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Molecular Studies On Fluoroquinolone Resistant *Mycoplasma gallisepticum* Isolates From Broiler Flocks

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ABSTRACT

Mycoplasma gallisepticum (*MG*) is considered the most important pathogenic species causing avian Mycoplasmosis. It is sensitive to antimicrobials whose action sites are other than the bacterial cell wall, such as tetracyclines, macrolides and quinolones. Resistance to fluoroquinolones is mainly due to chromosomal mutations in quinolone resistant determinig regions (QRDRs) of genes encoding the subunits of the drug's target enzymes, DNA gyrase and topoisomerase IV, which are essential for DNA replication. Our study designed for determination of minimum inhibitory concentration (MIC) of fluoroquinolones against 15 field isolates by the broth microdilution method also molecular detection of *MG* virulence gene (mgc2 gene) and mutation in QRDRs of Egyption fluoroquinolones resistant *MG* field isolates. Our results showed that two *MG* isolates were resistant to both veterinary-use and human-use fluoroquinolones. In addition, nucleotide sequence analysis revealed mutations in QRDR of *gyr*B (Ser-437→Leu and Asp- 549→ Asn).

INTRODUCTION

Mycoplasma gallisepticum (MG) is an avian pathogen involved in chronic respiratory disease (CRD) in chickens resulting in considerable economic losses in poultry production industries worldwide *(1)*. Actually, avian mycoplasmosis is caused by several pathogenic mycoplasmas, however *MG* is the most important and the only one that causes an OIE (office international des Epizootic) notifiable disease *(2)*. Three main approaches used for the diagnosis of avian Mycoplasmosis: isolation and identification, detection of antibodies, and molecular detection of the organism's nucleic acid by PCR *(3)*. Culture is the gold standard for direct detection of the organism, but pathogenic avian Mycoplasmas are slow growing, relatively fastidious organisms, and might require up to 3 weeks for detectable growth *(4)*. Polymerase chain reaction represents a rapid and sensitive alternative to traditional culture methods. The 16SrRNA PCR method is commonly used for confirmation of Mycoplasma infection in chickens. However, since it is based on the 16SrRNA gene, the identification of strains from the PCR product is not possible because of the conserved nature of this gene *(5)*. *M. gallisepticum* is characterized by a flask-shaped appearance and a specialized tip-like organelle which mediates cytadhesion to the tracheal epithelial cells through mgc2-cytadhisen encoding surface protein gene which encodes a cytadhesin protein which play a role in the attachment process to mucosal membranes and thus initiate infection *(6)*.

Control of *MG* infection by vaccination is limited because the lack of effective vaccine and so, chemotherapeutically control is necessary . *MG* is known to be susceptible to several antimicrobials *(7,8)* whose action sites are other than the bacterial cell wall, such as tetracyclines, macrolides and quinolones. *(9)*.

Fluoroquinolones inhibit DNA gyrase and topoisomerase IV activities which are involved in DNA replication *(10)*. DNA gyrase is a tetrameric enzyme composed of two GyrA and GyrB subunits, encoded by gyrA and gyrB genes, respectively, while topoisomerase IV is a tetrameric enzyme composed of two ParC and ParE subunits, encoded by parC and parE genes, respectively *(11)*. In *MG*, the major target for quinolones is DNA gyrase *(12)* . This enzyme belongs to type II topoisomerase family, which facilitates DNA unwinding at replication forks, while the main action of topoisomerase IV, is to decatenate or remove the interlinking of daughter chromosomes at the completion of a round of DNA replication and allows their segregation into daughter cell *(13, 14)*.

Extensive application of fluoroquinolones such as enrofloxacin , ciprofloxacin and danofloxacin was the main cause of fluoroquinolones resistantance *(15)*. However, the World Health Organization (WHO) currently considers fluoroquinolones to be critically important antimicrobials, proposing very restricted use in veterinary practice, and a number of countries such as those of the European Union have forbidden some related uses (i.e. use as growth promoters) *(16)*.

