Molecular Characterization of Mycoplasma Isolated From Chicken

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Abstract

In order to study the Molecular characterization of mycoplasma isolated from chicken. A total number of 200 samples collected from birds showing respiratory manifestations and apparently healthy chicken of different ages(two weeks: two months) from different localities(al-ismailia & sharkeya Governorates). These samples included 110 samples from respiratory organs(trachea -lung -air sac) & 75 swabs from nasal cleft and 15 samples from fluid of swollen joints. A trial for isolation and identification of different *Mycoplasma* was done using conventional and recent techniques. All the results were finally confirmed by polymerase chain reaction (PCR) which showed products of the amplified16S rRNA gene and mgc2 gene of MG, detection of 16s rRNA gene of MS and 16S rRNA gene of un typed *Mycoplasma* in chickens using a set of primers tested and compared according to reference strain .The PCR amplification products were electrophoresed on 1% agrose gel stained with ethidium bromide. All the examined field isolates were identified as *Mycoplasma* (gave a characteristic common band at different levels of bp fragment). Primary isolation of the microorganism on PPLO medium, which appeared as fried egg when examined by dissecting microscope yielded118 positive samples with a total incidence rate 59%. The highest recovery rate was from respiratory organs (72.7%) followed by swabs from nasal claft (46.7%) and samples from swollen joints (20%).Sequence analysis of two *Mycoplasma gallisepticum* (strains for mgc2 gene from chicken revealed high similarity with the homologous reference strains on Gene Bank. and take these experessions on Gene Bank as Man-Reh.1/Mg/CK/EG016 acc# KY404986 and Man-Reh.2/Mg/CK/EG016 acc# KY404987.

Introduction

Avian mycoplasmosis consitutes one of the major economic problems facing poultry industry allover the world because of its significant losses which are mainly due to reduced egg production , poor feed conversion and carcass condemination at processing ***(Yoder 1984 and Cassel et al ., 1985).***

Mycoplasma gallisepticum and *Mycoplasma synoviae* are

ismailia ,7 sharkeya Governorates). These samples include **110** samples from respiratory organs(trachea - lung -air sac) & **75** swabs from nasal cleft and **15** samples from fluid of swollen joints. as shown in table (1)

2- Polymerase chain reaction (PCR) for *Mycoplasma*:

a- DNA extraction *(Fan et al. 1995):*

Mycoplasma cultures were grown in Frey's broth, 5ml of 24hr broth cultures were centrifuged for 10 minutes at 12000 r.p.m. The pellet was washed twice in 1ml of phosphate buffered saline pH 7.2 (PBS) and suspended in 50 µl PBS. The cell suspension was heated directly at100°C for 10 minutes in a heat block to break the cell membranes, then cooled on ice for 5 minutes . Finally, the cell suspension was centrifuged for 5 minutes and the supernateant containing DNA was collected and stored at -20°C until use.

b-PCR ampilfication

The PCR mixture was composed of the following:

DreamTaq ™ Green Master Mix (2X)*--- 25µl; Forward primer--1µl; Reverse primer --1µl; Template DNA-- 5µl and Water, nuclease-free -- to 50 µl.

*** DreamTaq ™ Green Master Mix (2X) (Fermentas):** it is a ready to use solution containing DreamTaq ™ DNA polymerase, optimized DreamTaq ™ Green buffer (2X), 4mM $MgCl_2$ and dNTPs (dATP, dCTP, dGTP and considered to be the most important of the pathogenic mycoplasmas for chickens , and both occur world-wide ***(OIE, 2008).*** They spread vertically through infected eggs and horizontally by close contact ***(Bradbury et al ., 2001).***

More recently, laboratory testing of *Mycoplasms* using the polymerase chain reaction (PCR) has become standered method for early detection of *Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS).*

PCR detection was primarily developed for *mycoplasma* ***(Nascimento & Yamamoto, 1991; and Nascimento et al., 1993)*** and was accepted worldwide for detection of all avian Mycoplasmas in specific DNA amplification for diagnosis ***(Lauerman, 1998; Nascimento et al., 1998)*** polymerase chain reaction (PCR) test could detect positive swabs whereas attempted at culture were negative ***(Kempf ,1998)***.

