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 Egyptian 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 gyrB (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).
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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 resistance (15). However, the World Health Organization (WHO) currently considers fluoroquinolones to be critically important antimicrobials, proposing very restricted use in veterinary medicine, 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 gyrase B 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-dakaahia 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 Acholoplasma using Digitonin test (18). Biochemical characterization of Mycoplasma isolates by Glucose fermentation and arginine deamination 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 Committee 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 done for 15 Mycoplasma isolates (10 from El-sharkia and 5 isolates from El-dakaahia 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: 25 μl DreamTaq TM Green Master Mix (2X), 1 μl of each primer 10 pmol, 5 μl 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 mge2 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 gyrB 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 ORDR3

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 MEGAS 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-dakhia Governorates

Seventy two MG isolates out of four hundred examined specimens (57/273 M. gallisepticum isolates from El-sharkia Governorate and 15/127 M. gallisepticum isolates from El-dakhia 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 mge2 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).
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PCR amplification and DNA sequence analysis of quinolone resistant determining region (ORDR) of MG isolates

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

Solid-phase sequencing of the amplified DNA revealed amino acid replacement in the ORDR of gyrB 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-CGG, 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 gyrB gene sequences of M. gallisepticum isolate their percentage of identity are shown (Fig 5, 6).

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Sequence (5'-3')</th>
<th>Amplified Product Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16s RNA F</td>
<td>GAGCTAATCTGTAAGTGTTGGTC</td>
<td>185 bp</td>
<td>(5)</td>
</tr>
<tr>
<td>16s RNA R</td>
<td>GCCCTTTGGGTTAGCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgC2F</td>
<td>GCT TGT TGT TGT CGG GTG CTA</td>
<td>824 bp</td>
<td>(28)</td>
</tr>
<tr>
<td>MgC2R</td>
<td>CGG TGG AAA ACC AGC TCT TG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gyrB-F</td>
<td>CTGACGGTAGATAGCAAG</td>
<td>580-bp</td>
<td>(29)</td>
</tr>
<tr>
<td>gyrB-R</td>
<td>GACATCAGCATCGGTGATGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Isolation sites</th>
<th>Recovery rate of MG isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>El-sharkia Governorate</td>
</tr>
<tr>
<td>Choanal Cleft swab</td>
<td>15/62(24.19%)</td>
</tr>
<tr>
<td>Trachea</td>
<td>14/73(19.17%)</td>
</tr>
<tr>
<td>Lung</td>
<td>13/68(19.11%)</td>
</tr>
<tr>
<td>Air sac</td>
<td>15/70(21.42%)</td>
</tr>
<tr>
<td>Total</td>
<td>57/273(20.87%)</td>
</tr>
</tbody>
</table>
### Table 3. The MICs of antimicrobial agents used against field isolates of *M. gallisepticum* recovered from El-sharkia and El-dakahlia Governorates

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

MIC: minimum inhibitory concentration.
High lighted isolates: representing the resistant ones to fluoroquinolones agents.

### Table 4. MIC range, MIC<sub>50</sub> and MIC<sub>90</sub> of fluoroquinolones against *M. gallisepticum* isolates

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrofloxacin</td>
<td>≥ 2</td>
<td>0.063-4</td>
<td>0.25</td>
</tr>
<tr>
<td>Difloxacin</td>
<td>≥ 4</td>
<td>0.125-8</td>
<td>0.25</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≥ 2</td>
<td>0.063-4</td>
<td>0.125</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>≥ 8</td>
<td>0.063-8</td>
<td>0.125</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>≥ 8</td>
<td>0.063-8</td>
<td>0.125</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>≥ 8</td>
<td>0.063-8</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> The MIC below which treatment is likely to be successful, values are based on CLSI standards.
<sup>b</sup> The MIC at which 50% of the isolates are inhibited.
<sup>c</sup> 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 (mgc2 gene).
- M: 100 bp DNA ladder "Marker"
- Lanes (2, 3, 4, 5, 7, 17): positive field MG isolates.
- Lanes (9 & 11): negative control for *M. gallisepticum* mgc2 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

Fig. 4. Amino acids sequence similarities for *M. gallisepticum* gyrB of the fluoroquinolones resistant isolate under study and the reference *M. gallisepticum* strains. Dots indicate amino acid positions identical to the corresponding *M. gallisepticum* gyrB sequence. Amino acid positions conserved in all sequences are designated by asterisks. Numbers refer to the amino acid 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.
**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 mgc2 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 mgc2 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).

REFERENCES


دراسات جزيئية على الميكوبلازما جاليسيتكم المقاومة للفلوروكينولون والمعزولة من قطعان

بدارى التسمين

أحمد محمد عمار، عادل عامر محمد - نورهان خيرى عبادالعزيز -
ساليى حامد عبد الحفيظ - سحر السيد عودة

تتعدد الميكوبلازما جاليسيتكم من الاعشاب المسببة لميكوبلازما الطيور وفي الانتشار الرئيسي
المسبب لمرض الجهاز التنفسي المزمن في الطيور، ونحوه خسائر اقتصادية كبيرة في صناعات انتاج الدجاج
في جميع أنحاء العالم. تعد الميكوبلازما جاليسيتكم حساسة لجميع مضادات الحيوية عدا أوتامافون 32
جدر الخلية البكتيرية مثل الباراسيكلين والماكرويلدات والفينولون.

يرجع ظهور المقاومة للفلوروكينولونات أساساً إلى حدوث طفرات الكروموسومات في المناطق المحددة
لمقاومة الكينولون من الجينات المرمزه للانزيمات التي تؤثر عليها الكينولونات، وهي حيث تحتوي هذه
الحمض النووي. لذلك صممت Topoisomerase IV و DNA gyrase
الإنزيمات ضرورية لتكرار دراستنا لتحديد تركيز الجين الدائم المتبقي للفلوروكينولونات لمسة عشر معزولة حقيقية بواسطة التخليص
المصغر والكشف الجزيئي لجين الضراوه لهذه المعزولات. وأيضاً الكشف الجزيئي للطفرات الموجودة في
المناطق المحددة لمقاومة الكينولون في المعزولات المصرية المقاومة للكينولونات. وقد اظهرت النتائج عدد
معزولات مقاومة جين انواع الفلوروكينولونات ذات الاستخدام البيطرى والبشرى بالإضافة إلى ذلك تم
تسجيل طفرات في أماكن تأتي في الفلوروكينولونات مثل استبدال الحمض الأميني سيرين الى لوبيسين عند
الموقع 374 وايضاً الامبرتن إلى إسريجين عند الموقع 549 في

 gyrase