Unfortunately, data on the prevalence of antimicrobial-resistant veterinary pathogens are sparse, particularly in developing countries, including Egypt, where antimicrobials are overused in veterinary medicine and food animals. Additionally, due to fluoroquinolone resistance in our tested *MG* strains recovered from diseased chickens in Sharkia Province, Egypt and the limited amount of information on fluoroquinolone resistance in our country, we determined MICs of some veterinary-use and human-use fluoroquinolones and studied the the mutation that occurred in the DNA gyraseB in fluoroquinolone resistant *Mycoplasma gallisepticum* isolates from broiler flocks .

MATERIALS AND METHODS

Samples

Four hundred samples were collected from broiler flocks, with a history of respiratory manifestations from different localities (El-sharkia and El-dakahlia Governorates) and not respond to treatment. Samples included 300 tissue samples (air sacs, tracheas and lungs) and 100 Choanal cleft swabs from diseased chicken.

Isolation and identification of *Mycoplasma* species

Mycoplasmas were isolated on PPLO medium, as previously mentioned *(17)*. which was differentiated from Acholeplasma using Digitonin test *(18)*. Biochemical characterization of Mycoplasma isolates by Glucose fermentation and arginine deaminationas tests was carried out as previously described *(19)*.

In vitro susceptibility testing

In vitro susceptibility testing of 15 *M. gallisepticum* field isolates for fluoroquinolones as enrofloxacin (ENFX), ciprofloxacin (CPFX), difloxacin (DIFX), gatifloxacin (GFLX), levofloxacin (LVFX) and Ofloxacin (OFLX) (Oxoid, UK) was determined by the broth microdilution method *(20)*. Antimicrobial concentrations ranged from 0.016 to 16 µg/ml. MIC results were interpreted according to National Commitee for Clinical Laboratory Standards (NCCLS) institute *(21, 22)*, additionally, MIC50 and MIC90 were calculated using an orderly array method *(23)*.

Conventional Polymerase chain reaction (PCR)

DNA extraction of *M.gallisepticum (8)*

DNA extraction was dove for 15 Mycoplasma isolates (10 from El-sharkia and 5 isolates from El-dakahlia Governorates) recovered from broilers with respiratory manifestations. PCR was done using two sets of primers for the detection of 16S rRNA and mgc2 genes. In addition, gyrB amplified with gene specific primers designed on the basis of the genomic sequence of *M. gallisepticum* strain R (accession no. AE015450) *(24)*. The selected

primers from the published papers are shown in table 1.

PCR amplification and cycling protocol

DNA samples were amplified in a total of 50 µl of the following reaction mixture: 25µl DreamTaq TM Green Master Mix (2X), 1µl of each primers 10 pmol, 5µl template DNA and completed to 50 µl by water nuclease-free. PCR cycling program was performed in thermal cycler (PTC-100 TM programmable thermal cycler, Peltier-Effect cycling, MJ, Research, INC., UK) as following: initial denaturation at 94°C for 30 s, followed by 40 cycles each of denaturation at 93°C for 30 s, annealing at 55°C for 30 s, and extension at 72 °C for 60 s, followed by final extension at 72 °C for 5 min for *16S* RNA gene. Concerning to *mgc*2 gene amplification cycle was initial denaturation at 94 °C for 3 min , followed by 40 cycles each of denaturation at 94°C for 20 s, annealing at 58 °C for 40 s, and extension at 72°C for 60 s, followed by final extension at 72°C for 5 min. In case of *gyr*B gene, PCR cycling conditions consisted of initial denaturation at 95°C for 3 min, followed by 30 cycles each of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s, followed by final extension at 72°C for 10 min. The amplified PCR product was electrophoresed on a 1.5% agarose gel in Tris-acetate-EDTA buffer *(25)*. A 100-bp DNA ladder (Invitrogen, Carlsbad, CA) was used as a molecular weight marker.

Nucleotide sequence analysis of the QRDRs

PCR amplicon was purified from the gel using the QIAquick gel extraction kit (QIAGEN, Valencia, CA) and sequenced in both forward and reverse directions using the amplification primers. The sequencing reaction was performed in an automated sequencer (Macrogen Inc., Korea ABI 3730XL DNA analyzer). DNA sequence data were analyzed by comparison with published GenBank DNA sequences using the NCBI-BLAST program *(26)*. Alignment of the nucleotide sequences was performed by the use of MEGA5 program *(27)*, product version 5.1 (http://www.megasoftware.net). Translation of the nucleotide sequences to amino acid sequences was performed using the ExPASy (Expert Protein Analysis System) Translate Tool (http://us.expasy.org/, Swiss Institute of Bioinformatics SIB, Geneva, Switzerland). Lastly, amino acid sequences were aligned using the MEGA5 program.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this article have been deposited in the GenBank database under accession numbers KJ486460 .