This work was designed to study the application of PCR for detection of *Mycoplasma Spp.* isolated from chickens and Gene Sequencing of isolates of *Mycoplasma* spp and recorded on gene bank.

Material and Methods

1 Samples:

Two hundred samples were collected from birds showing respiratory manifestations and apparently healthy chicken of different ages(two weeks: two months from different localities(al-

e- Screening of PCR products by agarose gel electrophoresis *(Fan et al. 1995)*

Thirty microlitre aliquots of amplified DNAs were electroporesed in 2% agarose gels in TBE containing 0.5% ethidium bromide at 100 V. Five micrograms of 100bp DNA ladder (Pharmacia) were also run in each gel as standard for size determination of DNA fragments. The DNA was visualized under ultraviolet illuminator and photographed. Visualization was done in transiluminator (Spectroline, Model312A, 312 nm Ultraviolet, USA) and photographs were taken by UV camera (Polariod DS 34 direct screen instant camera, England).

3- DNA sequencing:

The amplified fragments were purified using Gene Jet PCR purification kit: Fermentas (cat no. KO701). Sequencing was performed at Macrogen Company (South Korea) . identification of homologies between nucleotide and amino acid sequences of the isolated MG strains were compared with other strains published on GenBank using BLAST 2.0 and PSI-BLAST search programs ,respectively. The obtained nucleotide sequences comprasion and their multiple alignments with refrence as well as the deduction of amino acid sequences were done using the Bio Edit sequence alignment editor (**Poumarat et al ., 1999)** . CLUSTALX software for dTTP, 0.4 mM each). Also, it contains a density reagent and two dyes for monitoring electrophoresis progress.

Primer selection

Oligonucleotide primers (100 pmol)

1-Primers for identification of *M. gallisepticum*

A set of primers were used for identification of MG targeting the 16S rRNA gene ,*mgc*2 gene and OIE primers. as shown in table 2,3.

Table (2), (3): Oligonucleotide primers used for identification of M. gallisepticum.

C- Polymerase Chain Reaction (PCR) procedure *(Fan et al. 1995)*

The reaction mixture (total volume of 50 µl) was 5 µl of 10 X reaction buffer (Applied Biosystem), 1.5 µl of mM MgCl2, 1 µl of nucleotides mix (10mM), (Sigma), DNA was added 5 µl(containing 50ng) and 1 µl primer. Then 2U of DNA Taq polymerase (Applied Biosystem) was added and the mixture was completed by ultra-pure distilled water to 50 µl. PCR was performed on progene "Programmable Thermal Controller" (UK).

D- PCR Cycling Protocol .***(Garcia et al., 2005)***

Amplification of a target sequence was performed using primers and accomplished with five cycles 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 30 cycles with the same sequence , except for final extension at 72°C for 15 minutes.

Higgins and Sharp (1989). multiple sequence alignment

Table(1): Types and Numbers of samples

Sample types	No. of samples
Respiratory organs	110
Swabs	75
Fluid of swollen joints	15
Total	200

Table (2) :*1- mgc2 primer*

Primer Designation	Sequence (5'-3')	Reference	Amplified Product Size
***MgC*2 gene Forward Reverse**	CGC AAT TTG GTC CTA ATC CCC AAC A TTC C TAA ACC CAC CTC CAG CTT TAT	Lysnyansky e*t al* .(2005)	**300 bp**

Table (3): *2- OIE primer*

Primer Designation	Sequence (5'-3')	Reference	Amplified Product Size
OIE Forward Reverse	GAG CTA ATC TGT AAA GTT GGT C GCT TCC TTG CGG TTA GCA AC	OIE Terrestrial manual (2008)	**185 bp**

2-Primers for identification of *M. synoviae*

Table (4): *Oligonucleotide primers used for detection 16S rRNAgene and identification of M. synoviae*

Primer Designation	Sequence (5'-3')	Reference	Amplified Product Size
OIE Forward Reverse	GAG AAG CAA AAT AGT GAT ATC A CAG TCG TCT CCG AAG TTA ACA A	OIE Terrestrial manual (2008)	**205-210 bp**

3-Primers for identification of *un typed Mycoplasma*

Table (5): *Oligonucleotide primers used for detection of 16S RNA gene of un typed Mycoplasma (Intra Space Region ISR Ramirez 2011)*

