BACTERIOLOGICAL AND SEROLOGICAL STUDIES ON AVIAN MYCOPLASMAS IN MENOFIA GOVERNORATE

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ABSTRACT

Traditional and recent techniques were used for diagnosis of avian mycoplasmas in chickens showing respiratory manifestations from different farms in Menofia Governorate. A total of 57 isolates were recovered from examined organs of 100 chickens at 45-50 days old. The recovered isolates were classified into 3 Acholeplasma and 54 Mycoplasma isolates, of which 52 isolates were identified as M. gallisepticum.

100 serum samples were collected from all chickens and examined by serum plate agglutination test (SPA) and 90 of these samples were tested by enzyme-linked immunosorbent assay (ELISA). The results of SPA test were, 65 positive, 9 suspected and 26 negative. The results of ELISA using M. gallisepticum coated plate (KPL kit) were, 57 positive, 16 suspected and 17 negative.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis and Western-blot technique were used to differentiate between two M. gallisepticum reference strains (F & S6) and two M. gallisepticum field isolates. The 4 strains of M. gallisepticum were similar in SDS-PAGE patterns and in their immunoreaction of proteins with minor differences.

Polymerase chain reaction (PCR) was used to compare between M. gallisepticum reference strains and M. gallisepticum field isolates. it gave two different results when two different primer sets were used, the first primer gave a specific band at 615 bp, which is specific for M. oral but not for M. gallisepticum.The second primer gave a specific band at 330 bp which is specific for M. gallisepticum.

INTRODUCTION

Mycoplasma gallisepticum infection in chickens is still an important veterinary problem caus-

ing decreased egg production, growth and feed conversion rates, increased mortality and condemnation rates of carcasses as well as indirect losses due to increased sensitivity of infected birds to management failures and associating agents, as infectious bronchitis, laryngotracheitis, or Newcastle disease viruses and *Escherichia coli* **(Carpenter et al., 1981)**.

Successful control of the disease, including eradication of *M. gallisepticum* depends very much on reliable diagnosis of infection. This can be done by culturing the agent and by detecting antibodies against *M. gallisepticum* by serological tests, such as serum plate agglutination test (SPA). This test is rapid and sensitive but often gives false positive reactions connected with antigen preparation techniques **(Opitz and Cyr, 1986 and Ahmad et al., 1989)**. The use of enzyme-linked immunosorbent assay (ELISA) has been proposed because of higher sensitivity **(Ansari et al., 1983; Avakian et al., 1987 and Stipkovits et al., 1993)**.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) was used for identification of different strains of MG **(Khan et al., 1987; Barbour and Newman, 1989 and Thongkamkoon et al., 1996)**. Western-blot technique was used for identification of different strains of MG **(Avakian and Kleven, 1990; Ellakany et al., 1997 and Salisch et al., 1999)**. Polymerase chain reaction (PCR) was chosen due to many advantages that were reported for this technique as it is very specific and sensitive **(Kempf et al., 1993; Fan et al.,1995 and Ren et al., 2000)**.

The purpose of this work was to determine the applicability and reliability of these recent techniques in diagnosis of *Mycoplasma gallisepticum* infection in chickens in Menofia Governorate.

MATERIAL AND METHODS

Two hundred samples including airsacs and lungs were collected from 100 chickens (at 45-50 days old), which showed respiratory mainfestations from different farms at Menofia Governorate.

Isolation and identification of members of the genus Mycoplasma (Razin & Tully, 1983):

About 0.5 g of tissue was aseptically removed into a sterile morter, chopped into small pieces and ground, 5 ml of broth were added to form an emulsion. From the emulsion, direct plating (Po) using a bent Pasteur pipette was done and about 0.2-0.3 ml were transferred to the broth (Bo) . On the third day it was transferred to plate (P1) and broth (B1) and on the sixth day, another plating was tried (P3) beside indirect plating (P2) from the orginal broth; on the 9th day, a last plating (P4) was done from (B1). Broth and agar plates were incubated at 37°C under reduced oxygen tension in humidified candle jar. Plates were examined for suspected colonies after 48 hours under dissecting microscope using slightly oblique light, then every other day up to 7-10 days for the appearance of growth of Mycoplasma on agar plates. Agar blocks with Mycoplas-

ma colonies were transferred into fluid PPLO medium. Serial dilutions of Mycoplasma broth cultures were prepared. After culturing each dilution separately into corresponding agar medium, a single colony was picked up from morphologically different colonies and this procedure was repeated for at least two to three times till a pure culture was obtained .

