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Genotypic Characterization of Mycoplasma Species Isolated From Turkey Flocks

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MYCOPLASMOSIS in turkeys is a major egg transmitted disease characterized by infra-orbital sinusitis, reduced hatchability, high embryonic mortality, reduced live body weight. Swollen infra-orbital sinus in turkeys resulting in misshapping, and difficult marketing as well as spread of infection to other poultry species. So, this study aimed to investigate Mycoplasmas involved in turkey sinusitis using molecular technique, their phylogenetic analysis versus GenBank database. Suspected turkey flocks (n=17) were investigated using PCR technique for aspirated fluid from infra-orbital sinus. Seven selected isolates of both identified *M. gallisepticum* (MG), and *M. meleagridis* (MM) were subjected for sequencing, and phylogenetic analysis. PCR results exhibited single infection of MG and MM in 8, and 3 out of 17 turkey flocks respectively, while mixed infection of both MG and MM were found in 4 flocks. Sequenced MG mgc2 gene of selected seven strains showed similar identity of 93.1-97.6 with each other, 90.4-97.3% with field strains and 84.9-95.9% with reference ones. On the other hand, sequenced 16s rRNA of all isolated seven MM strains revealed complete identity of 100% with each other, 99.7% with MM reference strains, and 83.1-93.1% with other Mycoplasmas spp Both MG, and MM isolates were registered on the GenBank with accession numbers; MW713803, MW713804, MW713805, MW713806, MW713807, MW713808, MW713809, MW700292, MW700293, MW700294, MW700295, MW700296, MW700297, MW700298. It was concluded that the increased incidence of MG in turkeys sinusitis, also, MM isolates were the 1st Egyptian field strains submitted to GenBank database. There were similarities between the recent isolates and reference vaccine strains as well as these molecular analysis and genetic diversity would play an important role in control strategy of Mycoplasmosis in poultry.

Keywords: *Mycoplasma gallisepticum, Mycoplasma meleagridis*, PCR, Sequencing, Turkey flocks.

Introduction

There were many mycoplasma species in poultry, the most important ones include *M. meleagridis (MM), M. gallisepticum* (MG), *M. iowae*, and *M. synoviae* that causing a disease called avian Mycoplasmosis with vertical and/or horizontal transmission [1].

MG was found in 56 bird species [2], and it was characterized by ocular and nasal discharges, respiratory rales, coughing, sneezing in chickens and conjunctivitis with infra orbital sinusitis in turkeys [3].

Mycoplasmosis is one of the costliest disease in chickens and turkeys production as a result of reduced egg production, increased embryonic mortality, and medication costs [4 , 5]

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MM is mainly recovered from turkeys and characterized by poor embryonic growth performance, skeletal abnormalities, and early mortality with infrequent infraorbital sinusitis [6,7].

The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory, urogenital tracts, and the joints in some animals.

Mycoplasmas are not only extracellular but also intracellular agents [8], with higher susceptibility to mutations [9].

Adherence of Mycoplasma to the epithelial cells of respiratory system resulting in inflammations and damaged cells [10]. Also, Mycoplasma produce biofilm increasing its resistance and chronicity of the disease [11,12], besides, secondary metabolites (Hydrogen peroxide, H_2O_2) were produced to damage the respiratory cilia and oxidize the lipids [13-15]. Additionally, Mycoplasma modulate the host immune response [16] and increase the birds susceptibility to other infection like *H. paragallinarum*, *E. coli,* and virus infection.

In Egypt, a few papers have discussed Mycoplasma in turkeys as well as the turkey herd is considered the hidden source of Mycoplasma infection in the poultry sector, as it is affected with sinusitis, which is the most common disease of turkeys that mainly caused by Mycoplasma and too difficult to treat, leading to infection dissemination among the other different poultry flocks. Also, Mycoplasmosis caused severe economic losses include body weight losses, carcass condemnations, medication costs, poor hatchability, and early embryonic mortality. So, this work aimed to focus on mycoplasma infection in turkeys in relation to turkey sinusitis using PCR technique and determine the gene diversity among the isolates.

Material and Methods

Samples collection and processing

Seventeen Turkey flocks suffering from infra-orbital sinusitis, 9 Bronze, 2 White Nicholas, and 6 Balady were investigated in Alexandria. The aspirated exudate under aseptic conditions were subjected for general bacteriological examination to affirm the co-infection then were transmitted to Reference laboratory for Veterinary Quality Control on Poultry Production, Agriculture Research Center, Egypt. for application of PCR against the available Mycoplasma primers (*M. gallisepticum*, and *M. meleagridis)*.

