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Molecular Typing of *Mycoplasma gallisepticum* (Mg) In Egypt Using Lipoprotein Gene

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Background: *Mycoplasma gallisepticum* (Mg) is the most pathogenic and economically important bacterial respiratory pathogen of poultry.
Objective: monitoring the incidence of Mg infection in Monofia and Gharbia Governorates by culture, PCR and molecular characterization of the isolates to assess their genetic relatedness with other selected MG isolates circulating in Egypt and other countries.
Methods: 194 tissue samples and 50 tracheal swabs from broiler chickens flocks from Monofia and Gharbia Governerates were cultured followed by molecular typing of lipoprotein gene of three Mg isolates.
Results: *Mycoplasma gallisepticum* incidence by culture method were (6.2%) & (4.4%) in Monofia and in Gharbia respectively. By using PCR, *Mycoplasma gallisepticum* incidence were (8.46%) & (7.02%) in Monofia and Gharbia respectively. Partial sequencing of lipoprotein gene for three Mg isolates was applied. The generated phylogenetic trees showed similarity with varying percentages between isolates MW689244, MW689245 and MW699359 and other selected isolates of *Mycoplasma gallisepticum* circulating in Egypt and other countries. Strain MW689244 showed 100% nucleotide and amino acid identity with Chinese strain QHDT-1 KY088057. The three isolates belong to one clade with Avipro vaccine CP028147.
Conclusion: PCR was more sensitive than culture. Typing of *Mycoplasma gallisepticum* using lipoprotein gene indicated that the circulated *Mycoplasma gallisepticum* strains are mutant type of Mg F vaccine strain.

Keywords: *Mg, PCR, Lipoprotein gene.*

Introduction

Mycoplasma gallisepticum (MG) causes chronic respiratory disease in domestic poultry, especially when flocks are stressed and/or other respiratory pathogens are present [1].

MG infection is especially important in chickens and turkeys because it causes respiratory disease and reduces meat and egg production [2]. Avian mycoplasma causes a significant economic load on the poultry industry [3].

Mycoplasma gallisepticum (MG) is the cause of respiratory diseases and is the most economically important avian mycoplasma [4], and also; reduces egg production and reduces feed conversion efficiency. Production losses of 10 to 20% have been reported in layers [5]. All ages of chickens and turkeys are susceptible to this disease, but young birds are more susceptible than adult birds [6]. *M. gallisepticum* weakens the immune system of poultry making them susceptible to other diseases and can survive in different tanks in poultry farms such as; food, water, hair, feces and dust [7]. Diagnosis of Mycoplasma infection is based on serology, isolation and identification of Mycoplasmas. Serological determination of Mycoplasma is a good screening tool on a large scale or in commercial poultry farms, but is considered nonspecific due to cross-reactivity of Mycoplasma spp. and often give false positive results [8], [9]. Isolation and identification of mycoplasmas is the gold standard for diagnosis, but it is time consuming due to the slower growth pattern of the organism [10].

The aim of this study was to determine the incidence of respiratory disease caused by *Mycoplasma gallisepticum* in chickens in Monofia and Garbia provinces, and to molecularly classify MG isolates and assess genetic relatedness of them

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along with other MG strains circulating in Egypt and worldwide**.**

Material and Methods

Clinical specimens

A total of two hundred and forty four samples consists of one hundred ninety four tissue specimen (Lungs, tracheas, and air sacs) and (fifty) tracheal swabs were collected from fourteen boiler flocks with age range1-8weeks and average flock size 1000-5000 birds with a history of respiratory manifestations that do not respond to treatment from different districts in Monofia & Gharbia governorates in Egypt table (1). Swabs and tissue specimen were placed into separate sterile containers and then transported to mycoplasma laboratory-Animal Health Research Institute, Egypt- in an ice tank within 24 hours for *Mycoplasma gallisepticum* diagnosis.

TABLE 1. Tissue specimens, swabs, numbers and sources

Specimens type	No./ Locality		Total No.
	Monofia governorate	Gharbia governorate	
Tracheas Lungs & Air sacs	100	94	194
Trachea swabs	30	20	50
Total no.	**130**	**114**	**244**

The Phenotypic characterization of Mycoplasma:

Approximately half grams of each tissue sample was cut into small pieces, ground with sterile sand, and cultured in agar-based medium containing pleuropneumoniae-like microorganism (PPLO) broth and mycoplasma-selective supplement as already mentioned, OIE[1]. Mycoplasma was distinguished from Ahcoleplasma using the digitonin test [11]. Biochemical characterization of Mycoplasma isolates was performed using glucose fermentation and arginine deamination [12]. Typing of Mycoplasma isolates using specific antisera was performed by the growth inhibition test described by [13].