RESULTS

Recovery rate of *M.gallisepticum* isolates from El-sharkia and El-dakhlia Governorates

Seventy two *MG* isolates out of four hundred examined specmines (57/273 *M.gallisepticum* isolates from El-sharkia Governorate and 15/127 *M.gallisepticum* isolates from El-dakahlia Governorate) were obtained from respiratory organs of broilers suffered from respiratory manifestation table 2.

Conventional PCR for confirming of *M. gallisepticum* isolates

The results showed that 16S rRNA gene was detected in all examined isolates and gave characteristic bands at 185bp, while mgc2 gene was detected in 6 examined isolates and gave characteristic bands at 824 bp (Fig. 1, 2).

MICs of fluoroquinolone against *M. gallisepticum* isolates

Among 15 *MG* isolates , 2 only (No.1 and No.6) were resistance for both veterinary and human-use fluoroquinolones. Gatifloxacin (GFLX) was most effective fluoroquinolone against all *MG* isolates. Additionally, MIC50 and MIC90 values were lower for the human - use fluoroquinolones as compared to the veterinary -use agents (Table 3, 4) .

PCR amplification and DNA sequence analysis of quinolone resistant determing region (QRDR) of *MG* isolates

From all tested *MG* strains, a 580 bp fragment covering the region of *gyr*B (Fig. 3), were obtained.

Solid-phase sequencing of the amplified DNA revealed amino acid replacement in the QRDR of *gyr*B at codon 437 (serine TCA → leucine CTC) and 549 (aspartate GAC → asparagine AAT). Interestingly, twelve silent mutations were recorded in such fluoroquinolone resistant *M. gallisepticum* isolate at nucleotides 1308 (ATC-ATA, both are isoleucine), 1317(AAG-AAA, lysine), 1329 (CCT-CCA, proline), 1347 (ACT-ACA, threonine), 1380 (GAA-GAG, glutamate), 1410 (TTA-TTG, leucine), 1548 (CCA-CCT, proline), 1671 (CCA-CCG, proline), 1680 (GGG-GGA, glutamate), 1767 (GGT-GGC, glutamate), 1773 (AAT-AAC, asparagine) and 1776 (CCT-CCC, proline), none of these resulted in amino acid substitutions (Fig. 4). The nucleotide and aminoacid sequences of *M. gallisepticum* gyrase B was deposited into GenBank.

Phylogenetic tree of nucleotides and aminoacids based on *gyr*B gene sequences of *M. gallisepticum* isolate their percentage of identity are shown (Fig 5, 6).

Table 1. Oligonucleotide primers used for detection 16S rRNA, mgc2 and gyrB genes of *M. gallisepticum* field isolates

Primer Designation	Sequence (5'-3')	Amplified Product Size	Reference
16s RNA F	GAGCTAATCTGTAAAGTTGGTC	185 bp	*(5)*
16s RNA R	GCTTCCTTGCGGTTAGCAAC		
MgC2F	GCT TTG TGT TCT CGG GTG CTA	824 bp	*(28)*
MgC2R	CGG TGG AAA ACC AGC TCT TG		
gyrB-F	CTGACGGTAAGATTAGCAAAG	580-bp	*(29)*
gyrB-R	GACATCAGCATCGGTCATGA		

Table 2. Recovery rate of *MG* isolates from El-sharkia and El-dakahlia Governorates

Isolation sites	Recovery rate of *MG* isolates	
	El-sharkia Governorate	El-dakahlia Governorate
Choanal Cleft swab	15/62(24.19%)	4/38(10.52%)
Trachea	14/73(19.17%)	2/27(7.40%)
Lung	13/68(19.11%)	5/32(15.62%)
Air sac	15/70(21.42%)	4/30(13.33%)
Total	57/273(20.87%)	15/127(11.81%)

Table 3. The MICs of antimicrobial agents used against field isolates of *M.gallisepticum* recovered from El-sharkia and El-dakahlia Governorates