Identification of *M. gallisepticum* isolates:

For the conventional identification, glucose fermentation and arginine deamination tests were done according to **Erno and Stipkovits (1973)** and growth inhibition test was performed as described by **Clyde (1964)**.

The serological diagnosis was done using serum plate agglutination test and Enzyme-Linked Immunosorbent Assay (ELISA) using KPL kits (Kirkegaard and Perry Laboratories Inc.)

The Sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE), mentioned by **Laemmli, (1970)** and Western Blot described by **Thomas and Sharp (1988)** were also used.

The Polymerase chain reaction for diagnosis of *M. gallisepticum* was applied as referred to by **Hempstead (1990)**, where two types of primer sets were used:

1- PCR primers according to **Innis and Gelfand (1990) and EL-Shater et al. (1995)** : Two oligonucleotide primers were selected as one right assigned (1) and one left assigned (2). The sequence of primer (1) was 5-TAA GAA TCC AGG GTG AGC AAT-3. The sequence of primer (2) was 5-TCC TCC ACT AAA TAA ATT GAC CCG-3

2- PCR primers according to **Kempf et al. (1993)** : The sequence of primer (1) was 5 -TAAC TAT CGC ATG AGAAT AAC -3. The sequence of primer (2) was 5- GTT ACT TAT TCAAA TGG TACAG -3.

Synthesis of these primers was done by (MWG - Biotech.AG, Germany).

RESULTS

Isolation and identification of *M. gallisepticum* :

As shown in Table (1), 57 isolates were recovered from 200 samples collected from 100 chickens in this study, of which 35 were obtained from airsacs and 22 from lungs; 54 of the isolates (94.7%) were sensitive to digitonin, ie. they belonged to the Family Mycoplasmataceae and 3 isolates were not sensitive to digitonin belonging to Family Acholeplasmataceae.

Identification of *Mycoplasma gallisepticum* :

From 54 Mycoplasma isolates, only 52 isolates were glucose + ve and arginin -ve (Table 2) and thus were identified as M. gallisepticum. The other 2 isolates were glucose - ve and arginin + ve i.e other Mycoplasma species. The growth inhibition test confirmed the biochemical reaction and substantiated the identification of M. gallisepticum.

Serological diagnosis of *M. gallisepticum* infection in chickens :

As revealed in Table 3, the examination of 100 serum samples collected from chickens at 45-50 days old by the serum plate agglutination test showed that, 65 samples were positive (65%), 9 samples were suspected (9%) and 26 samples were negative (26%). Enzyme linked immunosorbent assay (ELISA) using *M. gallisepticum* coated plate (KPL kit) was used to test 90 serum samples, of which 57 positive samples for *M.gallisepticum* were detected (63.3%) but 16 samples were suspected (17.8%) and 17 samples were negative (18.9%).

Diagnosis of *M. gallisepticum* by Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) :

The SDS—PAGE patterns of 4 strains of M.gallisepticum were similar with only minor differences. The approximate molecular masses of the protein fractions, as compared to those of the standard marker, are illustrated in Tables (4,5).

Diagnosis of M. gallisepticum by Western—Blot:

The SDS-PAGE fractions of proteins of *M.gallisepticum* strains (F strain, S6 strain, Field isolates (1,2) were analyzed by Western blot using hyperimmune serum of rabbits. Table (6) shows that the number of antigenically positive fractions with F-strain were 4 antigenic fractions with molecular weights ranged from 122.25 to 25.575 kDa . The S6 - strain had also 4 antigenic fractions, but their molecular weights ranged from 113.33 to 27.665 kDa. The field isolate no. 1 had 3 antigenic fractions with 71.227 to 30.228 kDa and field isolate no. 2, 4 antigenic fractions with molecular weights ranged from 65.644 to 20.850 kDa.