Primer Designation	Sequence (5'-3')	Reference	Amplified Product Size
16s RNA gene(common) Forward Reverse	CGT TCT CGG GTC TTG TAC AC CGC AGG TTT GCA CGT CCT TCA TCG	Ramirez *et al.* (2011)	**Different levels of bp**

Table(6) : *PCR Cycling Protocol*

Initial Denaturation	Actual Cycles Temperature/ Seconds	Final Extension
94°C for 3 minutes	35 cycles of: Denaturation 94 /20 s Annealing 58 /40 s Extension 72/ 60 s	72°C for 15 minutes

2-1-b- Result of specific PCR of 16S rRNA gene of *M.gallisepticum* using OIE primer:

The result of specific PCR was shown in photo (3). The PCR technique was used for the detection of 16S rRNA gene of **MG** in naturally infected chicken. Isolated strains from tracheal swabs were tested and compared with **MG** reference strain ***OIE Terrestrial manual (2008)***. The PCR amplification products were electrophoresed on 1% agarose gel stained with ethidium bromide. All the examined field isolates were identified as *M. gallisepticum* (gave a characteristic common band at 185 bp fragment).

2-2- Result of specific PCR of 16S rRNA gene of *M.synoviae*:

The result of specific PCR was shown in photo (4). The PCR technique was used for the detection of 16S rRNA gene of **MS** in

Results

1- Primary isolation of Mycoplasma from collected samples

2-Results of polymerase chain reaction PCR of *Mycoplasma* isolates:

2-1-a- Result of specific PCR of *mgc2* gene of *M.gallisepticum*:

The result of specific PCR was shown in photo (2). The PCR technique was used for the detection of mgc2 gene of **MG** in naturally infected chicken. Isolated strains from tracheal swabs were tested and compared with **MG** reference strain **Lysnyansky *et al* .(2005)**. The PCR amplification products were electrophoresed on 1% agarose gel stained with ethidium bromide. All the examined field isolates were identified as ***M. gallisepticum*** (gave a characteristic common band at 300 bp fragment).

strain ***Ramirez et al. (2011)***. The PCR amplification products were electrophoresed on 1% agrose gelstained with ethidium bromide. All the examined field isolates were identified as *mycoplasma* (gave a characteristic common band at different levels of bp fragment).

3- Results of Sequence analysis:

Sequence analysis of two *mycoplasma gallisepticum* (Man-Reh.1-Mg-CK-EG016 and Man-Reh.2-Mg-CK-EG016) strains for mgc2 gene from chicken revealed high similarity with the homologous reference strains on Gene Bank as shown in table (8) and take theses experessions on Gene Bank as Man-Reh.1/Mg/CK/EG016 acc# KY404986 and Man-Reh.2/Mg/CK/EG016 acc# KY404987. Sequence analysis of two samples of MS have non specific sequence.

naturally infected chicken. Isolated strains from tracheal swabs were tested and compared with **MS** reference strain ***OIE Terrestrial manual (2008)***. The PCR amplification products were electrophoresed on 1% agarose gel stained with ethidium bromide. All the examined field isolates were identified as *M. synoviae* (gave a characteristic common band at 205-210 bp fragment).

2-3- Result of specific PCR of 16s rRNA gene of *un typed Mycoplasma* using the common primer of Mycoplasma (ISR):

The result of specific PCR was shown in photo (5). The PCR technique was used for the detection of 16s rRNA gene of un typed *mycoplasmain* naturally infected chicken isolated strains from tracheal swabs were tested and compared according to reference

Table (7) *Recovery rate of Mycoplasma isolation from collected samples*

Site of isolation	No. examined	Isolation		Precentage
		+ve	-ve	
Respiratory organs	110	80	30	72.7%
Swabs	75	35	40	46.7%
Swollen joints	15	3	12	20%
Total	200	118	82	59%

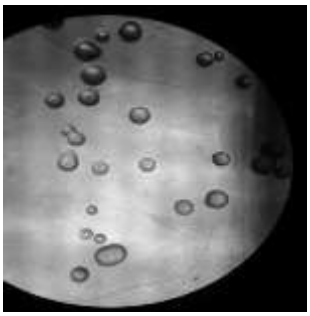


Photo (1) *characteristic morphological apperance of mycoplasma colonies on PPLO agar medium (fried egg apperance)* .