DNA extraction and purification

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract DNA by mixing of the sample suspension, 200 µl with proteinase K, 10 µl, and lysis buffer, 200 µl then incubated at 56°C for 10 min. After that, 200 µl, ethanol (100%) was added. The sample washed and centrifuged followed by nucleic acid elution.

Oligonucleotides Primers

All primers throughout current study were supplied by Metabion, Germany as illustrated in (table 2).

Polymerase chain reaction

In thermal cycler (Applied biosystem 2720) put a mixture of 12.5 µl, EmeraldAmp Max PCR Master Mix (Takara, Japan) with 1 µl from each primer (20 pmol concentration), 4.5 µl, water, and 6 µl, DNA template.

PCR analysis

At room temperature, 1.5% agarose gel in 1x TBE buffer was prepared for PCR electrophoresis. Put 20 µl, PCR product from each in each gel slot and 100 bp, ladder, computer software, gel documentation system were used.

Sequencing and Phylogenetic tree

QIAquick PCR Product extraction kit (Qiagen, Valencia), Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer), and Centrisep spin column were used to purify the PCR products of randomly selected Mycoplasma isolates (seven MG, and seven MM). The sequence reaction was analysed in Applied Biosystems3130 genetic analyzer (HITACHI, Japan). The sequence result was identified by Basic Local Alignment Search Tool analysis (BLAST®) [17] and recorded in GenBank. The phylogenetic tree was created by the Meg Align module of Laser gene DNA Star version 12.1 [18], and Phylogenetic analyses were performed using maximum likelihood, neighbor-joining, and maximum parsimony in MEGA6 [19].

Ethical approval

The Ethics of Animal Health Committee, Desert Research Center, Egypt affirmed This study.

TABLE 1. History of collected samples from infectious sinusitis in turkey flocks in Alexandria Governorate.

Farm No.	Area/ date	Number of sample tested separately/ Number of birds/ breed	Size of swelling	Eye involvement	Exudate color/ consistency
1	Kilo28 North coast/11/5/2020	3/100/White Nicholas	Moderate	involved	Clear liquid
2	Hawaria/22/5/2020	2/50/balady	Mild	Not involved	Clear liquid
3	Naseria-1/27/8/2020	2/200/Bronze	Large	involved	Turbid viscous
4	Amria-K/13/6/2020	1/50/Balady	Large	involved	Turbid viscous
5	Abees-1/9/8/2020	1/150/Balady	Moderate	Not involved	Clear liquid
6	Kilo21 North coast/9/6/2020	3/100/Balady	Mild	Not involved	Clear liquid
7	AmriaA/25/7/2020	2/150/Bronze	Large	involved	Turbid viscous
8	Nobaria/20/10/2020	3/200/Balady	Moderate	involved	Turbid viscous creamy
9	Abees-2/15/11/2020	2/50/Bronze	Moderate	involved	Turbid viscous
10	Naseria-2/6/12/2020	2/100/Bronze	Large	involved	Turbid viscous with caseation
11	Nobaria/23/12/2020	2/150/Bronze	Moderate	Not involved	Clear liquid
12	Kilo35Desert road/28/12/2020	1/50/Bronze	Mild	Not involved	Clear liquid
13	Kilo48North coast 3/1/2021	2/50/Balady	Large	involved	Turbid viscous with caseation
14	Kilo59Desert road 12/1/2021	3/200/White Nicholas	Moderate	involved	Turbid viscous
15	Maryout-1/15/1/2021	2/150/Bronze	Large	involved	Turbid viscous creamy
16	Maryout-2/15/1/2021	2/100/Bronze	Large	involved	Turbid viscous with caseation
17	Maryout-3/15/1/2021	2/100/Bronze	Large	involved	Turbid viscous creamy

TABLE 2. Target genes, primer sequence, and amplicon size for investigation of *M. gallisepticum and M. meleagridis* among turkeys

Target genes	Primers	Amplified segments (bp)	References
M. gallisepticum mgc2	CGCAATTTGGTCCTAATCCCCAACA TAAACCCACCTCCAGCTTTATTTCC	300	[20]
M. meleagridis 16S rRNA	CGA GCG AAG TTT TTC GGA AC GGTACC GTC AGG ATA AAT GC	422	[21]

Results

PCR Results

Within the fifteen positive flocks affected with infra-orbital sinusitis, MG detected alone in eight flocks, while MM detected alone in Three flocks. Both MG, and MM were positive in four out of 17 under current investigation–in 4 flocks. (Table 3 and Figures 1 and 2).