Diagnosis of *M. gallisepticum* by Polymerase chain reaction (PCR) :

Figure (1) shows that the reference strain (S6) and 11 field isolates of M.gallisepticum gave a PCR product of 615 bp which is specific for *M.oral* but not specific for *M.gallisepticum*, when a

primer set of the following sequence was used : Left : 5-TAA GAA TCC AGGGTGAGC AAT-3. Right :5 -TCCTCC ACT AAATAAATTGAC CCG-3.

Figure (2) shows that the reference strains (S6 and F-stains) and field isolates of *M. gallisepticum* gave a characteristic PCR product of 330 bp which is specific for *M. gallisepticum*. when a primer set of the following sequence was used : Left : 5-GTT ACT TAT TCA AAT GGTA CAG -3 Right : 5- TAA CTA TCG CAT GAG AAT AAC-3.

DISCUSSION

The present study was carried out to investigate the incidence of *M. gallisepticum* in chickens at 45-50 days old, which showed respiratory manifestations in different farms at Menofia Governorate. 52 (26%) *M. gallisepticum* isolates were successfully isolated out of 200 samples including airsacs and lungs. These findings were in agreement wit that of **Sokkar et al. (1986)**, who isolated *M. gallisepticum* from chickens with an incidence of (24%), and **Singab (1987)**, who isolated mycoplasma from chickens with a slightly higher rate (28%).

The prevalence rate of mycoplasma species in airsacs and lungs of chickens was 97.1% and 90.9% respectively. These organs are the predilection seat of mycoplasmas in chickens **(Freundt, 1976)**. However, the isolation rate was slightly higher in airsac samples. These findings were in agreement with others **(Shaker, 1991 and Roshdy, 1997)**, who used airsacs and lungs of diseased chickens as preferred sources of samples and found that the isolation of *M. gallisepticum* from airsacs was more than that isolated from lungs.

Serum plate agglutination test (SPA) was applied as a traditional serological test. 65 samples were positive (65%), 9 were suspected (9%) and 26 were negative (26%). These findings were nearly in agreement with that of **Saif - Edin (1997)**. The results of the SPA demonstrated that the *M. gallisepticum* SPA test is known to be very sensitive, detecting a high positive number of samples. However, the sensitivity may be on the cost of specificity. This may be due to that, SPA test often gives false positive reactions connected with antigen preparation techniques, bad quality of sera to be tested or use of oil emulsion poultry vaccines, also the presence of *M. synoviae* infection in flocks to be tested will give problem with cross-reacting antibodies in serological tests **(Opitz & Cyr, 1986 & Yoder, 1989)**.

The use of enzyme-linked immunosorbent assay (ELISA) has been proposed because of higher sensitivity and specificity **(Ansari et al., 1983; Opitz et al. ,1983; Avakian et al., 1987 and Panangala et al., 1990)**. ELISA results of serum samples using M. gallisepticum coated plates (KPL kit) for detection of M. gallisepticum antibodies showed 57 positive samples (63.3%), 16

suspected (17.8%) and 17 negative (18.9%). These results were in agreement with **Kempf et al. (1994)**.

Sodium dodecyl-sulfate polacrylamide gel electrophoresis can be used for differentiation of different strains of *M. gallisepticum*. The SDS-PAGE patterns of four strains of *M. gallisepticum* (F-strain, S6-strain and 2 field isolates) were similar, with only minor differences. These findings were in agreement with **Khan et al. (1987), Avakian & Kleven, (1990) and Eissa et al., (2000),** who reported the presence of similarity between SDS-PAGE protein profiles of *M. gallisepticum* reference strains and *M. gallisepticum* field isolates with minor differences. These differences were described as probably due to incomplete removal of growth-medium serum proteins **(Rhoades et al., 1973)**, or mutation or perhaps accidental mixing of cultures that had occurred during in vitro / or in vivo passage of these cultures in various laboratories. The former seems more plausible in view of only minor change in the overall protein. So the ability of SDS-PAGE to differentiate strains of *M. gallisepticum* should be a useful procedure in epidemiologic and other studies, where minor but unique differences in protein patterns may be used to identify a particular strains of *M. gallisepticum* **(Khan et al., 1987)**.

