light red or no color change indicated a negative test.

E) Film and spot formation **(Fabricant and Freundt, 1967).**

The film and spot formation test was used to detect the lipolytic activity of certain Mycoplasma species in which the test medium was seeded with tested organism, incubated at 37oC in a candle jar container for up to 14 days, and examined microscopically using reflected light. Production of a film was seen as iridescent or pearly area, usually on area of heavy growth. The medium sometimes showed some clearing around area of growth.

Oligonucleotide primers encoding mgc2gene:

These primers are specific for mgc2 gene of MG, have a specific sequence and amplify a specific product as shown in (Table 2).

Table (2): Oligonucleotide sequence of MG mgc2 gene detection **(Lysnyansky *et al.*, 2005).**

Primer name	PCR primer pair base (Base Sequence)	Amplified product
Mgc2 2F	**(3'CGCAATTTGGTCCTAATCCCCAACA'5)**	**300 (bp)**
Mgc2 2R	**(5'TAAACCCACCTCCAGCTTTATTTCC'3)**	

Methods for purification of the PCR Products.

QIAquik PCR product purification protocol:

Using QIAquick PCR Product extraction kit. **(Qiagen Inc. Valencia CA).**

1) 5 volumes of Buffer BP1 were added to 1 volume of the PCR sample and mixed.

2) Checked that the color of the mixture was yellow (similar to Buffer BP1 without the PCR sample).

3) A QIAquick spin column was placed in a provided 2 ml collection tube.

4) To bind DNA, the sample was applied to the QIAquick column, and centrifuged for 1 min at 8000 rpm.

5) Centrifugate was discarded and QIAquick column was placed back in the same collection tube.

6) For washing, 750 ul were added of buffer PE to QIAquick column and centrifuged for 1 min at 8000 rpm.

7) Centrifugate was discarded and QIAquick column was placed back in the same collection tube.

8) Centrifuged for additional 1 min at 8000 rpm.

9) QIAquick column was placed into a clean 1.5 ml micro centrifuge tube.

10) For eluting DNA, 30 µl of Buffer EB (10mM Tris.CL PH 8.5) or water (PH 7.0-8.5) were added to the center of the QIAquick membrane for 5 minutes then centrifugation for 1 min at 12000 rpm was done.

Mgc2 PCR Amplification for MG (Ferguson *et al.*, 2005):

The reaction mixture (total volume of 50µl) was 5µl of 10X PCR reaction buffer (Promega), 5µl 1 mMdNTP mix (Sigma), 4µl of 25 Mm $Mgcl_2$, 5µl DNA *Taq* polymerase, 1µl containing 5 Mm of each primer, 5µl of sample DNA. Then 0.25ml Go-*Taq* polymerase (Promega) added and the mixture was completed by sterile distilled water.

Using thermal cycler machine and MG Thermal cycler program; the amplification consisted of an.

Initial denaturation	**94°C**	**3 min**
Then 40 cycles of:		
Denaturation	**94°C**	**30 seconds**
Annealing	**55°C**	**30 seconds**
Extention	**72°C**	**1 minutes**
Final extention	**72°C**	**5 minutes**

A liquots of amplified products (10µl) were electrophoresed through 1% agarose gel and DNA was visualized by Ultraviolet transilluminator after ethidium bromide staining then photographed.

Screening of PCR products by agarose gel electrophoresis according to **(Sambrook *et al.*, 1989).** Two grams of agarose were added to 100ml Tris acetate EDTA (TAE) buffer. The agarose was autoclaved for 10 minutes and 0.5-µg / ml ethidium bromide was added and then left to cool to room temperature. The gel tray was tapped and the worm agarose was poured in. The comb was inserted in the agarose, which was left to polymerize. After hardening, the tray was untapped, the comb was removed and the gel was applied to electrophoresis cell. The cell was filled with TAE buffer.

10 µl of each of the PCR product sample were applied to the gel along with 5µL molecular weight marker after mixing each with 1 µL loading buffer on a piece of parafilm . Each mixture was applied to a slot using 10µL micropipette. The electrophoresis cell was covered and the power supply was adjusted at 10 volt/cm. the gel was taken out from the cell and examined under short wave UV transilluminator.

Photographing of the gel:

The gel was photographed in order to obtain permanent record using digital camera (Acer CR-5130, China).

Sequencing reaction:

A purified RT-PCR product was sequenced in the forward and/ or reverse directions on an applied Biosystems 3130 automated DNA Sequencer **(ABI, 3130, USA).**Using a ready reaction Big dye Terminator V3.1 cycle sequencing kit.(Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817.

A BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul *et al.*, 1990)** was initially performed to establish sequence identity to Gen-Bank accessions. The sequence reaction was carried out according to the instruction of the manufacture as following.

Table (3): *Preparation of master mix using Big dye Terminator V3.1 cycle sequencing kit.*

Amount	Reagent
2µl	**Big dye terminator v.3.1**
1µl	**Primer**
From 1 to 10 µl	**Template according to quality of band and concentration of DNA**
Complete till to total volume become 20µl	**Deionized water or PCR grade Water**
20µl (Mix well, spin briefly)	**Total volume**

Phylogenetic analysis:

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of Meg Align module of Laser gene DNA Star software Pairwise, which was designed by **Thompson *et al.*, (1994).** Sequence alignments and phylogenetic comparisons of the aligned sequences for the gene were also performed with

the Meg Align module of Laser gene DNA Star software to determine nucleotide and amino acid sequence similarities and relationships.

RESULTS

A total of (142) was characterized by PCR by using MG primer. The samples had amplified fragments at 300bp against MG.

The samples were divided into (22) samples as direct samples on PCR and (120) samples as indirect samples after cultivation on Frey's broth and agar. The results of PCR on direct samples were (8) samples while on indirect samples were (95) samples

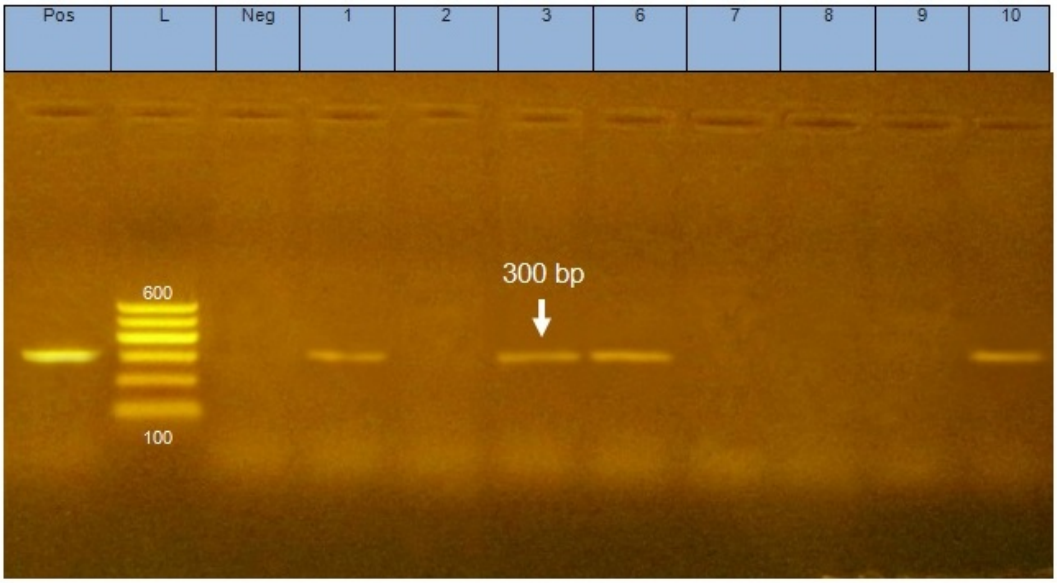

Fig. (1): Agarose gel electrophoresis showing amplification of 300bp fragment of MG.

DNA sequencing result:

Sequencing of mgc2 gene was conducted in both directions and a consensus sequence of 300 bp was used for nucleotides and deduced amino acid analysis. The original sequence was trimmed to remove indefinite nucleotides. Sequences usually exist in the beginning of the sequencing reaction. Four mgc2 sequences were submitted to Gen Bank database where obtained the accession numbers; MF496039 (2032138 seq 1), MG356828 (2032742 seq1), MG356829 (2058726 seq1) and MG356830 (2058734 seq1). Identification of homologies between nucleotide and amino acid sequences of the Egyptian MG strains and other strains published on Gen Bank was done using BLAST 2.0 and PSI-BLAST search programs (National Center for Biotechnology Information "NCBI" http://www.ncbi.nlm.nih.gov/),

