Advanced Studies On Mycoplasma Microorganisms In Ducks

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ABSTRACT

Seven hundred and eighteen samples were collected from farms and back-yard of ducks in Dakahlia and Sharkia Governorates were investigated to detect the incidence of natural infection of ducks with mycoplasma. The isolates were identified by phenotypic and genotypic. *Mycoplasma gallisepticum* (*MG*) was isolated from 76.92% of examined lungs, 33.33% of egg yolk and 25% of trachea. Also *M. gallinarum* was isolated from 75% of trachea, 36.36% of tracheal swabs. While, *M. anatis* was isolated from 66.67% and 23.08% of egg yolk and lungs, respectively. *Acholeplasma* species could be isolated only from 63.64% of tracheal swabs.

This study confirmed that Polymerase Chain Reaction (PCR) is rapid, simple and reproducible, and can detect 824 bp DNA fragment in all the examined *MG* field isolates (*mgc2* cytadhesin gene). Meanwhile, Random Amplified Polymorphic DNA (RAPD) can distinguished genetically *MG* field isolates with high similarity, composed with the reference strain, while DNA fingerprinting of *M. anatis*, *M. gallinarum* and *Acholeplasma* species was characteristic for each one of them.

INTRODUCTION

Mycoplasmas frequently have been isolated from domestic chickens and turkeys. Also, mycoplasmas have been recovered from domestic ducks through out the world ***(1-3)*** and from wild ducks in Spain ***(4)*** and the United States ***(5)***.

Many of these mycoplasmas cause clinical respiratory disease and joint synovia ***(6)***.

Isolation and identification of mycoplasma organisms are difficult. Therefore, serological assays such as the rapid serum plate agglutination (SPA) and the growth inhibition (GI) tests have been used.

Recently, molecular biology techniques such as Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) or the Arbitrarily primed PCR (AP-PCR) have been applied for identification of mycoplasmas. Mycoplasmal 16S rRNA sequences have been determined for many species and provide the basis for a systematic phylogenetic analysis of these organisms ***(7)***. An approach has been reported for assessing genetic relatedness among mycoplasmas based on 16S rRNA PCR in which primers are selected to detect *Mycoplasma* species ***(8)***.

The PCR techniques have been proven to be very specific and sensitive method for amplifying low amounts of nucleic acid to a level that can be easily detected. They have been used to detect *M. gallisepticum* ***(9 and 10)***.

The RAPD method has been used to study heterogeneity in closely related organisms ***(11)***. This method detects differences in the DNA sequences at sites in the genome that are defined by the primer used. The number and length reveal sequence variation of amplified products, which may be phylogenetically conserved. This method is advantageous for strain or isolate identification ***(12)***.

The present work was planned to detect the incidence of mycoplasmas among ducks. Evaluating of the conventional PCR and RAPD in diagnosis of mycoplasma infection in ducks. In addition, emphases of the isolates can be carried out by sequencing.

MATERIAL AND METHODS

Examined samples

Seven hundred and eighteen samples were collected from different localities (farms and back-yard) in Dakahlia and Sharkia Governorates, either diseased or freshly dead ducks suffering from respiratory troubles. The apparently healthy and diseased ducks were subjected to both clinical examination, postmortem and bacteriological examination. Meanwhile freshly dead ducks were subjected for postmortem and bacteriological examination. Five hundred and ninety eight samples were collected from tracheal swabs, lung and tracheal tissues, and one hundred and twenty samples were collected from egg yolk. The tracheal swabs were collected from living ducks but trachea and lung tissues were collected from freshly dead ducks. Table 1 shows the sources, recovery sites and number of the samples collected from each site.

Table 1. The sources, recovery sites and number of the collected samples.

Source of samples	Age/ Month	Recovery site	No. of examined samples
Farms	5-6	Tracheal swabs	300
	3-4	Lung	57
	3-4	Trachea	57
	--	Eggs	120
Back-yard	3-4	Tracheal swabs	120
	2-3	Lung tissue	32
	2-3	Trachea tissue	32
Total			718

Media used in this work for isolation and identification were as described by ***Frey et al. (13)***.

Characterization of mycoplasma on the media were as described by ***Erno and stipkovits (14)***.

The isolated mycoplasma strains were identified by growth inhibition test as described by ***World Health Organization (15)***.

Egg yolk extraction was tested for the detection of mycoplasma antibodies using serum plate agglutination test (SPA) according to ***Alder (16)***.

Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) were used for identification of *M. gallisepticum, M. anatis* and *M.gallinarum* as the following steps:

1- DNA extraction and purification:

a) DNA extraction by rapid method as described by *Fan, et al. (11).*

b) DNA Extraction using QIA amp: As described by manufacturer.

2- Primer selection and preparation

a) Conventional PCR primers *(10)*

Two specific oligonucleotide primers for identification of *M. gallisepticum* were used for detection of *mgc2* specific gene for *M. gallisepticum.*

The sequence of primer (F) was 5`- GCT TTG TGT TCT CGG GTG CTA - 3`.

The sequence of primer (R) was 5`- CGG TGG AAA ACC AGC TCT TG - 3`.

b) RAPD primers

The single primer was synthesized as described by ***Fan, et al. (11)***.

The sequence of the single primer was 5` AGGCAGCAGTAGGGAAT – 3`.

Synthesis of these primers was done in Germany (Sigma).

3-Polymerase Chain Reaction (PCR) procedure *(10)*

The reaction mixture (total volume of 50µl) was 5 µl of 10 X reaction buffer

(Applied Biosystem), 1.5µl of 25 mM $MgCl_2$, 1µl of nucleotides mix (10 mM), (Sigma), DNA was added 5µl (containing 50 ng) and 2µl primer (containing 400ng of forward and reverse primers). Then, 0.5 µl (2U) of DNA *Taq* polymerase (Applied Biosystem) was added and the mixture was completed by ultra-pure distilled water to 50µl. PCR was performed on Progene "Programmable Thermal Controller" (UK). Amplification was performed by heating the sample for 3 minutes at 94°C for initial denaturation. After this step forty cycles were performed as follows: Denaturation for 20 sec. at 94°C, annealing for 40 sec. at 55°C and extension for 1 minute at 72°C with the exception of final extension step was held for 5 minutes for final extension. The analysis of PCR amplified products was done by using 8µl of amplified PCR product, mixed with 2µl loading buffer and electrophoresed through 1% agarose gel and DNA was visualized by UV fluorescence after ethidium bromide staining, and then photographed.

4-Random Amplified Polymorphic DNA (RAPD) procedure *(11)*

The reaction mixture was done as described above. The reaction conditions were as follow 3 cycles of 15 seconds at 94°C (denaturation), 2 minutes at 28°C (annealing), 3 minutes at 74°C (extension) and then for 35 cycles of 15 seconds at 94°C, 2 minutes at 35°C, 3 minutes at 74°C. Ten microlitre aliquots of amplified DNAs were electrophoresed in 2% agarose gels in TBE containing 0.5% ethidium bromide at 100V. 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 performed in transiluminator (Spectroline, Model 312 A, 312 nm Ultraviolet, USA) and photographs were taken by Digital Camera (Canon, Japan). The amplified band were estimated by nacked eye.

5-Sequencing of *MG* isolates was done in Germany by Sigma Company.

RESULTS

1-Primary isolation of *Mycoplasma* from ducks

In farms twenty two tracheal swabs out of three hundred were positive for mycoplasma isolation with an incidence rate 7.33%. Five samples of lung tissue out of fifty seven were positive for mycoplasma isolation with an incidence rate 8.77% while seven samples of trachea out of fifty seven were positive for mycoplasma isolation with an incidence rate 12.28%. Three egg yolk samples out of one hundred and twenty were positive for mycoplasma isolation with an incidence rate 2.5%. In back-yard, eleven tracheal swab samples out of one hundred and twenty were positive for mycoplasma isolation with an incidence rate 9.16%. Three samples of lung tissue out of thirty two were positive for mycoplasma isolation with an incidence rate 9.37%. Six samples of trachea out of thirty two were positive for mycoplasma isolation with an incidence rate (18.75%). The incidence of mycoplasma detection from ducks in farms and back-yard are seen in Table 2.

Table 2. Primary isolation of *Mycoplasma* from samples collected from farms and back-yard of ducks.