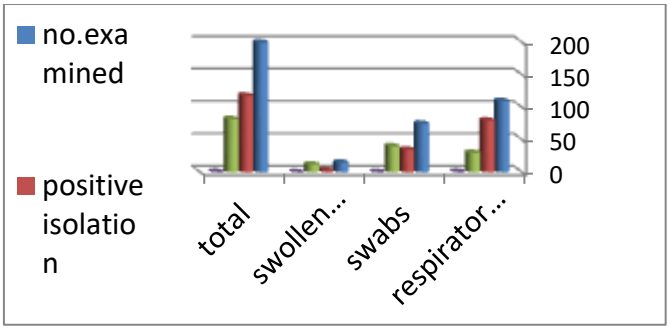


figure (1) *Recovery rate of **Mycoplasma** isolation from collected samples*

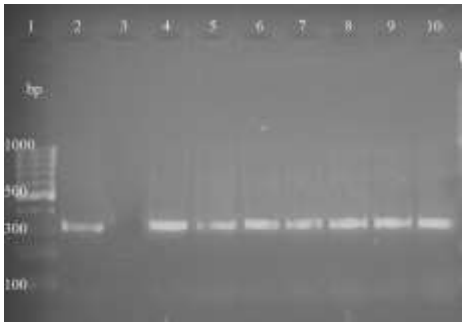


photo (2): *Agarose gel electrophoresis of Mycoplasm gallisepticum field isolated strains from tracheal swabs using mgc2 gene primer*

Lane 1: 100 bp Ladder
Lane 2: control positive
Lane 3: control negative
Lane 4-10: M. gallisepticum field isolate

photo (3): *Agarose gel electrophoresis of **Mycoplasm gallisepticum** field isolated strains from tracheal swabs using **OIE** primer*

Lane 1: 100 bp Ladder
Lane 2: control positive
Lane 3- 9-: M. gallisepticum field isolate
Lane 10: control negative

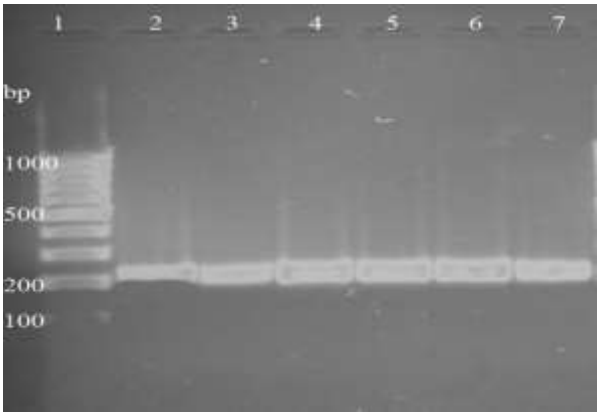


photo (4): *Agarose gel electrophoresis of Mycoplasm synoviae field isolated strains from tracheal swabs using OIE primer*

Lane 1: 100 bp Ladder
Lane 2: control positive
Lane 3- 7-: M. synoviae field isolate

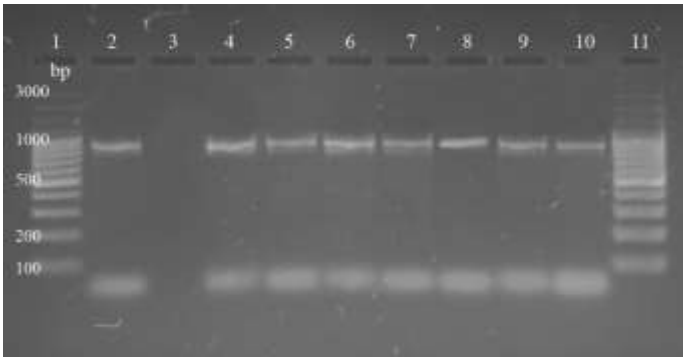


photo (5): *Agarose gel electrophoresis of un-typed* ***Mycoplasma*** *field isolates strains using 16s RNA primer* ***Ramirez et al., (2011)***

Lane 1, 11: 100 bp Ladder
Lane 2: control positive
Lane 3: Control Negative
Lane 4-10: M. field isolate