Mycoplasma isolates sequence and phylogenetic tree analysis

MG mgc2 ~~gene~~ of selected seven strains (Maryout-3, Amria-A, Nobaria, Naseria, Naseria-2, Hawaria, and Abees-2) was compared with that of Egyptian field, and reference vaccinal strains (S6, ts-11, and F). All isolates were

genetically related to Egyptian field strain with 89 bsv (bootstrap value), and among them with 73 bsv. Their sequence identity was 93.1-97.6% with each other and 90.4-97.3% with the Egyptian strains, and 84.9-95.9% with reference ones (Figures 3, and 4). Maryout-3 strain was achieved the higher similarity of 97.3% with field strain (Eid1.mg-TK-EG014) and 95.9% with reference strain (S6). 16s rRNA gene of other selected seven MM strains (Maryout-1, Maryout-2, Maryout-3, North-coast, Amria-k, Nobaria, and Desert road) were clustered with MM-ATCC-27764, MM-NCTC-10153, MM-NBRC-14852, and MM-17529 in the same branch of 99 bootstrap value and 63 bootstrap value with other *Mycoplasma* spp.. An amino acid sequence identity of all seven MM strains was 100% with each other, 99.7% with other MM spp., and 83.1-93.1% with other *Mycoplasma* spp. (Fig. 5 and 6).

GenBank accessions

Genes sequences of both MG and MM were registered in GenBank and the accession numbers were taken as follow, MW713803 for Hawaria, MW713804 for Naseria, MW713805 for Amria-A, MW713806 for Abees-2, MW713807 for Naseria-2, MW713808 for Nobaria, MW713809 for Maryout-3, MW700292 for Amria-k, MW700293 for Nobaria, MW700294 for Desert road, MW700295 for North-coast, MW700296 for Maryout-1, MW700297 for Maryout-2, and MW700298 for Maryout-3 (Table 3).

TABLE 3. Incidences of MG and MM isolated from turkeys as single and mixed infection.

No. of infection	%	Single infection				Mixed infection		Total
		MG	%	MM	%	MG & MM	%	
2	11.76%	8	47.06%	3	17.65%	4	23.53%	17

TABLE 4. Data of Mycoplasma isolates registered in GenBank.

Serial numbers	Identified codes	Area	Species	Breeds	Collection dates	Accession numbers
1	Hawaria	Alexandria	Turkey	Balady	22/5/2020	MW713803
2	Naseria			Bronze	27/8/2020	MW713804
3	Amria-A			Bronze	25/7/2020	MW713805
4	Abees-2			Bronze	15/11/2020	MW713806
5	Naseria-2			Bronze	6/12/2020	MW713807
6	Nobaria			Bronze	23/12/2020	MW713808
7	Maryout-3			Bronze	15/1/2021	MW713809
8	Amria-K			Balady	13/6/2020	MW700292
9	Nobaria			Balady	20/10/2020	MW700293
10	Desert road			Bronze	28/12/2020	MW700294
11	North-coast			Balady	3/1/2021	MW700295
12	Maryout-1			Bronze	15/1/2021	MW700296
13	Maryout-2			Bronze	15/1/2021	MW700297
14	Maryout-3			Bronze	15/1/2021	MW700298

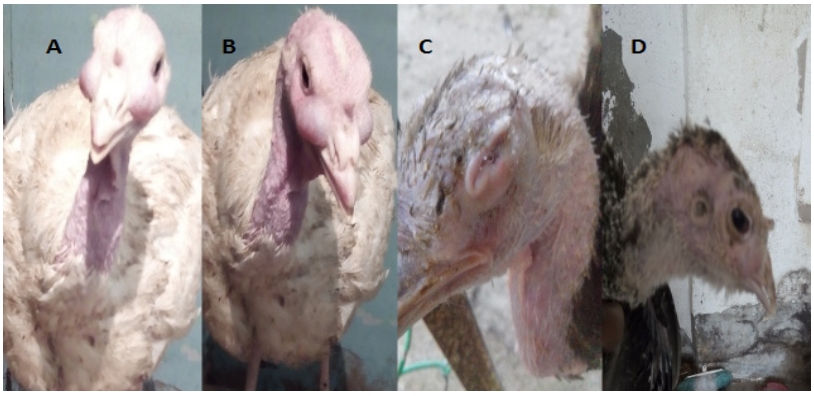

Fig. 1. Turkeys suffering from infra-orbital sinusitis. A: 100 White Nicholas turkeys, Kilo28 North coast, 4 months age collected at 11/5/2020. B: 200 White Nicholas turkeys, Kilo59Desert road, 3 months age, collected at 12/1/2021. C: 100 Bronze turkeys, Maryout-3, 3 months age, collected at 15/1/2021. D: 50 Balady turkeys, Hawaria, 2 months age, collected at 22/5/2020