Source of samples	Age/Month	Recovery Site	No. of examined samples	No. of positive samples	Incidence %
Farms	5-6	Tracheal swabs	300	22	7.33
	3-4	Lung	57	5	8.77
	3-4	Trachea	57	7	12.28
	--	Eggs	120	3	2.5
Back-yard	3-4	Tracheal swabs	120	11	9.16
	2-3	Lung	32	3	9.37
	2-3	Trachea	32	6	18.75
Total			718	57	7.93

2.Biochemical characterization and Serological identification of Mycoplasma isolated from ducks

In the present study, three mycoplasma species could be identified as *M. gallinarum, M. gallisepticum* and *M. anatis*. The highest recovery rate of *M. gallinarum* was 75% from trachea, followed by tracheal swabs 36.36%. *M. gallisepticum*, the isolation rate was high from lungs 76.92%, followed by egg yolk 33.33% and trachea 25%. On the other hand, *M. anatis* could be isolated from egg yolk and lungs 66.67% and 23.08%, respectively.

Acholeplasma species could be isolated only from tracheal swabs 63.64%. The results of biochemical and serological identification are seen in Table 3.

Table 3. Biochemical characterization and Serological identification of *Mycoplasma* isolated from ducks.

Recovery Site	No. of tested samples	Biochemical tests				Serological identification	No. of positive samples	Incidence %
		Digitonin	Glucos	Arginin	Film & spot			
Tracheal swabs	33	+	-	+	+	*M. gallinarum*	12	36.36
		-	+	-	-	*Acholeplasma species*	21	63.64
Trachea	8	+	+	-	-	*M. gallisepticum*	2	25
		+	-	+	+	*M. gallinarum*	6	75
Lung	13	+	+	-	+	*M. anatis*	3	23.08
		+	+	-	-	*M. gallisepticum*	10	76.92
Eggs	3	+	+	-	+	*M. anatis*	2	66.67
		+	+	-	-	*M. gallisepticum*	1	33.33
Total	57						57	

3.Polymerase chain reaction results (PCR)

The PCR results detected the presence of a characteristic common band at 824 bp in all isolated mycoplasma and the reference strains (Photo 1).

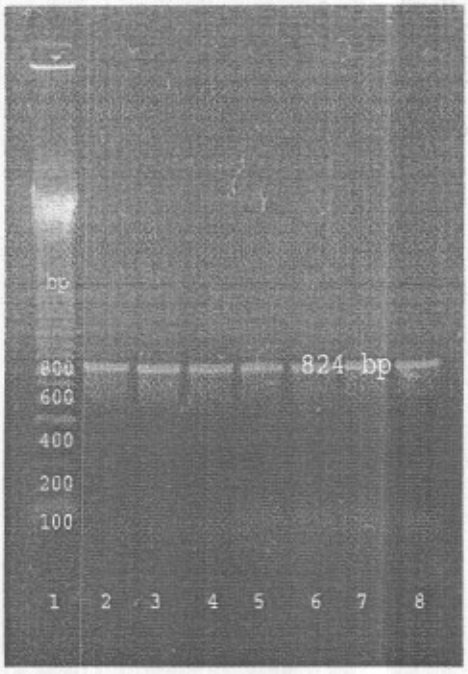


Photo (1): Agarose gel electrophoresis of PCR product from *Mycoplasma gallisepticum* reference strain and some field isolates.
Lane 1 : 100bp DNA ladder (Pharmacia),
Lane 2 : *M. gallisepticum* reference strain.
Lanes : 3-8 *M. gallisepticum* field isolates.