Western-blot technique was used to examine antigenic variability among *M. gallisepticum* reference strains (F & S6 strains) and M. gallisepticum field isolates (1 & 2). The Western blot analysis of antigens of four *M. gallisepticum* strains demonstrated both common and restricted patterns of immune recognition using polyclonal rabbit antisera (PCA). These results indicate the presence of similarity between immunoreactivity of the two *M. gallisepticum* reference strains (F & S6 strains) at 72 kDa and 33 kDa and a presence of similarity between immunoreactivity of the two *M. gallisepticum* field isolates (1 & 2) at 39 kDa. However, differences in immunoreactivity of *M. gallisepticum* reference strains (F & S6 strains) and *M. gallisepticum* field isolates (1 & 2) were demonstrated. These results were in agreement with **Ellakany et al. (1997)**.

Polymerase chain reaction (PCR) as a recent technique was employed in this study. In the polymerase chain reaction two primers were used that gave two different results. The first primer **(El-Shater et al., 1995)** gave a characteristic PCR product of 615 bp which is specific for *M. orale* but not for *M. gallisepticum*. However, this primer was reported by **El-Shater et al. (1995)** to be specific for *M. gallisepticum*. The repeated application of this primer gave the same result. This result led us to suspect the identification of the *M. gallisepticum*, which was rather reconfirmed by the repeated biochemical and serological methods. On the other hand, when the second primer was used according to **Kempf et al., (1993)** to compare between two *M. gallisepticum* reference strains (F & S6 strains) and two *M. gallisepticum* field isolates (1 & 2) it gave a characteristic PCR product of 330 bp which is specific for *M. gallisepticum*, exactly as reported by **Kempf et al., (1993)**. Such discrepancies in results could not be explained. Perhaps the se-

quence of the first primer may share some similarity with DNA sequence of *M. oral* or as mentioned by **Gassen et al. (1994)**, the minimum changes occurred in its parameter could lead to completely other results. Moreover, **Razin (1994)** summarized the disadvantages of PCR. In that it may give false negative results due to inhibitors in extracted DNA, faulty reagents or the procedure may be too sensitive yielding results due to contamination of PCR reagents with target DNA, also quantitation of organisms in clinical sample may be difficult and PCR procedure is still too complex to be carried out in a routine diagnostic laboratory .

Table (1) : Results of isolation and identification of Mycoplasma and Acholeplasma from chickens at 45-50-days-old .

Samples	Number examined	Number positive	Differentiation of isolates by digitonin sensitivity test			
			Mycoplasma	%	Acholeplasma	%
Air sac	100	35	34	97.1	1	2.9
Lung	100	22	20	90.9	2	9.1
Total	200	57	54	94.7	3	5.3

Table (2) : Identification of *M. gallisepticum* isolated from chickens at 45-50- days-old .

Samples	Number of examined isolates	Biochemical tests				Growth inhibition +
		Digitonin		G+ A-	G - A+	
		+	-			
Air sac	35	34	1	32	2	32
Lung	22	20	2	20	_	20
Total	57	54	3	52	2	52

Table (3) : Serological examination of sera collected from chickens at 45–50 days old using *M. gallispeticum* antigen

Test	No. of samples	No. of positives	No.of suspected	No. of negatives
SPA	100	65 (65%)	9 (9%)	26 (26%)
ELISA	90	57 (63.3%)	16 (17.8%)	17 (18.9%)

Table (4) : SDS-PAGE characteristic of ommon bands for different protein profiles of reference strains and field isolates of *M.gallisepticum* .