Molecular identification of Mycoplasma

DNA Extraction:

DNA extraction was applied on swabs and tissue samples from (Monofia and Gharbia Governorates). A DNA extraction kit (Qiagen, GmbH, Germany) is used according to the manufacturer's instructions. Finally, the purified DNA was stored at −20 °C until amplification. PCR was applied using three sets of primers the first set of generic primers common for Mollicutes according to Van Kuppeveld *et al.* [14], the second set of primers for 16S rRNA gene for *Mycoplasma Synovaie* (MS) according to Laureman, [15] and the third set of primers for lipoprotein gene according to Nascimento *et al.* [16], Table (2).

TABLE 2. Oligonucleotide primers used for detection of Mycoplasma

Primer designation	Sequence (5'-3')	Reference	Amplified Product Size
Generic primers for Mollicutes	MGSO 5'TGC ACC ATC TGT CAC TCT GTT ACC CTC3' GPO-3 5'GGG AGC AAA CAG GAT TAG ATA CCC T3'	Van Kuppeveld *et al.* [14]	280 bp
MS primers	MS-f 5'GAG AAG CAA AAT AGT GAT ATC A3' MS-r 5' CAG TCG TCT CCG AAG TTA ACA A3'	Lauerman, [15]	207bp
MG primers	MG- f 5'GGA TCC CAT CTC GAC CAC GAC AAA A 3' MG-r 5'CTT TCA ATC AGT GAG TAA CTG ATG A 3'	Nascimento *et al.* [16]	732bp

PCR amplification and cycling protocol.

PCR was carried out using (GTC96) thermo cycler Cleaver scientific Ltd.

Total reaction volume is 50 µL. The reaction mixture consisted of 25 µL of DreamTaq Green Master Mix (2X) (Fermentas, Waltham, USA), 1 µL of 10 pmol of each primer (Sigma-Aldrich, St.Louis, USA), 5 µL of template DNA, and up to nuclease-free 50 µL of water.The PCR amplification program was applied as follows: Initial denaturation at 94°C for 5 min, followed by 40 cycles each of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 minutes.

Electrophoresis

PCR products were detected by electrophoresis on a 1.5% agarose gel in Tris-boric-EDTA buffer combined with markers of appropriate size (100 bp DNA ladder), subsequently examined under ultraviolet light

Genotypic characterization and identification of *Mycoplasma gallisepticum* isolates:

Sequencing data were verified by NCBI Blast search and assembled into edited chromate plots using Bio Edit software version 7.1.5.The edited sequences of the Mycoplasma gallisepticum isolate were characterized using BLAST n for Nucleotides or BLAST p for protein analysis (http://www.ncbi.nlm.nih.gov/BLAST/).To determine the similarity between the isolates and other selected reference isolates, an amino acid sequence identity matrix was calculated. Phylogenetic trees were generated using the distance-based neighbor-joining (NJ) method using MEGA version 7 to evaluate the genetic relatedness between the isolates and other selected reference isolates.

Accession number on GenBank:

The GenBank accession numbers of the generated nucleotide sequences of the *Mycoplasma gallisepticum* isolates reported in this study are (MW689244, MW689245, and MW699359) for the MG lipoprotein gene segments SH1, SH2, and SH3, respectively.

Lipoprotein gene sequence analysis:

Phylogenetic tree generated out of the nucleotide and amino acid identity tables shows similarity to varying percentages between isolates MW689244, MW689245 and MW699359 and other selected isolates of *Mycoplasma gallisepticum* circulating in Egypt and other countries around the world (Table 5, Figure 5).

Results and Discussion

Significant production losses in poultry sector due to egg production losses and downgrading of meat type birds are caused by avian mycoplasmosis Mg and Ms infection ,this infection may be Chronic Respiratory Disease (CRD) or in apparent silent infection [1]. The recovery rate of *Mycoplasma gallisepticum* isolates from Monofia and Gharbia governorates were 6.2% (8/130) and 4.4% (5/114) respectively indicating high prevalence of Mg in Monofia than Gharbia by bacterial isolation (Table 3, Fig. 1).