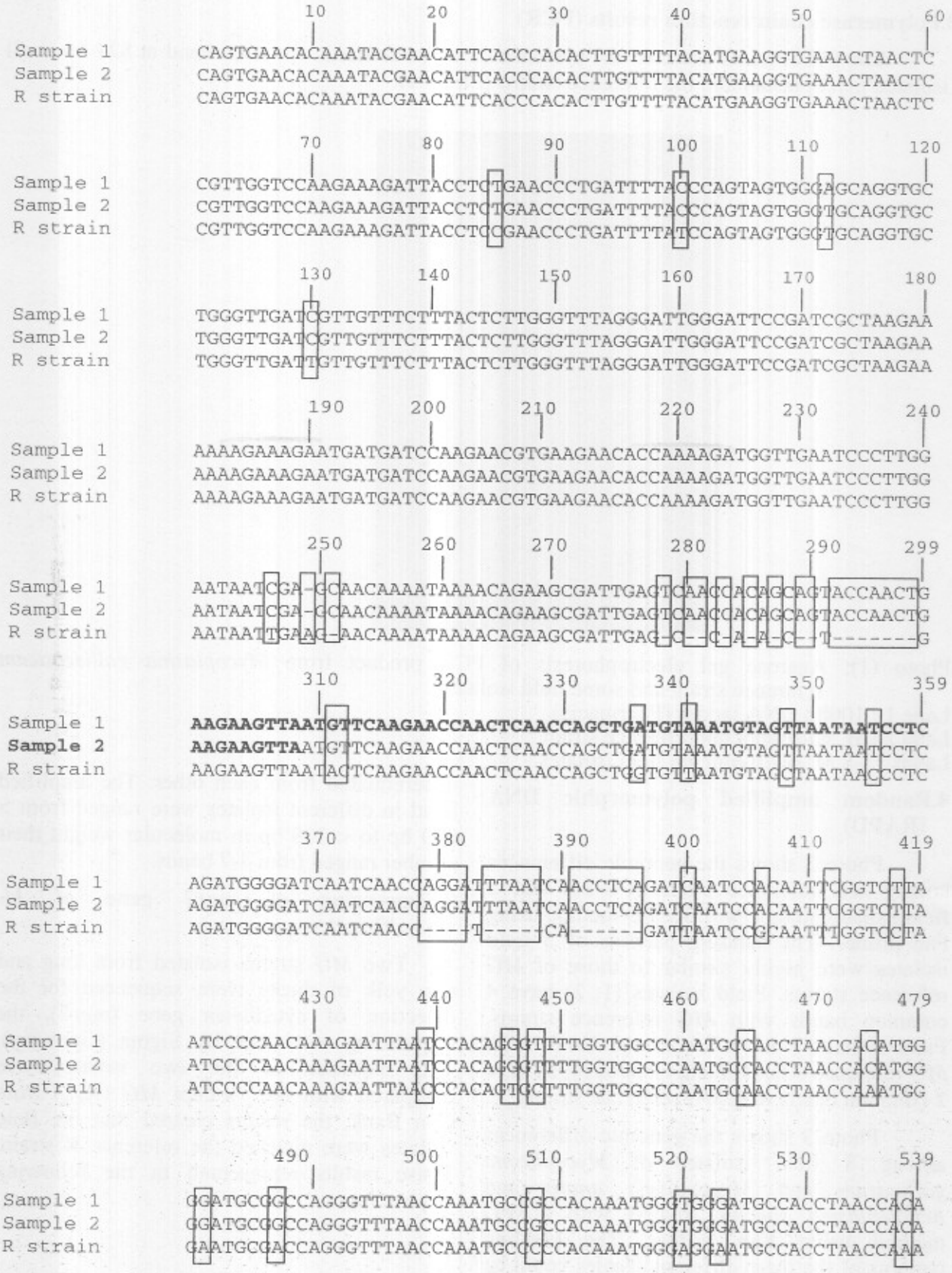
4.Random amplified polymorphic DNA (RAPD)

Photo 2 shows the genomic differences among *MG* reference strain (PG-31) and 5 field isolates studied with RAPD method using Fan primer. The banding patterns of 5 field isolates were highly similar to those of *MG* reference strains. Field isolates (1, 2) have 4 common bands with *MG* reference strains. Field isolates 3 have 6 common bands with *MG* reference strains. Field isolates (4, 5) have 2 common bands with *MG* reference strains.

Photo 3 shows the genomic differences among 8 field isolates of *Mycoplasma gallinarum* and *Mycoplasma anatis* and *Acholeplasma species* studied with RAPD method using **Fan** primer. The banding patterns of the three different species could be differentiated from each other. The amplified band in different isolates were ranged from > 800 bp to < 100 bp in molecular weight their number ranged from 4-7 bands.

5.Sequencing of *mgc2* gene of *M. gallisepticum*

Two *MG* strains isolated from lung and egg yolk of ducks were sequenced for the detection of cytadhesin gene (*mgc2*), the sequencing was done by Sigma (Germany). The sequence of the two isolates was compared with the virulent ***MG*** (R_{low}) from Gen Bank, the results cleared that the field isolates were matched the reference R strain. These results represented in the following sequencing:


10 20 30 40 50 60
Sample 1 CAGTGAACACAAATACGAACATTCACCCACACTTGTTTTACATGAAGGTGAAACTAACTC
Sample 2 CAGTGAACACAAATACGAACATTCACCCACACTTGTTTTACATGAAGGTGAAACTAACTC
R strain CAGTGAACACAAATACGAACATTCACCCACACTTGTTTTACATGAAGGTGAAACTAACTC
70 80 90 100 110 120
Sample 1 CGTTGGTCCAAGAAAGATTACCTCTGAACCCTGATTTTACCCAGTAGTGGGAGCAGGTGC
Sample 2 CGTTGGTCCAAGAAAGATTACCTCTGAACCCTGATTTTACCCAGTAGTGGGTGCAGGTGC
R strain CGTTGGTCCAAGAAAGATTACCTCGGAACCCTGATTTTATCCAGTAGTGGGTGCAGGTGC
130 140 150 160 170 180
Sample 1 TGGGTTGATCGTTGTTTCTTTACTCTTGGGTTTAGGGATTGGGATTCCGATCGCTAAGAA
Sample 2 TGGGTTGATCGTTGTTTCTTTACTCTTGGGTTTAGGGATTGGGATTCCGATCGCTAAGAA
R strain TGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTAGGGATTGGGATTCCGATCGCTAAGAA
190 200 210 220 230 240
Sample 1 AAAAGAAAGAATGATGATCCAAGAACGTGAAGAACACCAAAAGATGGTTGAATCCCTTGG
Sample 2 AAAAGAAAGAATGATGATCCAAGAACGTGAAGAACACCAAAAGATGGTTGAATCCCTTGG
R strain AAAAGAAAGAATGATGATCCAAGAACGTGAAGAACACCAAAAGATGGTTGAATCCCTTGG
250 260 270 280 290 299
Sample 1 AATAATCGA-GCAACAAAATAAAACAGAAGCGATTGAGTCAACCACAGCAGTACCAACTG
Sample 2 AATAATCGA-GCAACAAAATAAAACAGAAGCGATTGAGTCAACCACAGCAGTACCAACTG
R strain AATAATTGAAG-AACAAAATAAAACAGAAGCGATTGAG-C--C-A-A-C--T-------G
310 320 330 340 350 359
Sample 1 **AAGAAGTTAATGTTCAAGAACCAACTCAACCAGCTGATGTAAATGTAGTTAATAATCCTC**
Sample 2 **AAGAAGTTA**ATGTTCAAGAACCAACTCAACCAGCTGATGTAAATGTAGTTAATAATCCTC
R strain AAGAAGTTAATACTCAAGAACCAACTCAACCAGCTGGTGTTAATGTAGCTAATAACCCTC
370 380 390 400 410 419
Sample 1 AGATGGGGATCAATCAACCAGGATTTAATCAACCTCAGATCAATCCACAATTCGGTCTTA
Sample 2 AGATGGGGATCAATCAACCAGGATTTAATCAACCTCAGATCAATCCACAATTCGGTCTTA
R strain AGATGGGGATCAATCAACC----T-----CA------GATTAATCCGCAATTTGGTCCTA
430 440 450 460 470 479
Sample 1 ATCCCCAACAAAGAATTAATCCACACGGTTTTGGTGGCCCAATGCCACCTAACCACATGG
Sample 2 ATCCCCAACAAAGAATTAATCCACACGGTTTTGGTGGCCCAATGCCACCTAACCACATGG
R strain ATCCCCAACAAAGAATTAACCCACACTGCTTTGGTGGCCCAATGCAACCTAACCAAATGG
490 500 510 520 530 539
Sample 1 GGATGCGGCCAGGGTTTAACCAAATGCCGCCACAAATGGGTGGGATGCCACCTAACCACA
Sample 2 GGATGCGGCCAGGGTTTAACCAAATGCCGCCACAAATGGGTGGGATGCCACCTAACCACA
R strain GAATGCGACCAGGGTTTAACCAAATGCCCCCACAAATGGGAGGAATGCCACCTAACCAAA

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                    550        560        570        580        590        599
Sample 1  TGGGGATGCGGCCAGGGTTTAACCAAATGCCACCTAACCAAATGGGTGGAATGCCACCAA
Sample 2  TGGGGATGCGGCCAGGGTTTAACCAAATGCCACCTAACCAAATGGGTGGAATGCCACCAA
R strain  TGGGAATGCGACCAGGGTTTAACCAAATGCCCCC-A-C-AAATGGGAGGAATGCCACCAA