Lanes : Bands	Marker	F-strain	S6 – strain	Field (1) isolate	Field (2) isolate
1	200	122.25	113.33	71.227	65.644
2	97.40	72.057	72.057	39.035	39.578
3	68	39.578	38.499	34.469	34.708
4	43	33.069	33.0298	30.228	31.946
5	29	30.648	30.861	25.575	25.176
6	14.30	25.575	27.665	13.858	20.850
7		21.515	21.856		14.756
8		14.526	18.10		
9			14.990		

- Values indicated the approximate molecular weights of protein fractions expressed in kiloDalton (kDa). Molecular weights were calculated by comparison with those of the size marker fractions.

Table (5) : SDS-PAGE characteristic and percentage of amount of the different proteins of reference strains and field isolates of *M.gallisepticum* .

Lanes : Bands	Marker	F-strain	S6 – strain	Field (1) isolate	Field (2) isolate
1	2.4187	12.610	21.171	35.823	1.4996
2	0.85856	22.139	15.884	43.290	69.890
3	12.166	37.801	30.778	1.7609	5.9491
4	24.063	3.4126	4.0786	1.3764	4.7539
5	41.977	1.6688	5.7743	10.299	5.2414
6	18.517	13 694	5.2524	7.1394	10.996
7		8.1268	0.56013		0.62325
8		0.23553	3.3819		
9			12.841		
Sum	100	99.688	99.722	99.688	98.954

Values indicated the percentage of amount of the different proteins

Table (6) : Antigenic fractions detected with Western Blot of reference strains and Field isolates of . *M. gallisepticum* .

Lanes : Bands	Marker	F-strain	S6 – strain	Field (1) isolate	Field (2) isolate
1	200	122.25	113.33	71.227	65.644
2	97.40	72.057	72.057	39.035	3.578
3	68	33.069	33.0298	30.228	25.176
4	43	25.575	27.665		20.850
5	29				
6	14.30				
7					
8					
9					

Values indicate the approximate molecular weights of protein fractions expressed in kilo Dalton (kDa). Molecular weight were calculated by trace – back comparison to size of marker fractions.

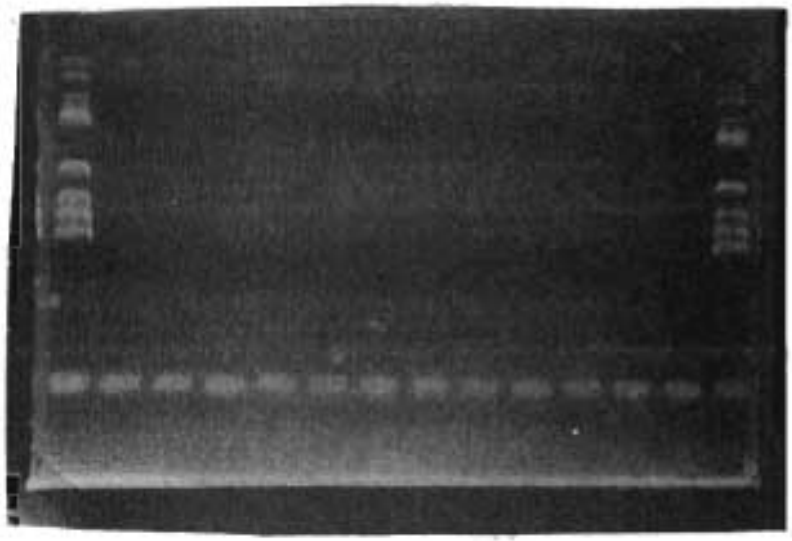

Fig. (1) : Agarose gel electrophoresis of PCR products for *M. gallisepticum* reference strain and Field isolates Lane 1 : 174 bp ladder Lane 2 : *M.gallisepticum* (S6 strain) Lane 3 -13 : *M.gallisepticum* (Field isolates).