TABLE 3. The Phynotypic characterization of Mycoplasma from the examined bird flocks in Monofia and Gharbia governorates:

Isolation Sites	% of primary isolation		Digitonin test				Mycoplasma typing using specific antisera				Un typed mycoplasma	
			% of *Mycoplasma Positive isolates*		% of *Acholeplasma*		% of glucose positive Mycoplasma using specific antisera for Mg		% of glucose positive Mycoplasma using specific antisera for Ms			
	Monofia governorate	Gharbia governorate	Monofia governorate	Gharbia governorate	Monofia governorate	Gharbia governorate	Monofia governorate	Gharbia governorate	Monofia governorate	Gharbia governorate	Monofia governorate	Gharbia governorate
Tracheal swabs (150)	12/30 (%40)	4/20 (%20)	11/30 (%36.6)	3/20 (%15)	1/30 (%3.3)	1/20 (%5)	2/30 (%6.7)	1/20 (%5)	3/30 (10%)	0/20 (0%)	6/30 (20%)	2/20 (10%)
Lung& Air sac (104)	/100 32 (%31)	/94 16 (%17)	28/100 (%28)	14/94 (%14.9)	4/100 (%4)	2/94 (%2.1)	6/100 (%6)	4/94 4.3) (%	5/100 (5%)	3/94 (6.4%)	17/100 (17%)	7/94 (7.5%)
Total (244)	/130 44 33.08) (%	/114 20 17.5) (%	39/130 (%30)	17/114 (%14.9)	5/130 (%3.85)	3/114 (%2.63)	8/130 (%6.2)	/114 5 4.4) (%	8/130 (6.2%)	3/114 (2.6%)	23/130 (17.7%)	9/114 (7.9

Total Mycoplasmas recovered were (33.08%) and (17.5%) in Monofia and Gharbia respectively, while *Mycoplasma gallisepticum* prevalence were (6.2%) & (4.4%), and *Mycoplasma synoviae* prevalence were (6.2%) & (2.6%) in Monofia and Gharbia respectively.

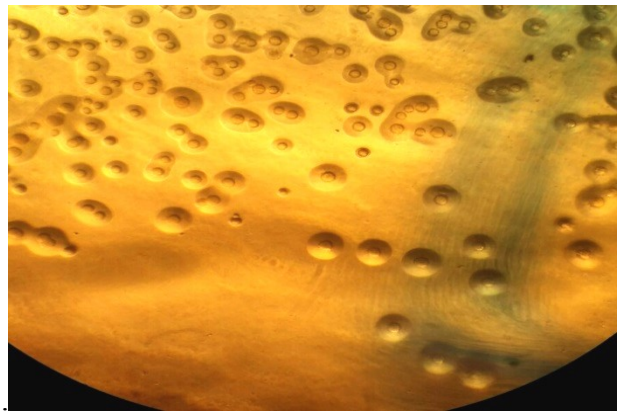

Fig.1. Mycoplasma isolates under sterio microscope showing typical fried egg appearance.

Molecular typing of Egyptian MG field genotypes provides a deeper understanding of the diversity and epidemiology of circulating strains.

16S rRNA-based Mycoplasma group-specific PCR assay: This method amplifies Mollicute species of the genera Mycoplasma, Acholeplasma, Ureaplasma, and Spiroplasma, but not other prokaryotic sequences [14] and [17] Figure (2), Table (4).

After amplification by PCR using primers specific for the lipoprotein gene of *Mycoplasma gallisepticum*, only (10^{-6}) picograms of MG DNA, a fraction of the total chromosomal content of the cell, was detected [15] Figure (3) .

Molecular typing by PCR indicates higher prevalence for Mollicuts in Monofia than Gharbia with lower incidence for *Mycoplasma gallisepticum* than *Mycoplasma synoviae*, Figure (4)

These results are similar to those from Italy, where commercial farms had a lower prevalence of MG than MS in the studied farms [18].This study relies on surface lipoprotein gene detection and target gene sequencing for genotyping of *M. galliseticum* strains.