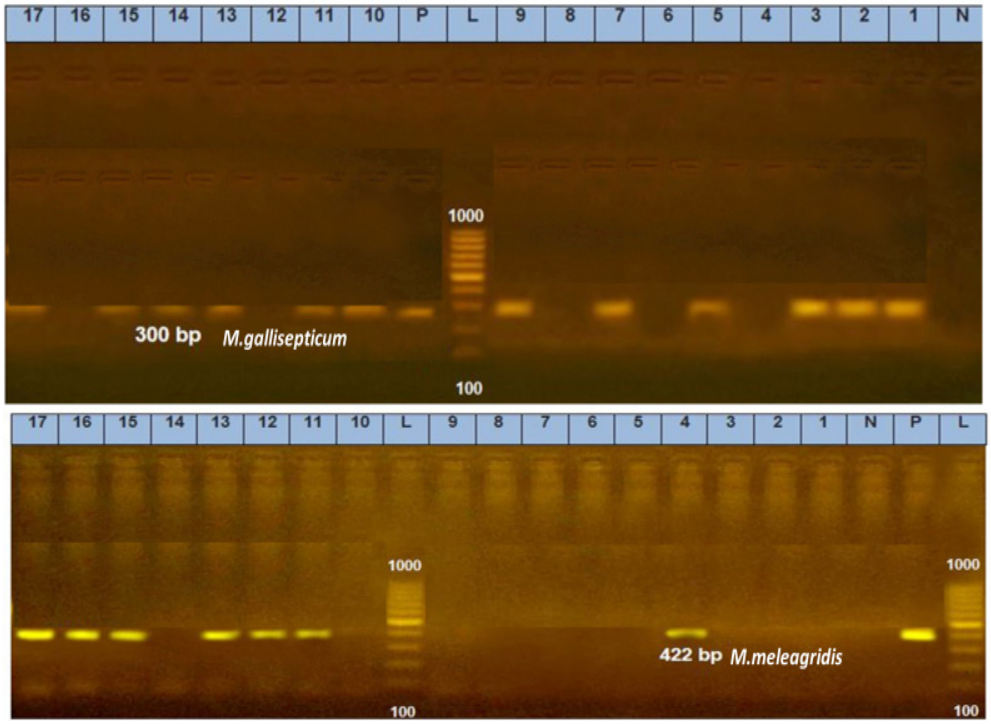

Fig. 2. Electrophoresis exhibited that *M. gallisepticum* detected specific bands at 300 bp in lanes 1, 2, 3, 5, 7, 9, 10, 11, 13, 14, 15, 17 and *M. meleagradis* detected at 422 bp in lanes 4, 11, 12, 13,15, 16, 17. Lane P is positive control, Lane N is negative control, Lane L is loader.