                    610        620        630        640        650        659
Sample 1  GACCAAACTTCCCTAACCAAATGCCTAATATGAATCAACCAAGACCAGGTTTCAGACCAC
Sample 2  GACCAAACTTCCCTAACCAAATGCCTAATATGAATCAACCAAGACCAGGTTTCAGACCAC
R strain  GACCAAACTTCCCTAACCAAATGCCTAATATGAACCAACCTAGACCAGGTTTCAGACCAC

                    670        680        690        700        710        718
Sample 1  AACCTGGTGGTGGGGT-CTCGATGGGAAATAAAGCTGGAGGCGGGTTTAATCACCCAGGT
Sample 2  AACCTGGTGGTGGGGT-CTCGATGGGAAATAAAGCTGGAGGCGGGTTTAATCACCCAGGT
R strain  AACCTGGTGGTGGGGTGC-CGATGGGAAATAAAGCTGGAGGTGGGTTTAATCACCCAGGT

                    730        740        750        760        769
Sample 1  GCACCAATGGGTCCAAACCGCATGAACTTCCCTCATCAAGGAATGAATCAG
Sample 2  GCACCAATGGGTCCAAACCGCATGAACTTCCCTCATCAAGGAATGAATCAG
R strain  ACACCAATGGGTCCAAACCGCATGAACTTCCCTAATCAAGGAATGAATCAG
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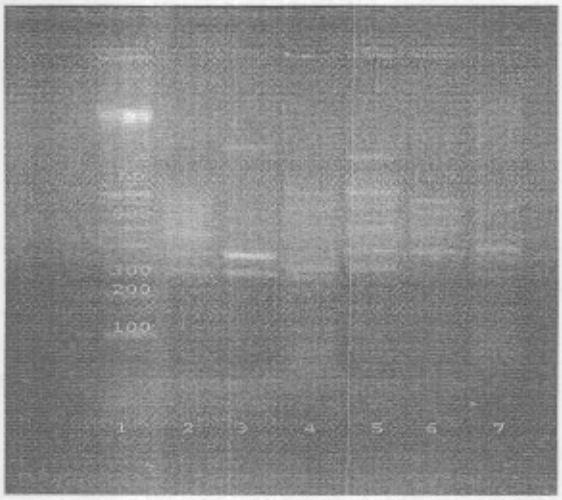
Random amplified polymorphic DNA (RAPD)



Photo(2):Electrophoretic analysis of RAPD patterns of ***Mycoplasma gallisepticum*** reference strain and some field isolates.
Lane 1: 100bp DNA ladder
Lane 2: ***M. gallisepticum*** reference strain.
Lanes: 3-7 ***M. gallisepticum*** field isolates.

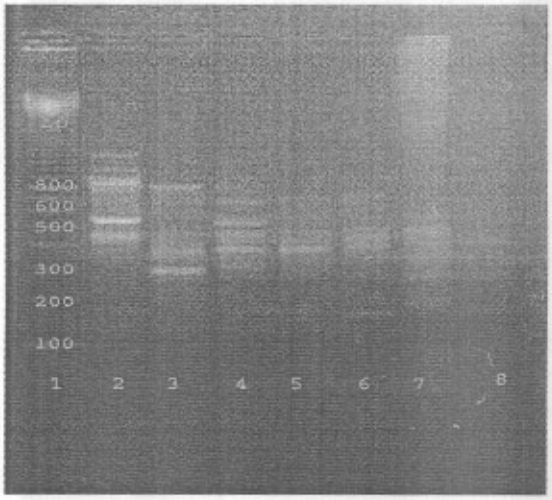


Photo (3):Electrophoretic analysis of RAPD patterns of ***Mycoplasma gallinarum, Mycoplasma anatis*** and ***Acholeplasma species***.
Lane 1: 100bp DNA ladder
Lane 2: ***M. gallinarum***,
Lane 3: ***M. anatis*** reference strain.
Lanes: 4-6 ***M. anatis*** field isolates.
Lanes: 7,8: ***Acholeplasma species***.

DISCUSSION

Mycoplasma anatis and ***Mycoplasma gallisepticum*** (***MG***) are the most common species of mycoplasma to be recovered from ducks *(17)*. Natural infection with ***MG*** have an uncertain pathogenicity *(18)*. It has been suggested that ***MG*** might cause air sacculitis in one-day-old ducklings inoculated via the air sacs *(19)*, though only subclinical infection without clinical signs or gross lesion was reported following intranasal exposure of 24- and 180-day-old ducks to this infection *(20)*.