Isolation and identification of mycoplasma:

Molecular detection of *Mycoplasma gallisepticum* using PCR:

Molecular typing of the Mycoplasma using Mycoplasma common primers at 280bp:

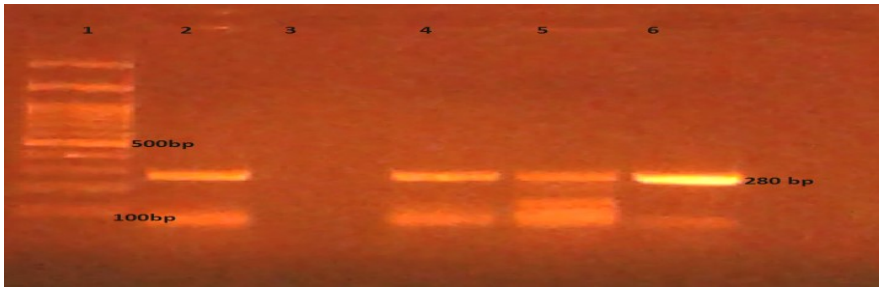

Fig. 2. Agarose gel electrophoresis of common 16SrRNA gene for Mollicutes at 280bp
Lane1:100bp DNA ladder, .lane2: control positive Mycoplasma strain, lane3: control negative and Lanes:4&5- Positive clinical samples

Molecular typing of the Mycoplasma using Lipoprotein gene at 732bp:

TABLE 4 . The recovery rate of Mycoplasma from the examined bird flocks in Monofia and Gharbia governorates by PCR:

Isolation Sites	Common PCR for Mollicutes		Specific PCR for Ms depend on 16S RNA gene		Specific PCR for Mg depends on Lipoprotein gene	
	Monofia governorate	Gharbia governorate	Monofia governorate	Gharbia governorate	Monofia governorate	Gharbia governorate
Tracheal swabs	(13/30) (43.33%)	(4/20) (%20)	5/30 (%16.67)	1/20 (%5)	4/30 (%13.3)	2/20 (%10)
Trachea , Lung& Air sac	(34/100) (34%)	17/94 (18.08)	10/100 (%10)	5/94 (%5.32)	7/100 (%7)	6/94 (%6.4)
Total	47/130 (36.15%)	21/114 (18.42)	15/130 (%11.54)	6/114 (%5.26)	11/130 (%8.46)	8/114 (% 7.02)

By using common PCR for mollicutes the incidence were (36.15%) & (18.42%) *Mycoplasma gallisepticum* incidence were (8.46%) & (7.02%) while *Mycoplasma synoviae* prevalence were (11.54%)&(5.26) in Monofia and Gharbia, respectively.

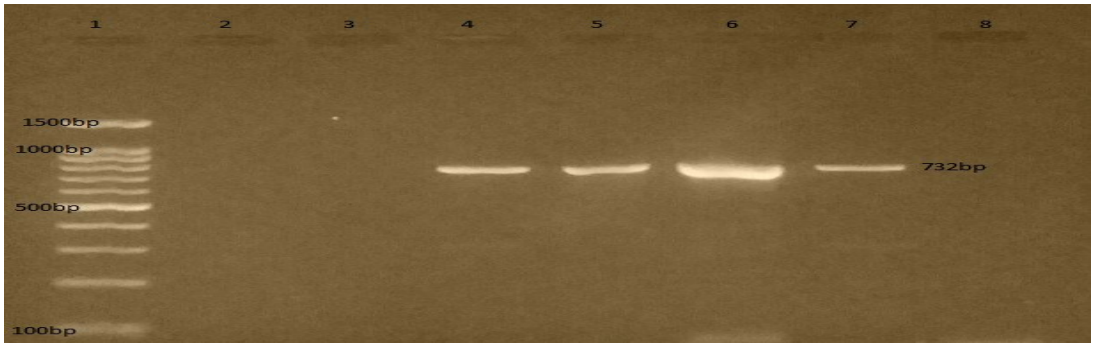

Fig. 3. Lane 1:100 bp DNA ladder , Lanes 2,&3: negative samples , Lanes 4,5and 6 positive samples for lipoprotein gene. Lane 7: control positive Mg, lane 8: control negative.