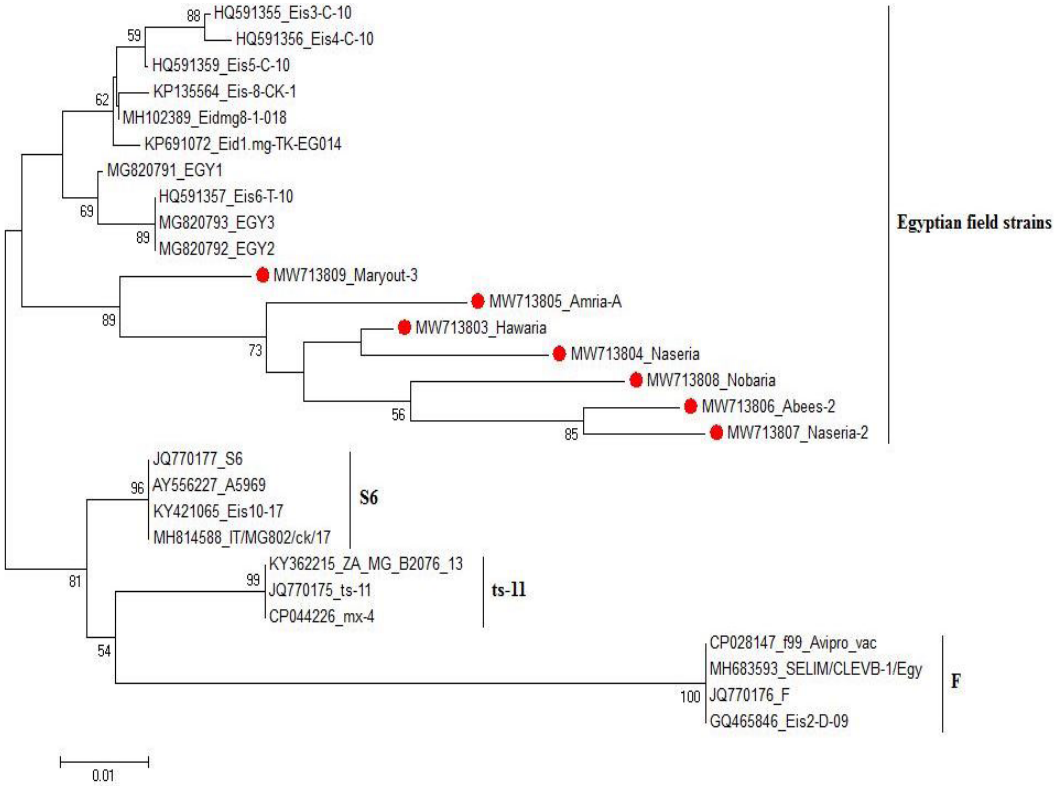

Fig. 3. Genetic relationships among *Mycoplasma gallisepticum* isolated strains (indicated by red dots), Egyptian field, and reference vaccine strains.

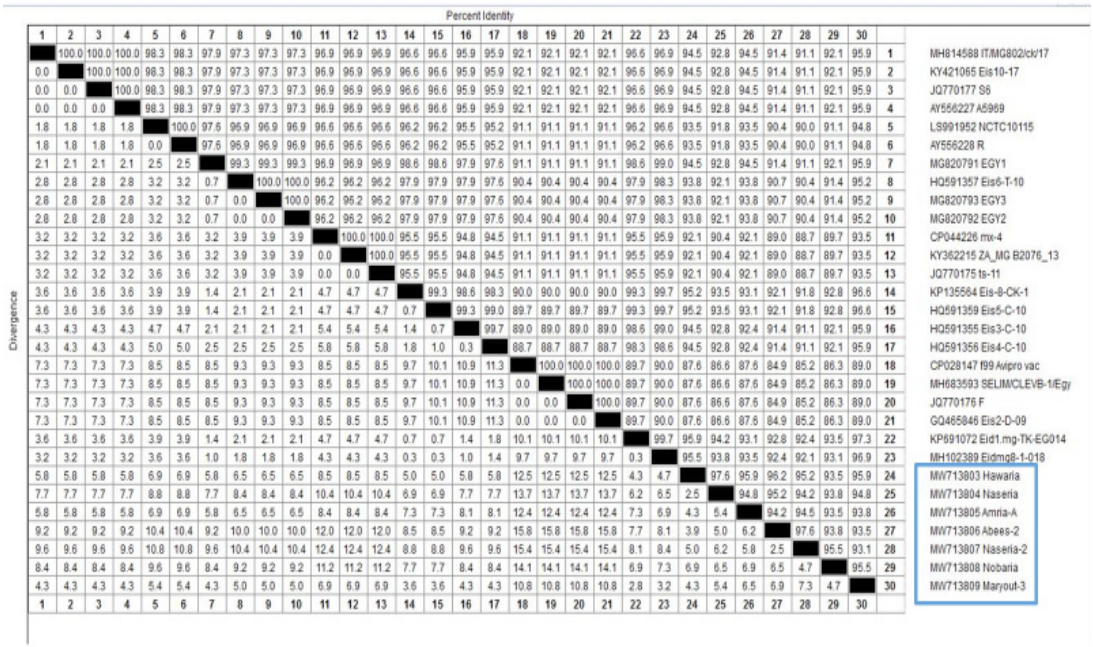

Fig. 4. Genetic identity among the selected *Mycoplasma gallisepticum* isolates (blue text box) , Egyptian field, and reference vaccine strains.