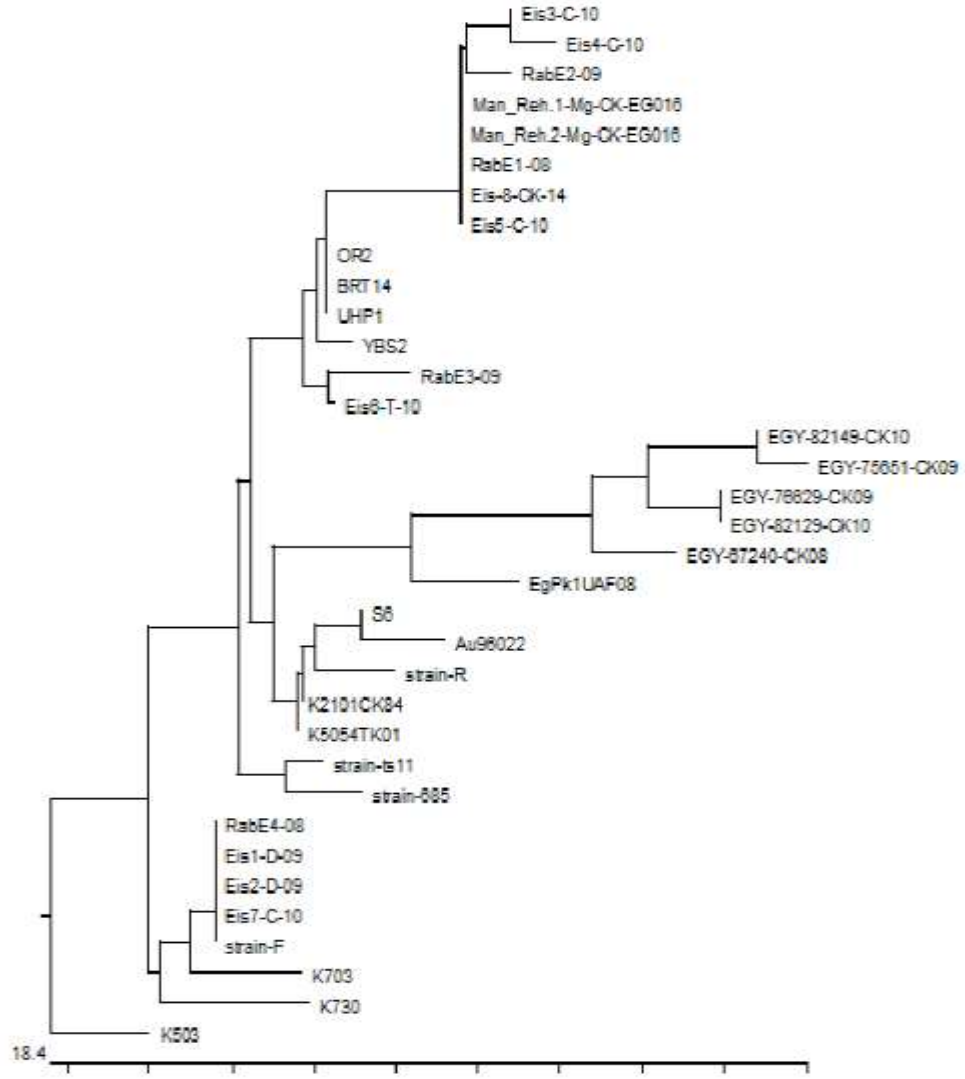