respectively. Comparisons of the obtained nucleotide sequences as well as the deduced amino acid of Egyptian MG strains and other strains published on Gen Bank were carried out using the BioEdit sequence alignment editor, ClustalW software for multiple sequence alignment, ClustalV and MegAlign, DNASTAR, Lasergene®, Version 7.1.0, USA. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on ClustalW. Sequence divergence and identity their percent was calculated by MegAlign. Protean™ (DNASTAR, Laser gene® and Version 7.1.0 identified the structural character of mgc2 protein sequence. USA) by measuring of antigenicity index. The 4 samples showed 73.1% maximum identity to the MG - strain - F - Mgc2 strain (Accession no. KJ364633), 76.7% maximum identity to the MG - strain - Eis 10 - 17 (Accession no. KY421065) and MG – strain - KS2 (Accession no. AY556293), 76.4% maximum identity to the MG - strain - R - Mgc2 (Accession no. AY556228), 77.4 % maximum identity to the MG - strain - ts - 11 - Mgc2 (Accession no. JQ770175) and 99.3% maximum identity to the MG - strain - VH1 - Mgc2 (Accession no. JX981926), MG - strain - MGS 1169 (Accession no. KP300759) and MG - strain – 6 - 85(Accession no. KP318741).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12		
1		92.8	91.5	91.8	91.8	72.8	72.8	72.8	73.1	73.1	73.1	73.1	1	KJ364633.1-MG-strain-F-Mgc2
2	5.9		97.7	98.4	97.0	76.7	76.7	76.7	76.7	76.7	76.7	76.7	2	KY421065.1-MG-strain-Eis10-17
3	7.4	2.4		97.4	96.7	76.7	76.7	76.7	76.7	76.7	76.7	76.7	3	AY556293.1-MG-strain-KS2
4	7.0	1.7	2.7		96.7	76.4	76.4	76.4	76.4	76.4	76.4	76.4	4	AY556228.1-MG-strain-R-Mgc2
5	7.0	3.0	3.4	3.4		77.4	77.4	77.4	77.4	77.4	77.4	77.4	5	JQ770175.1-MG-strain-ts-11-Mgc2
6	6.7	3.4	3.5	3.9	2.6		100.0	100.0	99.3	99.3	99.3	99.3	6	JX981926.1-MG-strain-VH1-Mgc2
7	6.7	3.4	3.5	3.9	2.6	0.0		100.0	99.3	99.3	99.3	99.3	7	KP300759.1-MG-strain-MGS1169
8	6.7	3.4	3.5	3.9	2.6	0.0	0.0		99.3	99.3	99.3	99.3	8	KP318741.1-MG-strain-6-85
9	6.2	3.5	3.5	3.9	2.6	0.8	0.8	0.8		100.0	100.0	100.0	9	M1
10	6.2	3.5	3.5	3.9	2.6	0.8	0.8	0.8	0.0		100.0	100.0	10	M3
11	6.2	3.5	3.5	3.9	2.6	0.8	0.8	0.8	0.0	0.0		100.0	11	M6
12	6.2	3.5	3.5	3.9	2.6	0.8	0.8	0.8	0.0	0.0	0.0		12	M10
	1	2	3	4	5	6	7	8	9	10	11	12		

Fig. (2): Sequence distance between the samples.

Phylogenetic analysis of the samples:

The tree indicates that, the 4 sequenced isolates in this study are closely related to each other and placed in one group with the vaccine strain 6/85 and ts11 strain. They are distinct from other field isolates from Egypt and other countries from Ks2 strain and far from the recently identified isolates from the Middle East from R strain and the original vaccine strain F.