Molecular typing of the Mycoplasma using 16SrRNA gene for Ms at 207bp:

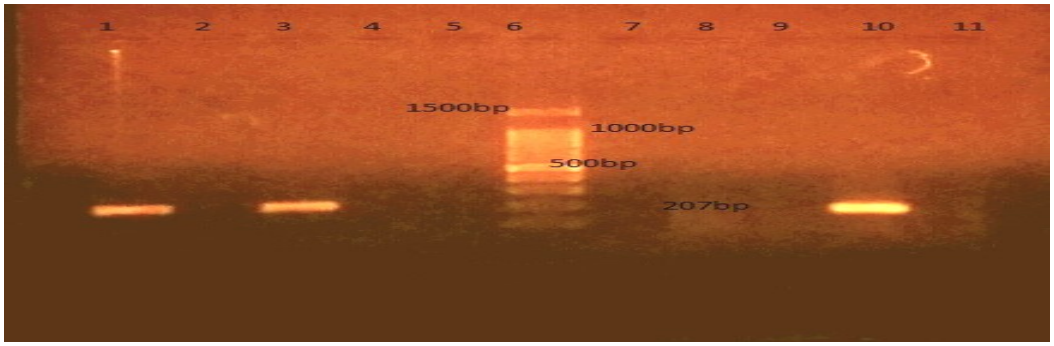

Fig. 4. Lanes 1&3 Positive Ms samples, lanes 2,4,5,7,8 and 9 negative MS samples. Lane 10 control positive Ms. Lane 11 control negative lane 6 :100bp DNA marker.

The lipoprotein gene regions analyzed in this study are more conserved than the mgc2 gene [19]. The generated phylogenetic tree diagram (Figure 5) and nucleotide and amino acid identity (Table 5) are shown for the MW689244, MW689245, and MW699359 isolates and other selected strains circulating in Egypt and other countries around the world showed different percentages of similarity between *Mycoplasma gallisepticum* isolates. The generated phylogenetic tree diagram (Fig. 5) and nucleotide and amino acid identity (Table 5) are shown for the MW689244, MW689245, and MW699359 isolates and other selected strains circulating in Egypt and other countries around the world showed different percentages of similarity between *Mycoplasma gallisepticum* isolates.

The molecular identification of Mycoplasma gallisepticum and bioinformatics analysis of

lipoprotein gene revealed that; the study Strain MW689244 SH1 had 100% nucleotide and amino acid identity with Chinese strain QHDT-1 KY088057.1 and 98%, 97% nucleotide and amino acid identity respectively with f99Avipro vaccine CP028147.1. While MW699359 SH3 strain was 58% and 65% nucleotide and amino acid identity with Egyptian MW689245 SH2. MW689245 strain had 54%, 56% nucleotide identity with f99Avipro vaccine CP028147.1 and Chinese strain QHDT-1 KY088057.1 and amino acid identity 55%, 57% each. From phylogenetic tree, the strains used in this analysis arranged into 2 branches each branch divided into 2 clades. This study isolates MW689244, MW689245 and MW699359 belong to one clade with Avipro vaccine CP028147 which is a type of F strain vaccine and to the Chinese strain QHDT-1 KY088057.1. Vaccination against the F strain is recommended in production areas where virulent wild MG predominates and is likely to replace the virulent MG strain in commercial herds [20]. Despite the disadvantages associated with the F strain being pathogenic and transmitted to broilers and turkeys [21], its vaccination program is still in place. Furthermore, in Italy, M. gallisepticum strains isolated from broiler chickens and their parents were indistinguishable from the vaccine strain ts-11 by the genotyping methods used and were referred to as strains.“ts-11-like” isolate.» [22].

The epidemiology of the outbreaks, together with genotypic and pathogenicity results, suggests that increased virulence and vertical transmission of the ts-11 vaccine has occurred and that the isolates ts-11 strains are most likely revertant strains derived from the ts-11 vaccine. Many reports [22, 23, 24] In Egypt, found that *Mycoplasma gallisepticum* is a pest of the poultry industry [25] discovered by sequencing the mgc2 gene of four closely related isolates close together and placed in a group that has the vaccine strain 6/85 and strain ts11.They also mentioned that litter is a constant source of Mycoplasma contamination and that treatment with appropriate disinfectants before disposal is necessary to limit the spread of Mycoplasma. Global trade in poultry products and waste without taking necessary precautions promotes the spread of new bacterial strains between different countries. Mutations located in the mgc2 region lead to changes in the antigenic index of this region of the mgc2 protein compared to other published MG strains, as mentioned by Eissa [26]. Continuous mutations can lead to the emergence of new virulent strains of Mycoplasma gallisepticum.