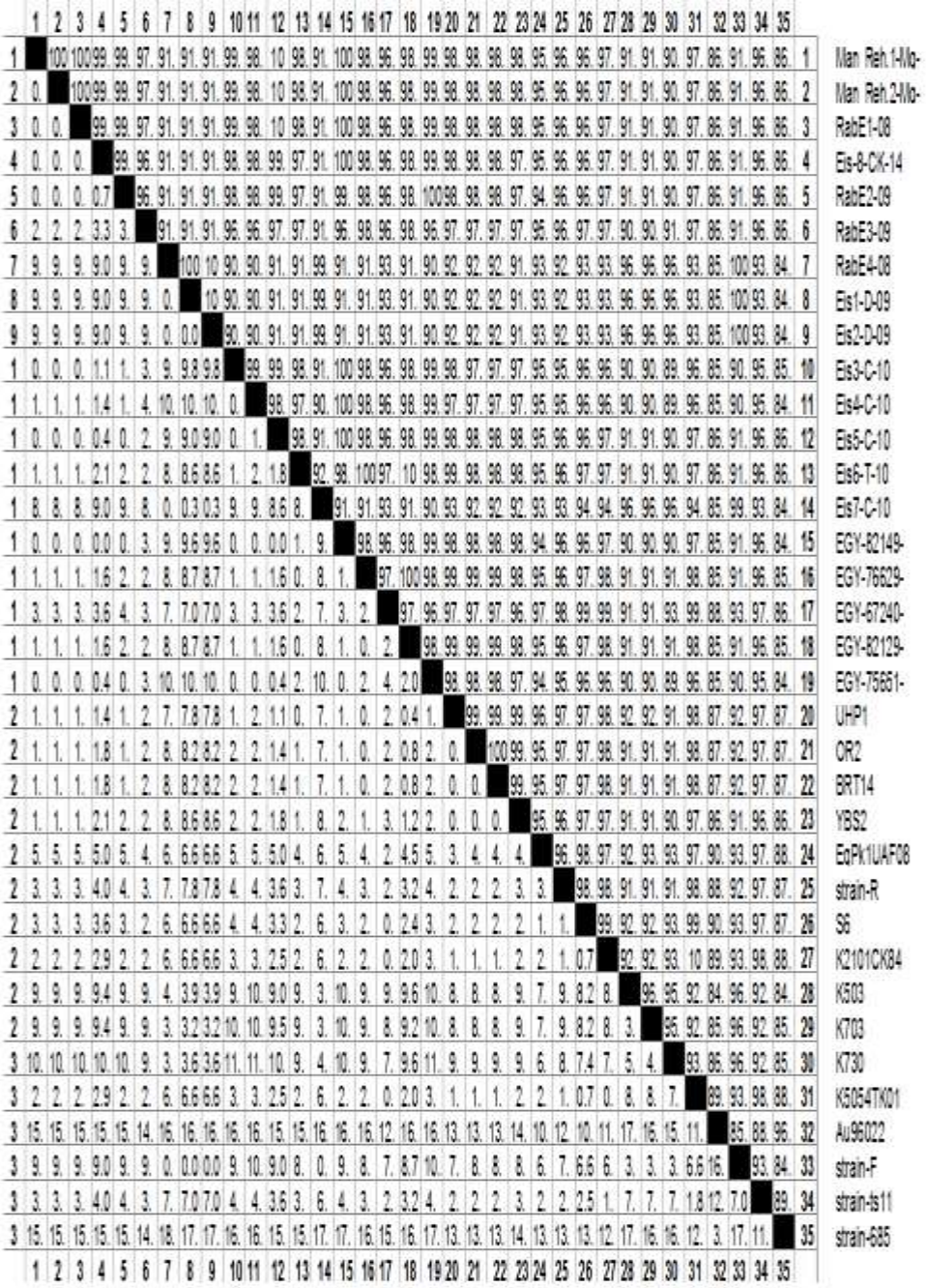