Location of recovered isolates Recovered	Isolates number	MIC(µg/ml)					
		ENFX	DIFX	CPFX	GFLX	OFLX	LVFX
El-sharkia	1	4	8	4	8	8	4
	2	0.25	0.125	0.125	0.063	0.063	0.25
	3	0.25	0.125	0.063	0.063	0.063	0.063
	4	0.25	1	0.125	0.063	0.125	0.125
	5	0.063	0.5	0.063	0.063	0.125	0.063
	6	4	8	4	8	8	8
	7	1	0.125	0.125	0.063	0.125	0.125
	8	0.25	0.5	0.063	0.063	0.125	0.25
	9	0.25	0.5	0.063	0.063	0.063	0.063
	10	0.25	0.25	0.125	0.063	0.125	0.25
El-dakahlia	11	0.125	0.063	0.125	0.063	0.125	0.125
	12	0.5	0.125	0.125	0.125	0.25	0.25
	13	0.5	0.125	0.125	0.25	0.25	0.25
	14	0.125	1	0.125	0.25	0.125	0.125
	15	0.25	0.125	0.125	0.125	0.125	0.25

MIC: minimum inhibitory concentration.
ENFX: Enrofloxacin, CPFX: ciprofloxacin, DIFX: difloxacin, GFLX: gatifloxacin, LVFX: levofloxacin and OFLX: Ofloxacin.
High lighted isolates: representing the resistant ones to fluoroquinolones agents.

Table 4. MIC range , MIC_{50} and MIC_{90} of fluoroquinolones against *M.gallisepticum* isolates

Antimicrobial agent	MIC(ug /ml)			
	Break point[a]	Range	MIC_{50}[b]	MIC_{90}[c]
Enrofloxacin	≥ 2	0.063- 4	0.25	0.5
Difloxacin	≥ 4	0.125- 8	0. 25	1
Ciprofloxacin	≥ 2	0.063- 4	0.125	0.125
Gatifloxacin	≥ 8	0.063 – 8	0.063	0.25
Ofloxacin	≥ 8	0.063 – 8	0.125	0.25
Levofloxacin	≥ 8	0.063 – 8	0.25	0.25

a The MIC below which treatment is likely to be successful, values are based on CLSI standards.
b The MIC at which 50% of the isolates are inhibited
c The MIC at which 90% of the isolates are inhibited.
*Both b and c were calculated by an orderly array method (23).

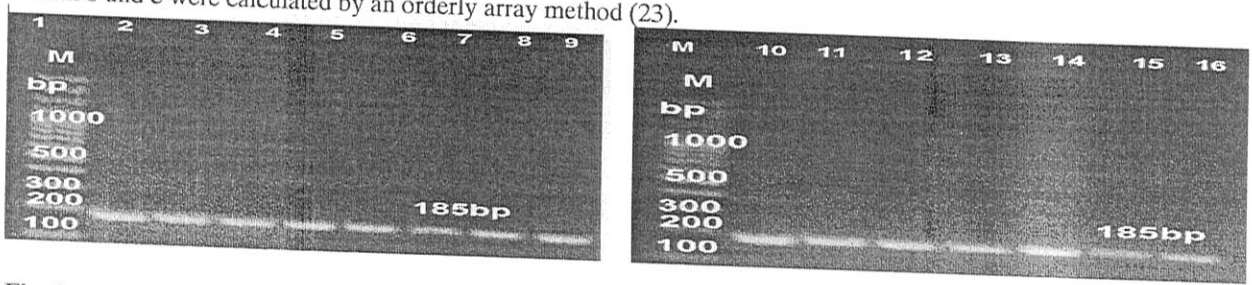

Fig. 1. Agarose gel electrophoresis of *M. gallisepticum* PCR product (16S rRNA gene).
M: 100 bp DNA ladder "Marker".
Lanes (2:16): positive for 16S rRNA gene of *M. gallisepticum* isolates from broilers showed respiratory manifestation.