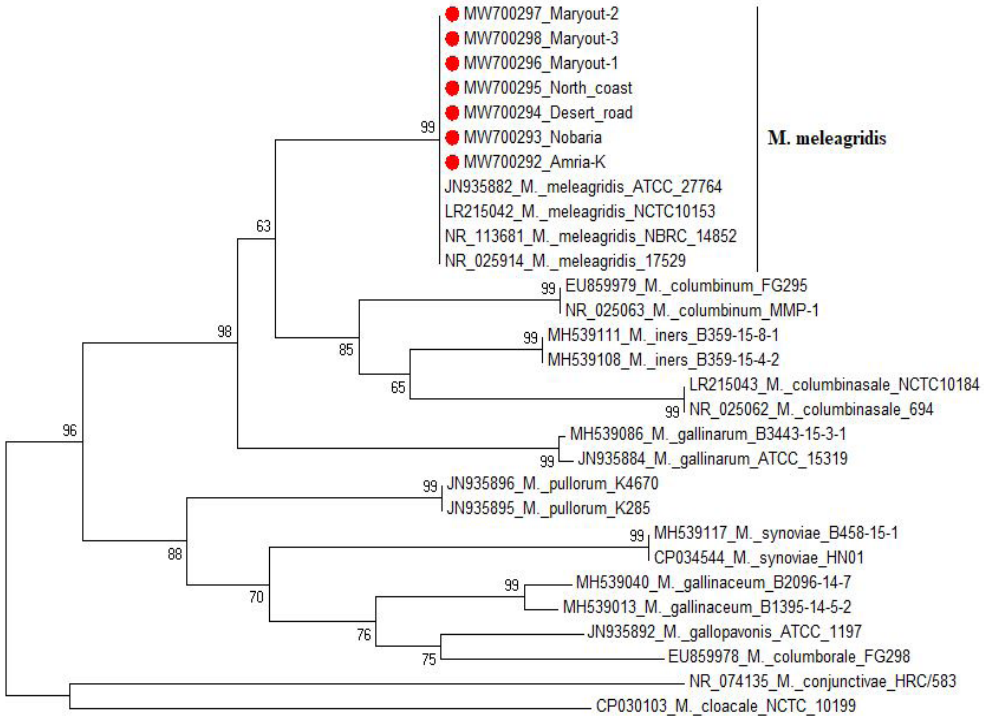

Fig. 5. Genetic relationships among *Mycoplasma meleagridis* isolated strains (indicated by red dots), reference strains, and other *Mycoplasma* spp.

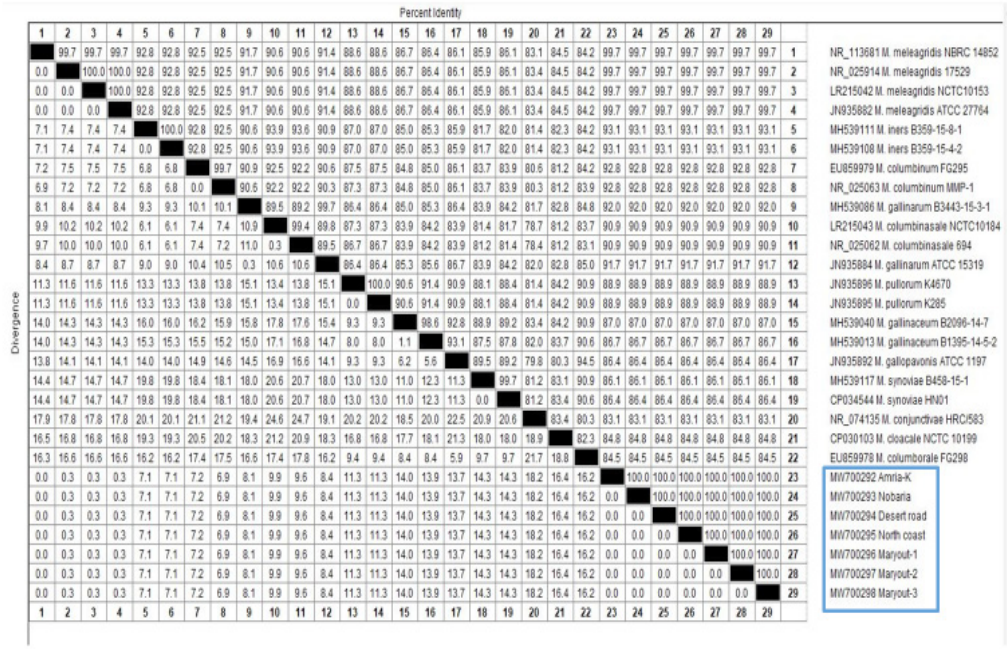

Fig. 6. Genetic identity among the selected *Mycoplasma meleagridis* isolates (blue text box), reference strains, and other *Mycoplasma* spp.