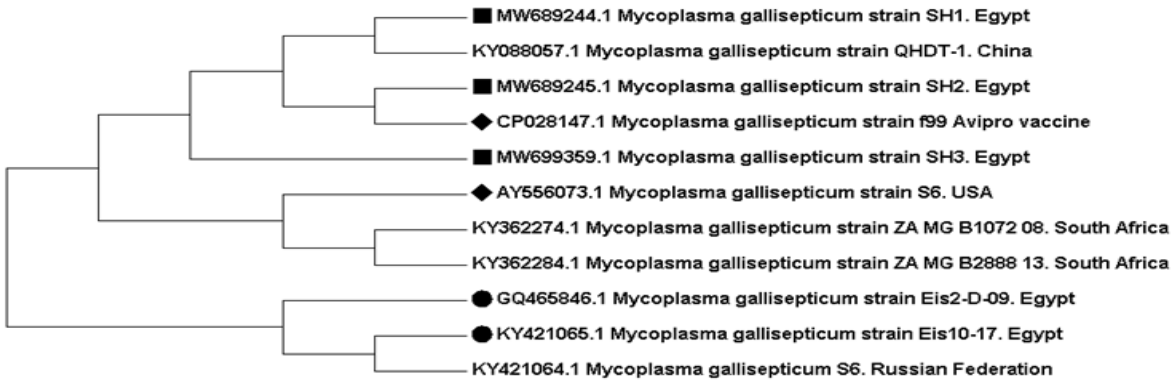

Fig. 5. The tree was constructed using the maximum likelihood method in MEGA 7.
■ This study sequence of *Mycoplasma gallisepticum* on GenBank with accession numbers MW689244, MW689245 and MW699359.
●Other sequences of Egyptian *Mycoplasma gallisepticum* on GenBank.
♦ vaccine strain f99Avipro vaccine CP028147.1.

TABLE 5. Nucleotide and amino acid identities of study isolets MW689244, MW689245, and MW699359 with selected references, Egyptian strains and vaccine sequences.

Seq	>MW689244.1 Mycoplasma gallisepticum strain SH1. Egypt	>MW699359.1 Mycoplasma gallisepticum strain SH3. Egypt	>MW689245.1 Mycoplasma gallisepticum strain SH2. Egypt	>KY421065.1 Mycoplasma gallisepticum strain Eis10-17. Egypt	>GQ465846.1 Mycoplasma gallisepticum strain Eis2-D-09. Egypt	>KY421064.1 Mycoplasma gallisepticum S6. Russian Federation	>AY556073.1 Mycoplasma gallisepticum strain S6. USA	>CP028147.1 Mycoplasma gallisepticum strain f99 Avipro vaccine	>KY088057.1 Mycoplasma gallisepticum strain QHDT-1. China	>KY362274.1 Mycoplasma gallisepticum strain ZA_MG B1072_08. South Africa	>KY362284.1 Mycoplasma gallisepticum strain ZA_MG B2888_13. South Africa
>MW689244.1 Mycoplasma gallisepticum strain SH1. Egypt	ID	32%	56%	37%	36%	37%	38%	98%	100%	38%	38%
>MW699359.1 Mycoplasma gallisepticum strain SH3. Egypt	38%	ID	58%	16%	15%	16%	17%	32%	32%	17%	17%
>MW689245.1 Mycoplasma gallisepticum strain SH2. Egypt	57%	65%	ID	22%	21%	22%	22%	54%	56%	22%	22%
>KY421065.1 Mycoplasma gallisepticum strain Eis10-17. Egypt	13%	6%	7%	ID	93%	100%	39%	37%	37%	39%	39%
>GQ465846.1 Mycoplasma gallisepticum strain Eis2-D-09. Egypt	10%	5%	6%	84%	ID	93%	37%	35%	36%	37%	37%
>KY421064.1 Mycoplasma gallisepticum S6. Russian Federation	13%	6%	7%	100%	84%	ID	39%	37%	37%	39%	39%
>AY556073.1 Mycoplasma gallisepticum strain S6. USA	14%	6%	7%	16%	13%	16%	ID	38%	38%	100%	100%
>CP028147.1 Mycoplasma gallisepticum strain f99 Avipro vaccine	97%	36%	55%	13%	10%	13%	14%	ID	98%	38%	38%
>KY088057.1 Mycoplasma gallisepticum strain QHDT-1. China	100%	38%	57%	13%	10%	13%	14%	97%	ID	38%	38%
>KY362274.1 Mycoplasma gallisepticum strain ZA_MG B1072_08. South Africa	14%	6%	7%	16%	13%	16%	100%	14%	14%	ID	100%
>KY362284.1 Mycoplasma gallisepticum strain ZA_MG B2888_13. South Africa	14%	6%	7%	16%	13%	16%	100%	14%	14%	100%	ID