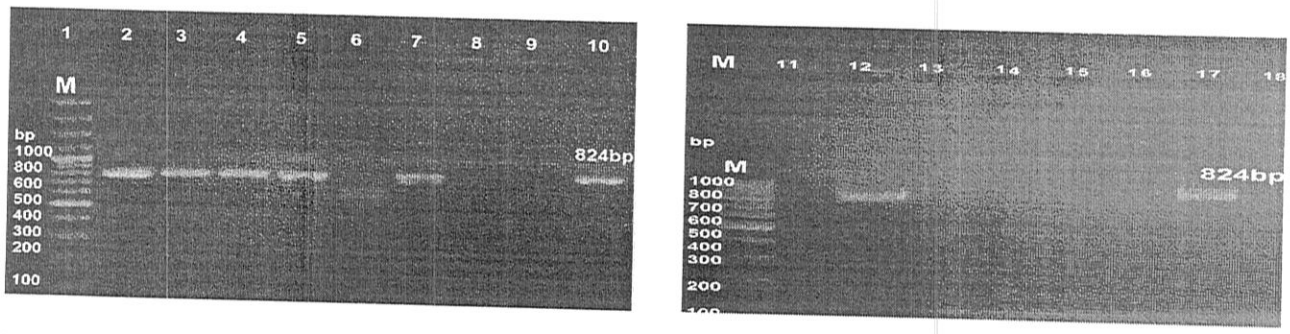

Fig. 2. Agarose gel electrophoresis of *M. gallisepticum* PCR product (*mgc*2 gene).
M: 100 bp DNA ladder "Marker
Lanes(2,3,4,5,7, 17): positive feild MG isolates.
Lanes (9 & 11): negative control for *M. gallisepticum mgc*2 gene.
Lanes (10 &12): positive control (Reference MG strain (PG31)).

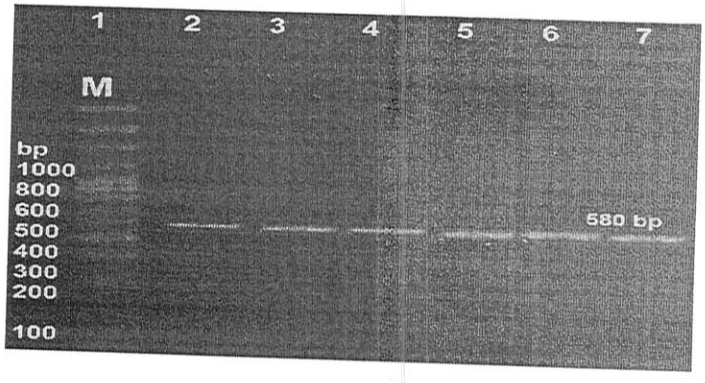

Fig. 3. Agarose gel electrophoresis of *M. gallisepticum* PCR product (*gyrB* gene).
Lane (1)M: 100 bp DNA ladder "Marker".
Lanes (2:7):positive isolates

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M.galliseticum wild strain,NC004829.2 ISIKHSEPQYQGQTKDRLGNTEVREFTNSVVSELLERFFLENPEEAAKITAKAVSAMFSRKRSEAALESARKSPFESASLPGKLADCTTKDMEISELYIV
                                      ---------+---------+---------+---------+---------+---------+---------+---------+---------+
                                     436                                                                                       536
M.gallisepticum-1-Egypt-2014          .L.......
EU597289-M.gallisepticum-MDE-3-gyrB-gen .......
EU597287-M.gallisepticum-SYR-2-gyrB-gen .......
EU595620-M.gallisepticum-YDK-4-gyrB-gen .......
EU595622-M.gallisepticum-MKT-3-gyrB-gen .......
EU595618-M.gallisepticum-JL-10-gyrB-gen .......
EU595611-M.gallisepticum-KBR-3-gyrB-gen .......
EU595619-M.gallisepticum-MT-13-gyrB-gen .......
EU597288-M.gallisepticum-MYZ-8-gyrB-gen .......
                                         ********************************************************************************