Discussion

Single infection with either MG or MM was 47.06% or 17.65% respectively, while mixed infection with both MG and MM was 23.53% of investigated turkey flocks affected with infectious sinusitis. These results were supported by Marouf et al., who recorded the higher incidence of *M. gallisepticum* in turkeys when compared with chickens [22], also, Hassan et al., reported the higher incidence of *M. gallisepticum* (59.9%) followed by *M. gallinarum* (30.3%), and untyped *Mycoplasma* (9.8%) in different turkey flocks of Sohag and Al-Minia governorates [23]. Abd El-Rahman revealed 31.7% of the examined turkey flocks were positive for *M. gallisepticum* [24]. Rasoulinezhad et al., showed MG incidence of 48.38%, and 16.66% in backyard and commercial turkey farms of Iran, respectively [25]. García et al., detected MM in 10 turkey flocks out of 46 (21.7%) as a single infection while it found mixed with *M. Iowae* (MI) in 9 flocks (19.6%) [26]. Eissa et al., diagnosed MG in 20% of investigated turkeys (1/5) [27]. On the contrary, Ongor et al., isolated simultaneously MM and MG from 2 out of 624 turkey flocks (0.32%) [28]. Jefferey et al., identified MM in 20 out of 48 turkey flocks (41.7%) [29]. The current study, on the level of sequencing, mgc2 gene in all selected seven MG strains, was related to that of other previously investigated Egyptian field strains with identity ranged from 90.4% (Naseria-2 with EGY-2, EGY-3, and Eis-6T-10) to 97.3% (Maryout-3 with Eid1. mg-TK-EG014). While when compared with reference vaccine strains the identity ranged from 84.9% (Abees-2 with Avipro-vac, 1/Egy, 0176-F, and Eis 2-D-09) to 95.9% (Maryout-3 with S6, A5969, Eis10-17, and CK/17). Although MM was the most isolated *mycoplasmas* from turkeys, in this study all 7 identified MM strains were recorded as the 1st ones submitted to GenBank database in Egypt and when compared with reference MM and other *mycoplasma* spp, they revealed 99.7% similarity with reference MM strains (ATCC-27764, NCTC-10153, NBRC-14852, and 17529), as well as 83.1% with *M.conjunctivae* HRC/583, and 93.1% with *M.iners* B359-15-8-1, and *M.iners* B359-15-4-2. Our data on identified MG isolates were matched with these of Marouf et al., that ~~showed~~ similarity of MG with EIS-6T-10 (2011 turkey local strain) ranged from 85% to 95.4% [30]. Also, Loolmani et al., found MG was identical to Indian strain (KP279742.1MG-9B) [31]. Marouf et al., recorded the Four sequenced strains were closely related to 6/85 and ts11, vaccine strains [22]. Rasoulinezhad et al., indicated high sequence similarity with Indian and Pakistanian MG isolates [25]. Eissa et al., submitted to GenBank data base under the accession number, HQ591357.1 and designation, MG-EIS-6T-10 with similarity of 97-99% with MG Israel strains, 96-98% with MG U.S strains, and 94-96.5% with Pakistan MG strains [27].

Conclusion

MG isolates were recorded the highest incidence than MM in turkeys, MM isolates were registered on GenBank database as the 1st Egyptian MM field strains and revealed no genetic diversity among them and similarity of 99.7% with MM reference strains. The increased similarity among the identified MG and/or MM isolates with reference vaccine strains might illustrate the possibility of high protection percent when such similar vaccine strains were applied like MG-S6 with identified MG-Maryout-3 (95.9%) and MM-ATCC-27764 with all identified MM isolates (99.7%). On controlling of Mycoplasmas in poultry industry in Egypt, turkey populations should be taken in consideration as a hidden source of *Mycoplasma* infection, so further molecular studies were required in turkey flocks to control Mycoplasmosis.

Declarations

Acknowledgement

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Competition interest

There is no any Competing interests among the authors.

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Ethical considerations

The authors affirmed that there is no any Ethical issues including plagiarism, misconduct, data fabrication and/or falsification, consent to publish, double publication and/or submission, and redundancy.