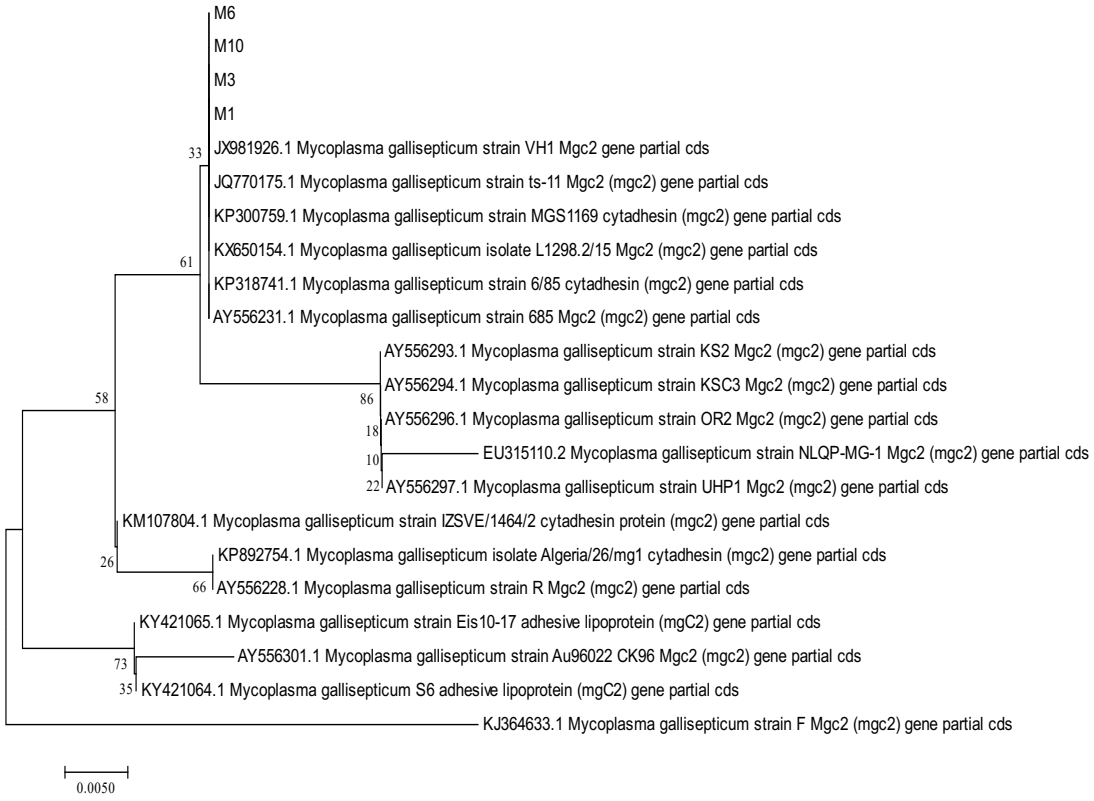

Fig. (3): Phylogenetic tree of the examined 4 samples.

DISCUSSION

MG is a pathogenic species within the genus *Mycoplasma* of the family Mycoplasmataceae. Mycoplasmas (or mollicutes) are eubacteria devoid of cell walls and the smallest self-replicating (can be grown on artificial cell-free media) prokaryotes **(Razin, 1992)**.

It is very important for poultry producer to be able to detect MG – infected birds in early courses of infection **(Kempf *et al.*, 1994).**

MG is an important avian pathogen causing high economic losses within the poultry industry **(Evans and Leigh 2008)**.

In the present study, conventional methods of diagnosis of Mycoplasmas were used. At first, the characteristic fried egg colony of Mycoplasmas was recorded by all isolates. The characteristic colony was tiny, smooth circular, translucent masses with a dense raised central area **(Yoder, 1984 and Quinn *et al.,* 2002).**

Although PCR technique has several advantages, but it also has some disadvantages like serious contamination problem, as a result from improper handling of the DNA tested sample inducing false results **(Levisohn and Kleven, 2000). Salisch *et al.,* (1999)** concluded that parallel to the PCR procedure, the conventional cultural methods should be performed. PCR and culture methods detect MG from 97 and 67% of the suspected samples, respectively. Culture method detects MG on day 26 PI, whereas PCR show positive results on day 54 PI **(Kempf *et al.,* 1993, 1994).** This difference of detection percentage could be due to fastidious nature of the organism and high sensitivity of PCR **(Frey *et al.,* 1968; Chanie *et al.,* 2009).** The molecular characterization of MG field genotypes has allowed for a better understanding of the diversity and epidemiology of the poultry pathogen worldwide. The use of GTS has also allowed the characterization of MG genotypes without the need for cultivation and a wide array of control isolates otherwise found in more *Mycoplasma*-specialized laboratories. In addition, the novel genotypes may help explain the failed vaccinations reported throughout the Egyptian poultry industry. Thus, the identification of novel genotypes present in the different geographical areas has implications for improved control strategies of the disease.

In the current study, the Mgc2 gene was the target gene for the DNA sequencing as the Mgc2 was characterized by the presence of nucleotide insertions/deletions, which may be helpful for differentiation of strains **(Ferguson *et al.,* 2005).**

The 4 sequenced isolates in this study are closely related to each other and placed in one group with the vaccine strain 6/85 and ts11 strain. They are distinct from other field isolates from Egypt and other countries from Ks2 strain and far from the recently identified isolates from the Middle East from R strain and the original vaccine strain F.

The 4 Egyptian isolates those are sequenced on this study (M1, M3, M6 and M10) were closely related to each other (100% identity) and very close to MG strain 6/85 (99.3%) and far from ts11 strain (77.4%) and from F strain of vaccine (73.1%).

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