M. galliseticum wild strain, NC004829.2 EGDSAGGSAKSGRDRFYQAILPLRGKVLNVEKANHEKIFKNEEIRTLITAIGAGVNPEFSLDKIRYNKIIIMTDADV
                                        ---------+---------+---------+---------+---------+---------+---------+---------+---------+
                                       537                                                                                       612
M.gallisepticum-1-Egypt-2014          ............N.
EU597289-M.gallisepticum-MDE-3-gyrB-gen ............N.
EU597287-M.gallisepticum-SYR-2-gyrB-gen ............N.
EU595620-M.gallisepticum-YDK-4-gyrB-gen ............N.
EU595622-M.gallisepticum-MKT-3-gyrB-gen ............N.
EU595618-M.gallisepticum-JL-10-gyrB-gen ............N.
EU595611-M.gallisepticum-KBR-3-gyrB-gen ............N.
EU595619-M.gallisepticum-MT-13-gyrB-gen ............N.
EU597288-M.gallisepticum-MYZ-8-gyrB-gen ............N.
                                        ************ ************************************************************
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Fig.4. Amino acids sequence similarities for *M. gallisepticum gyr*B of the fluoroquinolones resistant isolate under study and the reference *M. gallisepticum* strains. Dots indicate amino acid positions identical to the corresponding *M.gallisepticum gyr*B sequence. Amino acid positions conserved in all sequences are designated by asterisks. Numbers refer to the aminoacid positions in the *M.gallisepticum gyrB* sequence. The Ser-437 and Asp-549 in which mutations associated with fluoroquinolone resistance are found, are indicated by the solid bars.

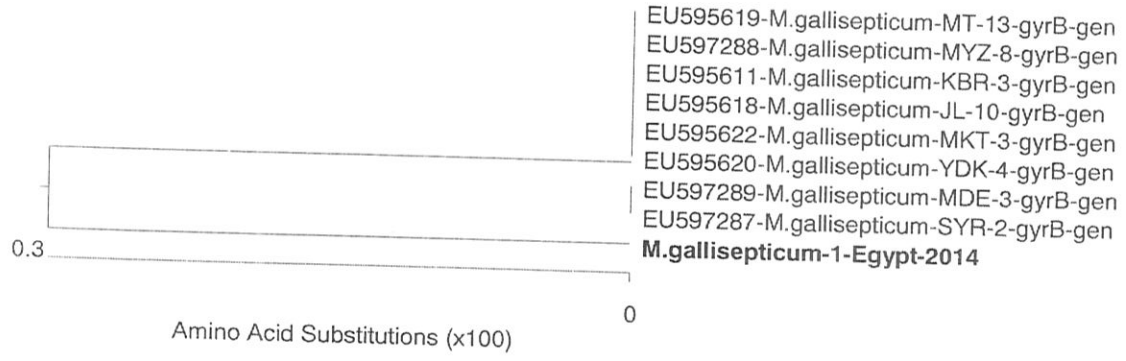

Fig.5. Phylogenetic tree of aminoacids based on *gyr*B gene sequences of *M. gallisepticum* isolate.

Percent Identity

	1	2	3	4	5	6	7	8	9	
1		98.9	98.9	98.9	98.9	98.9	98.9	98.9	98.9	1
2	0.6		100.0	100.0	100.0	100.0	100.0	100.0	100.0	2
3	0.6	0.0		100.0	100.0	100.0	100.0	100.0	100.0	3
4	0.6	0.0	0.0		100.0	100.0	100.0	100.0	100.0	4
5	0.6	0.0	0.0	0.0		100.0	100.0	100.0	100.0	5
6	0.6	0.0	0.0	0.0	0.0		100.0	100.0	100.0	6
7	0.6	0.0	0.0	0.0	0.0	0.0		100.0	100.0	7
8	0.6	0.0	0.0	0.0	0.0	0.0	0.0		100.0	8
9	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0		9
	1	2	3	4	5	6	7	8	9	

M-gallisepticum-1-Egypt-2014
EU597289-M-gallisepticum-MDE-3-gyrB-gen
EU597287-M-gallisepticum-SYR-2-gyrB-gen
EU595620-M-gallisepticum-YDK-4-gyrB-gen
EU595622-M-gallisepticum-MKT-3-gyrB-gen
EU595618-M-gallisepticum-JL-10-gyrB-gen
EU595611-M-gallisepticum-KBR-3-gyrB-gen
EU595619-M-gallisepticum-MT-13-gyrB-gen
EU597288-M-gallisepticum-MYZ-8-gyrB-gen

Fig.(6): Identity % of amino acids for *gyr*B gene sequences of *M. gallisepticum* isolate.