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التوصيف الجيني لأنواع الميكوبلازما المعزولة من قطعان الرومي

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يعتبر مرض الميكوبلازما في دجاج الرومي من الأمراض التي تنتقل عن طريق العدوي الرأسية من خلال البيض الي الأجنة ويتميز بالتهابات الجيوب الأنفية وتورمها ونقص معل الفقس وارتفاع نفوق الأجنة مع انخفاض الأوزان. تورم الجيوب الأنفية في دجاج الرومي ينتج عنه عدم قبول شكل الطائر لدي المربي والمستهلك وصعوبة التسويق بالإضافة الي تسببه في سهولة نقل المرض الي باقي أنواع الطيور المجاورة لذلك جاءت هذه الدراسة لفحص واستبيان الميكوبلازما في الجيوب الأنفية المتورمة في دجاج الرومي باستخدام التكنولوجيا الجزيئية لتفاعل البلمرة المتسلسل وتسجيل المعزولات في بنك الجينات ومقارنتها في الشجرة الوراثية بباقي عترات الميكوبلازما المسجلة حقليا وعترات اللقاح المرجعية مما يسهل متابعة الخريطة الوبائية للميكوبلازما في دجاج الرومي وامكانية اختيار أقرب عترة لقاح لاستخدامها حسب كل معزولة ودرجة قرابتها بكل منطقة فتزداد درجة المكافحة والوقاية من مرض الميكوبلازما. تم فحص عدد 17 قطيع رومي بمناطق مختلفة في محافظة الأسكندرية وكانت القطعان مصابة بتورم الجيوب الأنفية مشتملا أو غير مشتملا للعينين وتم سحب السوائل من داخل الجيوب الأنفية بسرنجة معقمة لكل جيب أنفي ووضع العينات علي ثلج وارسالها الي المعمل المرجعي للرقابة علي الانتاج الداجني - معهد بحوث صحة الحيوان - مركز البحوث الزراعية بالدقي في مصر لفحص وجود الميكوبلازما باستخدام تفاعل البلمرة المتسلسل طبقا للبرايمر المتواجد بالمعمل. تم اختيار عدد 7 معزولات من ميكوبلازما جاليسبتكم وعدد 7 معزولات من ميكوبلازما ميلياجريديز التي تم استبيانهم خلال الدراسة لعمل التتابع الجيني لهم وتسجيلهم في بنك الجينات مع مقارنتهم في الشجرة الوراثية بالعترات الحقلية المعزولة والمسجلة سابقا وعترات اللقاح المرجعية. نتائج تفاعل البلمرة المتسلسل أظهرت حدوث عدوي الميكوبلازما جاليسبتكم في عدد 12 قطيع دجاج رومي (8 قطيع مصاب بعدوي منفردة و4 مصاب بعدوي مختلطة بنوعي الميكوبلازما) وحدوث عدوي الميكوبلازما ميلياجريديز في عدد 7 قطيع دجاج رومي (3 قطيع مصاب بعدوي منفردة و4 مصاب بعدوي مختلطة بنوعي الميكوبلازما). عند عمل التتابع الجيني لعترات الميكوبلازما جاليسبتكم حدث تطابق بنسبة 93.1-97.6% فيما بينهم ونسبة 90.4-97.3% مع العترات الحقلية و84.9-95.9% مع عترات اللقاح المرجعية بينما كان تطابق الميكوبلازما ميلياجريديز بنسبة 100% فيما بينهم ونسبة 99.7% مع العترات الحقلية و83.1-93.1% مع عترات اللقاح المرجعية. تم تسجيل معزولات الميكوبلازما جاليسبتكم في قاعدة بيانات بنك الجينات تحت أرقام ، MW713808 ، MW713809 ، MW713803 ، MW713804 MW713805 ، MW713806, MW713807 و تسجيل معزولات الميكوبلازما ميلياجريديز تحت أرقام MW700297 ، MW700296 ، MW700295 ، MW700293 ، MW700294 ، MW700292 MW700298 ، وأخيرا كانت نسبة حدوث الميكوبلازما جاليسبتكم في دجاج الرومي أعلي من الميكوبلازما ميلياجريديز وتم تسجيل الميكوبلازما ميلياجريديز كأول عترة حقلية مصرية في قاعدة بيانات بنك الجينات كما أن التقارب والتشابه بين عترات الدراسة وعترات اللقاح المرجعية سيلعب دورا هاما في مكافحة مرض الميكوبلازما في قطاع الدواجن.

الكلمات الدالة: الميكوبلازما جاليسبتكم والميكوبلازما ميلياجريديز وتفاعل البلمرة المتسلسل والتتابع الجيني ودجاج الرومي.