Seven hundred and eighteen samples were collected from ducks of farms and back-yard in Dakahlia and Sharkia Governorates with isolation of ***M. gallisepticum*** was high from lungs 76.92%, followed by egg yolk 33.33% and trachea 25%. While the highest recovery rate of ***M. gallinarum*** was 75% from trachea, followed by tracheal swabs 36.36%. Concerning ***M. anatis***, it could be isolated from egg yolk and lungs 66.67% and 23.08%, respectively.

M. gallisepticum, M. anatis, ***M. gallinarum*** and ***Acholeplasma laidlawii*** were isolates from ducks suffering from sinusitis, dead in shell embryos and infertile eggs *(21-25)*.

In the present investigation, Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) or Arbitrarily Primed PCR (AP-PCR) were evaluated for rapid detection and identification of mycoplasma isolated from ducks. Two oligonucleotide primers were used for the detection of ***mgc2*** gene encodes a cytadhesin protein of ***M. gallisepticum***. The primers successfully detected the gene in all the examined ***M. gallisepticum*** field isolates and reference strain (824 bp fragment). Fragment of 824 bp could be detected in all tested ***MG*** strains *(10)*.

RAPD technique was used to detect the similarity and difference among ***MG*** field isolates compared with the reference strain. High similarity of DNA patterns of field isolates to those of ***MG*** reference strain showed 2-6 common DNA bands. The similarity between ***MG*** isolates from different sources was previously determined *(11)*. On the other hand, ***M. anatis***, ***M. gallinarum*** and ***Acholeplasma*** species showed different DNA patterns which could be differentiated from each other. The molecular diversity among the examined strains may refer to the variation in the source and geographic origin. The obtained results could be used in exploration of epidemiological problems of mycoplasma species.

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الملخص العربى

دراسات متقدمة على ميكروبات الميكوبلازما فى البط

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***المعمل الاقليمى- فرع المنصورة

أجريت هذه الدراسة على ٧١٨ عينة جمعت من مزارع وحظائر مختلفة للبط من محافظتى الدقهلية و الشرقية لعزل و تصنيف ميكروب الميكوبلازما فى البط. تم عزل و تصنيف الميكوبلازما جاليسبتكم من الرئة بنسبة ٧٦,٩٢% ومن البيض بنسبة ٣٣,٣٣% ومن القصبة الهوائية بنسبة ٢٥% ،وأيضا تم عزل و تصنيف الميكوبلازما جالينيرم من القصبة الهوائية بنسبة ٧٥% ومن مسحات القصبة الهوائية بنسبة ٣٦,٣٦% ، بينما تم عزل و تصنيف الميكوبلازما أناتس من البيض و الرئة بنسبة ٦٦,٦٧% ، ٢٣,٠٨% على التوالى. تم تأكيد التصنيف باختبار تفاعل انزيم البلمرة المتسلسل حيث أعطى حزمة مميزة عند ٨٢٤ قاعدة مزدوجة للميكوبلازما جاليسبتكم (*mgc2* gene) وذلك بمقارنتها بالعترة المرجعية التى أعطت نفس الحزمة.

تم إجراء اختبار تفاعل إنزيم البوليمريز العشوائي على خمسة عترات من الميكوبلازما جاليسبتكم المعزولة و مقارنتها بالعترة المرجعية . وقد وجد تشابه كبير بين أنماط حزمات الحامض النووي الموجودة في عترات الميكوبلازما جاليسبتكم المعزولة من الحقل و العترة المرجعية. تم أيضا إجراء اختبار تفاعل إنزيم البوليمريز العشوائي و قياس درجة التشابه بين سلالات الميكوبلازما أناتس والميكوبلازما جالينيرم والأكيلوبلازما تبين وجود اختلافات مميزة بالرغم من و جود حزم مشتركة بينهم و تبين أن هذا الاختبار بسيط و سريع و يمكن به دراسة و قياس درجة التشابه بين سلالات الميكوبلازما من مصادرمختلفة فى الدراسات الوبائية لأنه يحدد الاختلاف الجينى بين العترات.

وتم إجراء اختبار التتبع النيوكلتيدى لعينات الميكوبلازما جاليسبتكم المعزولة من البط ومقارنتها بالعترة المرجعية (R) *MG* ووجد تشابه بنسبة ٩٠ % .