DISCUSSION

Mycoplasma, belonging to the class Mollicutes, is a small free living highly fastidious and slow growing micro-organism, ***(30)***. Avian Mycoplasmosis is considered as one of the major economic problems facing poultry industry all over the world because of its significant losses which are mainly due to poor feed conversion and carcass condemnation at processing ***(31)***. In fact, one important feature of *M. gallisepticum* infection is that it can persist in the bird during all live, even in the presence of the humoral antibodies ***(32)***.

In the present investigation, recovery rate of *MG* isolation (20.87%) from El-sharkia Governorate and (11.81%) from El-dakahlia Governorate as show in table (2). These results agree with that recorded by several authors ***(33 - 38)***.

In this study the MIC values for fluoroquinolones of tested *MG* clinical isolates ranged from 0.063-8ug/ml as reported by other investigator ***(39)***. Also resistant isolates represented cross resistant to both human and veterinary- use fluoroquinolones as mentioned before ***(40 , 15)***.

In this research PCR was applied to amplify 16S rRNA gene of *M. gallisepticum* isolates and *mgc*2 gene which is an important virulence factor as it provides the pathogen for resisting host defenses, selective antibiotic therapy and establishing chronic infection ***(41)***. Results revealed that all examined *M. gallisepticum* isolates had 16S rRNA, which gave a characteristic band at 185 bp when visualized under UV transilluminator ***(5)***. In addition, 6 tested isolates only were positive for *mgc*2 gene and gave characteristic bands at 824 bp ***(28)*** .

Resistance to fluoroquinolones typically arises by stepwise acquisition of target mutations in the QRDRs of DNA gyrase and topoisomerase IV ***(42)***. Genetic analysis of *gyrB* revealed double mutations in the QRDR at codon 437 was reported by other investigator ***(29)*** and at codon 549 (aspartic acid → asparagine) which is considered as first report in this study and not recorded previously ***(15)*** . Mutations in *gyrB* have been associated with quinolone resistance ***(43);*** however, the mutation frequency is much lower compared to those for *gyrA* and *parC* ***(42 , 44, 45)*** Also mutations in *ParC* or *ParE* were observed only in mutants bearing at least one mutation in *gyrA* or *gyrB*, and exhibiting an increase in the MIC of enrofloxacin ***(46)***.

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الملخص العربى

دراسات جزيئيه على الميكوبلازما جاليسيبتكم المقاومه للفلوروكينولون والمعزوله من قطعان بدارى التسمين

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تعتبر الميكوبلازما جاليسيبتكم من اهم الانواع المسببه لميكوبلازما الطيور و هى العامل الرئيسى المسبب لمرض الجهاز التنفسى المزمن و الذى ينتج عنه خسائر اقتصاديه كبيره فى صناعات انتاج الدواجن في جميع أنحاء العالم. تعد الميكوبلازما جاليسيبتكم حساسة لجميع المضادات الحيوية عدا التي تؤثر على جدار الخلية البكتيرية مثل التتراسيكلين و الماكروليدات والكينولون.

يرجع ظهور المقاومة للفلوروكينولونات أساساً إلى حدوث طفرات الكروموسومات في المناطق المحدده لمقاومة الكينولون من الجينات المرمزه للانزيمات التى تؤثر عليها الكينولونات وهى حيث تعتبر هذه الانزيمات ضرورية لتكرار DNA gyrase وtopoisomerase IV الحمض النووي. لذلك صممت دراستنا لتحديد تركيز الحد الأدنى المثبط من الفلوروكينولونات لخمسة عشر معزولة حقلية بواسطه التخفيف المصغر والكشف الجزيئي لجين الضراوه لهذه المعزولات وايضا الكشف الجزيئي للطفرات الموجوده فى المناطق المحدده لمقاومه الكينولون في المعزولات المصريه المقاومه للكينولونات. وقد اظهرت النتائج عدد معزولتين مقاومتين لجميع انواع الفلوروكينولونات ذات الاستخدام البيطرى والبشرى بالاضافه الى ذلك تم تسجيل طفرات فى اماكن تاثير الفلوركينولونات مثل استبدال الحمض الامينى سيرين الى ليوسين عند الموقع٤٣٧ وايضا الاسبرتيت الى اسبرجين عند الموقع٥٤٩ فى gyrase. B