Conclusions

PCR is more sensitive than culture. Molecular typing of Mg using the lipoprotein gene indicates that the circulated Mg strains are mutant type of Mg F vaccine strain. In addition, the system of using live attenuated immunizations for Mycoplasma gallisepticum should be reconsidered and its role in the spread of mycoplasma is known.

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Conflict of interest: There is no competing of financial interests exists.

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Ethical approval: This study was conducted with the approval of the Agricultural Research Centre Institutional Animal Care and Use Committee (ARC-IACUC) and was organized and operated in accordance with the International Office for Communicable Diseases (OIE) and the Guide to Animal Epidemiology, 8th edition.

Care and Use of Laboratory Animals (2011).IACUC Protocol Number:

ARC	AHRI	23	33

Authrs's Contributions:

Abdelhassieb H. collecting samples share in isolation and identification of Mycoplasma and writing the manuscript. Attia , S. shared in putting the idea of work and follow up the work and Ouda, S. putting the idea of work, Mycoplasma isolation, identification ,molecular identification and sequencing of mycoplasma isolates analysis of data and writing manuscript.

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التصنيف الجزيئي لميكروب الميكوبلازما جاليسبتكم في مصر باستخدام جين البروتين الدهني

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الخلفية: الميكوبلازما جاليسيبتكم (Mg) هي أكثر مسببات الأمراض التنفسية البكتيرية أهمية اقتصادية للدواجن.

الهدف: رصد حالات الإصابة بالميكوبلازما جاليسيبتيكم في محافظتي المنوفية والغربية عن طريق التوصيف البكتيرى بالزرع والتوصيف الجزيئى باستخدام تفاعل انزيم البلمرة المتسلسل وعمل التتابع الجينى الجزئى للعزلات لتقييم ارتباطها الوراثي بعزلات أخرى مختارة من الميكوبلازما جاليسيتيكم المنتشرة في مصر ودول أخرى

الطرق: 194 عينة نسيجية و 50 مسحة من القصبة الهوائية من دجاج التسمين. تمت اخذ العينات من قطعان الدجاج من محافظتي المنوفية والغربية متبوعة بالتصنيف الجزيئي لجين البروتين الدهني لثلاثة من معزولات الميكوبلازما جاليسبتكم.

النتائج: بلغت نسبة الإصابة بالمايكوبلازما جاليسيبتيكم بطريقة الاستزراع (6.2%) و (4.4%) في المنوفية والغربية على التوالي. وباستخدام تفاعل انزيم البلمرة المتسلسل بلغت نسبة حدوث الميكوبلازما جاليسيبتيكم (8.46%) و(7.02%) في المنوفية والغربية على التوالي. تم تطبيق التسلسل الجزئي لجين البروتين الدهني لثلاث عزلات من الميكوبلازما جاليسبتكم . أظهرت أشجار النشوء والتطور المولدة تشابه بنسب متفاوتة بين العزلات MW689244 وMW689245 وMW699359 وعزلات مختارة أخرى من الميكوبلازما جاليسيبتيكم المنتشرة في مصر والدول الأخرى. وأظهرت السلالة MW689244 تشابه هوية النيوكليوتيدات والأحماض الأمينية بنسبة 100% مع السلالة الصينية QHDT-1 KY088057. تنتمي العزلات الثلاث إلى فرع واحد مع لقاح Avipro CP028147

الاستنتاج: كان تفاعل البوليميراز المتسلسل أكثر حساسية من الزرع البكتيرى.. يشير تصنيف الميكوبلازما جاليسيبتيكم باستخدام جين البروتين الدهني إلى أن سلالات الميكوبلازما جاليسيبتيكم المنتشرة هي نوع متحور من سلالة لقاح Mg F.

الكلمات الافتتاحية: الميكوبلازما جاليسبتكم Mg، تفاعل انزيم البلمرة المتسلسل، جين البروتين الدهني.