Figure (2) *Nucleotide and amino acid identities of mgc2 of Mycoplasma strains analyzed (marked) tree. (Phylogenetic tree of Untitled ClustalW (Slow/Accurate, Gonnet)*

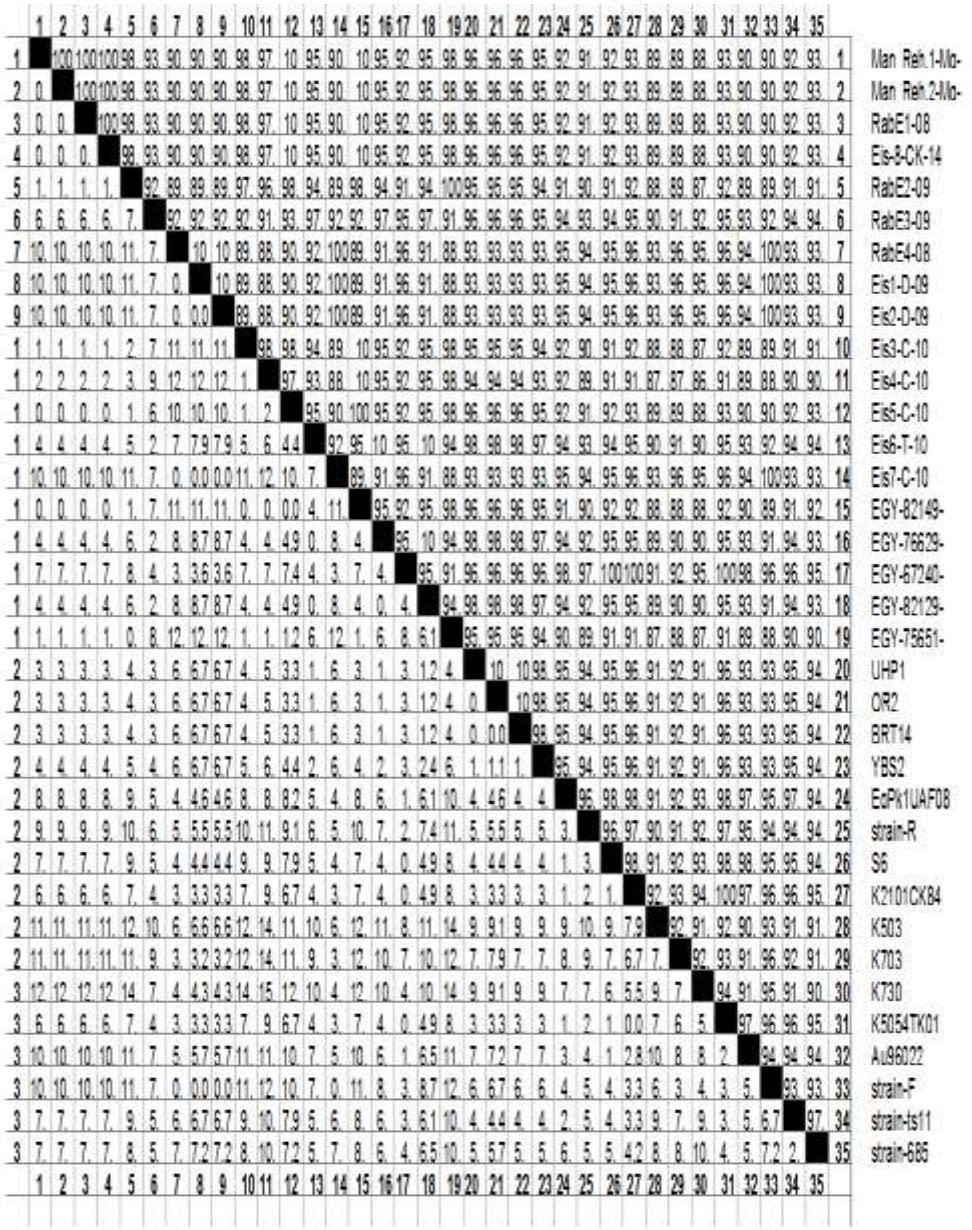
Table(8) Amino acid identities of mgc2 of ***Mycoplasma*** strains analyzed (marked) in this study in comprasion with others from different countries published in GenBank(Sequence pair distances of Untitled ClustalW(Slow/Accurate,Gonnet).

Precent identity



Table(9) *Nucleotide identities of mgc2 of* ***Mycoplasma*** *strains analyzed (marked) in this study in comprasion with others from different countries published in Gen Bank(Sequence pair distances of Untitled Clustal (Slow/Accurate,IUB).*

Precent identity



al., 2001 and Mcauliffe et al., 2003).

PCR proved more specific than culture method for identification of ***Mycoplasma*** from field samples. Samples collected were PCR positive, whereas, the same samples were culture positive on specific medium ***(Stanely et al., 2001, Finklin and Kleven, 2006).***

PCR technique has several advantages, but it also has some disadvantages like serious contamination problem as a result from improper handling of the DNA tested sample inducing false results ***(OIE, 2000)***. ***Salisch et al., (1999)*** concluded that parallel to the PCR procedure, the conventional cultural methods should be performed. ***Lee young (2003)*** suggested that the PCR technique is a valuable tool for the diagnosis of *M.synoviae*.

