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

Hesham S. Abdelhassieh¹, Saad Attia² and Sahar E. Ouda³*

¹General Organization for Veterinary Services, Giza, 12618, Egypt
²Faculty of Veterinary Medicine Cairo University, Giza, 12618, Egypt
³Mycoplasma Department, Animal Health Research Institute, ARC, Giza, 12618, Egypt

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 Governorates 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]. Mycoplasma 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.
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**

<table>
<thead>
<tr>
<th>Specimens type</th>
<th>No./ Locality</th>
<th>Monofia governorate</th>
<th>Gharbia governorate</th>
<th>Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracheas</td>
<td></td>
<td>100</td>
<td>94</td>
<td>194</td>
</tr>
<tr>
<td>Lungs &amp; Air sacs</td>
<td></td>
<td>30</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Tracheal swabs</td>
<td></td>
<td>130</td>
<td>114</td>
<td>244</td>
</tr>
</tbody>
</table>

**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 Synoviaie* (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**

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Sequence (5'-3' )</th>
<th>Reference</th>
<th>Amplified Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGSO</td>
<td>5'TGC ACC ATC TGT CAC TCT GTT ACC CTC3'</td>
<td>Van Kuppeveld et al. [14]</td>
<td>280 bp</td>
</tr>
<tr>
<td>GPO-3</td>
<td>5'GGG AGC AAA CAG GAT TAG ATA CCC T3'</td>
<td>Lauerman, [15]</td>
<td>207bp</td>
</tr>
<tr>
<td>MG SO</td>
<td>5'GGA TCC CAT CTC GAC CAC GAC AAA A 3'</td>
<td>Nascimento et al. [16]</td>
<td>732bp</td>
</tr>
<tr>
<td>MS</td>
<td>5'GAG AAG CAA AAT AGT GAT ATC A3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG r</td>
<td>5'CAG TCG TCT CCG AAG TTA ACA A3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG SO</td>
<td>5'GGA TCC CAT CTC GAC CAC GAC AAA A 3'</td>
<td>Nascimento et al. [16]</td>
<td>732bp</td>
</tr>
<tr>
<td>MG r</td>
<td>5'CAG TCG TCT CCG AAG TTA ACA A3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**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-borate-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 Phenotypic characterization of Mycoplasma from the examined bird flocks in Monofia and Gharbia governorates:**

<table>
<thead>
<tr>
<th>Isolation Sites</th>
<th>% of Primary Isolation</th>
<th>Digitonin test</th>
<th>Mycoplasma typing using specific antisera</th>
<th>Un typed mycoplasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of Mycoplasma Positive isolates</td>
<td>% of Acholeplasma</td>
<td>% of glucose positive Mycoplasma</td>
<td>% of glucose positive Mycoplasma</td>
</tr>
<tr>
<td></td>
<td>Monofia governorate</td>
<td>Gharbia governorate</td>
<td>Monofia governorate</td>
<td>Gharbia governorate</td>
</tr>
<tr>
<td>Swab samples</td>
<td>12/30 (40%)</td>
<td>4/20 (20%)</td>
<td>11/30 (36.6%)</td>
<td>3/20 (15%)</td>
</tr>
<tr>
<td>Lung samples</td>
<td>/100 (5%)</td>
<td>/94 (6%)</td>
<td>/28/100 (30%)</td>
<td>/149/49 (4%)</td>
</tr>
<tr>
<td>Air samples</td>
<td>/130 (50%)</td>
<td>/114 (6%)</td>
<td>/39/130 (30%)</td>
<td>/17/114 (14.9%)</td>
</tr>
<tr>
<td>Total samples</td>
<td>/33/108 (50%)</td>
<td>/17.5%</td>
<td>/17/114 (14.9%)</td>
<td>/100 (5%)</td>
</tr>
</tbody>
</table>

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.
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).

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:**

**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:

<table>
<thead>
<tr>
<th>Isolation Sites</th>
<th>Common PCR for Mollicutes</th>
<th>Specific PCR for Ms depend on 16S RNA gene</th>
<th>Specific PCR for Mg depends on Lipoprotein gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monofia governorate</td>
<td>Gharbia governorate</td>
<td>Monofia governorate</td>
</tr>
<tr>
<td>Tracheal swabs</td>
<td>(13/30)</td>
<td>(4/20)</td>
<td>5/30</td>
</tr>
<tr>
<td></td>
<td>(43.33%)</td>
<td>(%20)</td>
<td>(%16.67)</td>
</tr>
<tr>
<td>Trachea, Lung &amp; Air sac</td>
<td>(34/100)</td>
<td>17/94</td>
<td>10/100</td>
</tr>
<tr>
<td></td>
<td>(34%)</td>
<td>(18.08)</td>
<td>(%10)</td>
</tr>
<tr>
<td>Total</td>
<td>47/130</td>
<td>21/114</td>
<td>15/130</td>
</tr>
<tr>
<td></td>
<td>(36.15%)</td>
<td>(18.42)</td>
<td>(%11.54)</td>
</tr>
</tbody>
</table>

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, 5, and 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.
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.

Acknowledgements: Dr. Alaa Saad is appreciated for helping in phylogenetic analysis.

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

Funding Statement: Self-funding

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:

<table>
<thead>
<tr>
<th>ARC</th>
<th>AHRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>33</td>
</tr>
</tbody>
</table>

Authors’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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Molecular Typing of Mycoplasma gallisepticum (MG) in Egypt Using …

Hesham Salah 1, Saeed Abd El-Hady 2 and Sawsan El-Sayed 3

1. The National Veterinary Services, Giza, 2618, Egypt
2. Faculty of Veterinary Medicine, Cairo University, Giza, 2618, Egypt
3. Mycoplasma Research Division, Animal Health Research Center, Giza, 2618, Egypt

Background: Mycoplasma gallisepticum (MG) is one of the most important respiratory bacterial pathogens of poultry.

Objective: To monitor the occurrence of Mycoplasma gallisepticum infections in the provinces of Menoufia and Qalyubia by bacterial culture and molecular typing using the polymerase chain reaction enzyme-linked amplification technique (PCR-ELAT) and partial sequencing of the fatty acid protein gene of three isolates of Mycoplasma gallisepticum. The genetic relationships between MG isolates in Menoufia and Qalyubia and MG isolates from selected strains circulating in Egypt and other countries were evaluated.

Methods: 194 poultry tissue samples and 50 respiratory swabs from fattening chickens were collected from the provinces of Menoufia and Qalyubia. The fatty acid protein gene partial sequencing of three Mycoplasma gallisepticum isolates was performed. A distance tree was generated using the Nei-Gojobori genetic similarity method and the neighbor-joining algorithm. The lengths of the nucleotide and amino acid sequences were compared with the sequences of previously reported isolates from China (QHDT-1 KY088057).

Results: The percentage of MG isolation by culture was 6.2% (12 of 194) and 4.4% (21 of 476) in Menoufia and Qalyubia, respectively. The PCR-ELAT technique detected 8.46% (17 of 194) and 7.02% (34 of 476) MG infections in Menoufia and Qalyubia, respectively. The genetic similarity and genetic distance of the partial sequences of the fatty acid protein gene of the three Mycoplasma gallisepticum isolates were compared with other selected isolates. The genetic similarity of the three Mycoplasma gallisepticum isolates to the Avipro CP028147 vaccine strain was 100%.

Conclusion: The PCR-ELAT technique was more sensitive than bacterial culture and can detect Mycoplasma gallisepticum infections in poultry. The results obtained suggest that Mycoplasma gallisepticum strains circulating in Egypt and other countries are genetically similar to the Avipro CP028147 vaccine strain.