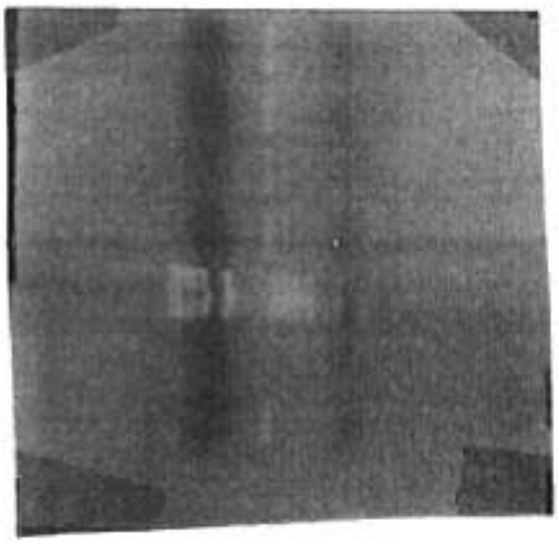

Fig. (2) : Agarose gel electrophoresis of PCR products for *M.gallisepticum* reference strains and field isolates Lane 1 -2 : M.gallsepticum (Field isolates) Lane 3 : *M.gallisepticum* (S6 - strain) Lane 4 : *M.gallisepticum* (F-strain) Lane 5 : Hae III marker . Lane 6 : - Ve laboratory contorl Lane 7 : +ve laboratory control.

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الملخص العربى

دراسات بكتيريولوچية وسيرولوچية على ميكوبلازما الدواجن فى محافظة المنوفية

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تم إستخدام الطرق التقليدية والطرق الحديثة لتشخيص الميكوبلازما جالسيبتكم فى الدجاج عند عمر ٤٥-٥٠ يوم والتى أظهرت أعراض تنفسية من المزارع المختلفة فى محافظة المنوفية، كان إجمالى عدد العترات المعزولة ٥٧ عترة والتى تم عزلها من الأكياس الهوائية والرئتين من ١٠٠ دجاجة وباختبار الحساسية للديجتونين أمكن تقسيمهم إلى ٥٤ عترة ميكوبلازما و ٣ عترات اكيلوبلازما وباستخدام الاختبارات البيوكيميائية واختبار المانع للنمو كانت النتيجة ٥٢ عترة ميكوبلازما جالسيبتكم.

تم تجميع ١٠٠ عينة سيرم وتم فحصها باختبار تلازن المصل على الشريحة و ٩٠ عينة سيرم وتم فحصها باختبار الاليزا كانت نتيجة إختبار تلازن المصل على الشريحة ٦٥ إيجابى و ٩ اشتباه و ٢٦ سلبى وكانت نتيجة اختبار الإليزا باستخدام كيتس - كى - بى - إل، ٥٧ إيجابى و ١٦ اشتباه و ١٧ سلبى.

تم إستخدام الترسيب الكهربى لجيلات البولى اكريلاميد سلفات الصوديوم دود سيل للتفريق بين عترات الميكوبلازما جاليسيبتكم المختلفة التى تصيب الدجاج وكان هناك تشابه بين أشكال البروتين للعترات المختلفة مع اختلاف بسيط.

تم استخدام اختبار الويسترن بلوت للتفريق أيضاً بين التفاعل المناعى للبروتين لعترات الميكوبلازما جاليسيبتكم المختلفة التى تصيب الدجاج وكان هناك أيضاً تشابه بين التفاعل المناعى لبروتين عترات الميكوبلازما جالسيبتكم مع اختلاف بسيط.

تم استخدام اختبار سلسلة تفاعل إنزيم البوليمريز ولكنه أعطى نتائج مختلفة عند استخدام نوعين مختلفين من البريمر حيث وجدت حزمة عند ٦١٥ زوج من القواعد وهى خاصة بالميكوبلازما أورال عند إستخدام البريمر الأول ووجدت حزمة عند ٣٣٠ زوج من القواعد فى الميكوبلازما جالسيبتكم عند استخدام البريمر الثانى.