In present study, MG PCR assays targeted the *mgc2* gene, which encodes cytadhesin protein of *M. gallisepticum* and also known to play a role in the attachment process. The all tested strains gave a characteristic fragment at 300bp. This result agreed with ***Garcia et al. (2005)*** who mentioned that the use of *mgc2* forward and reverse primers could identify the expected size of amplification products was varied in range of 236-302 bp. MG PCR assays targeted the *16S rRNA* gene. The all tested strains gave a characteristic fragment at 185bp. This result agreed with ***Mahmoud Hossam et al. (2016)*** who detected PCR could easily pick up *M.*

Discussion

Mycoplasma is a small free living highly fastidious and slow growing micro-organism, ***(Nicolas and Ayling, 2003).*** 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 reduced egg production, poor feed conversion and carcass condemnation at processing ***(Yoder, 1984).***

The most economically significant mycoplasma pathogen of poultry is *M. gallisepticum* ***(Kleven and Levisohn., 1996).*** *Mycoplasma synoviae (MS)* is recognized as pathogen in chickens and turkeys and is responsible for infectious synovitis ***(Kleven., 1997).*** Infection with *M. synoviae* causes a respiratory disorder and infectious synovitis in chicken especially further highlight the economic significance of these bacteria in commercial poultry ***(Feberwee et al ., 2009).***

In practice, confirmation of infection by conventional culture procedures is time consuming, laborious, expensive and required sterile conditions and personal skills ***(Hirsh and Zee, 1999)*** Therefore, the amplification of DNA of MG in the laboratory using PCR has been performed as very sensitive, specific and rapid method requiring less than 24 h for detection and identification of the organism ***(Khan and Kleven, 1993; Marios et***

accurate identification of the *Mycoplasma.*

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Finklin M and Kleven ,S.H. (2006): Evaluation of Diagnostic Methods for ***Mycoplasma*** *gallisepticum* through targeting 16S rRNA specific sequence at 185 bp and the results are shown in Photo (5). **MS** PCR assays targeted the *16S rRNA* gene. The all tested strains gave a characteristic fragment at 205-210bp and These findings have also been supported from the observation of ***(Bradbury 1998)***.So, the PCR , seems to be alternative method to difficult and time consuming techniques of culturing MG and MS thanks to its speed and reliability in routine diagnosis. ***(Bagcgl and Ilgaz, 2005)***.

The sequence of the ***mgc2*** gene had a 100% nucleotide sequence identity with recently isolated MG field strain of Gene Bank *Mycoplasma gallisepticum* strain Nouh-C-15-mgC2 and *Mycoplasma gallisepticum* strain Eis-8-CK-14 and a 99% nucleotide sequence identity with *Mycoplasma gallisepticum* strain Eid1.mg-TK-EG014 and *Mycoplasma gallisepticum* strain Eis5-C-10 as shown in figure (2).

In this study it was be concluded that Mycoplasmas are world wide pathogen in chickens and turkeys causing great economic losses. These results strongly support the use of this PCR assay as an efficient alternative or supplement to culture and serological identification, which are labor-intensive, extremely time-consuming, and often provide confusing results. Overall, it is suggested that the PCR could be an alternative method for

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الملخص العربى

تعتبرالميكوبلازما من ميكروبات واسعه الانتشارفي مزارع الدواجن المختلفة مسببه خسائر اقتصاديه كبيره للمربين و اصحاب المزارع وتتميز بانها تنتقل من الام المصابه الي الكتاكيت (انتقال رأسي). وهى ايضا تهيئ الطيور للاصابه الميكروبات الأخرى حيث ان العدوى تؤدى لقة الانتاج.
وفي خلال هذا العمل:
1- تم جمع عدد 200 عينه من الدجاج الذي يظهر عليه الاصابه باعراض تنفسيه من عدد من المحافظات(الاسماعيليه والشرقيه والقاهره) تشمل مسحات حلقيه واجزاء من الاعضاء التنفسيه والمفاصل المتورمه كالتالي
الاعضاء التنفسيه 110 عينه والمسحات الحلقيه 75 عينه و المفا صل المتورمه 15 عينه
2- تم اجراء اختبار البلمرة المتسلسل (PCR) لعترات ممثله من المجموعات التي تم عزلها لتأكيد العزل والتصنيف .
3- تم اجراء اختبار التسلسل الجيني لعترات معزوله تمثل الميكوبلازما جاليسبتكم ومقارنتها مع بنك الجينات ووجد تماثل كبير مع بعض العترات الموجوده في بنك الجينات .