

DIAGNOSTIC INVESTIGATION ON MYCOPLASMA GALLISEPTICUM INFECTIONS IN DIFFERENT EGYPTIAN BREEDER AND BROILER CHICKEN FLOCKS

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SUMMARY

This study was carried out to detect serologically the presence of antibodies against *Mycoplasma gallisepticum* (MG) in the serum of non MG vaccinated broiler and layer breeder, day-old and broiler chicken flocks using serum plate agglutination (SPA) test. Also, a trail for isolation and identification of different MG strains was done in the pervious flocks using conventional and recent techniques. The breeder flocks revealed positive SPA in 253/305 (82.95%), however, day-old and 4 week-old broiler flocks showed 30/70 (42.85%) positive reaction. In the breeder chickens, the total primary cultural isolation of mycoplasmas reached 405/510 (79.41%), while in day-old and 4 week-old broiler chicken flocks it was 105/135 (77.77%). Out of 405 samples in the breeder chickens 317 (78.27%) proved to be digitonin test positive (mycoplasma not acholeplasma) and out of 105 samples in the broiler chickens, 85 (80.95%) were positive digitonin. The biochemical (glucose fermentation test) and the serological (growth inhibition test) identifications of suspected mycoplasmas cultures in the breeder chicken flocks confirmed that percentages of 40.95%, 47.27%, 46.66%, 52.63% and 42.10% from tracheal swabs, nasal swabs, lung tissues, air-sacs and ovaries, respectively were positive to MG. Moreover, in broiler chickens, MG strains were isolated from the tracheal swabs, lungs and air-sacs in percentages of 57.14%, 58.0% and 52.63%; respectively. All the results were finally confirmed by polymerase chain reaction (PCR) which showed products of the amplified 16 SrRNA gene of MG from the tested positive cultures with a common band at 183 base pair (bp) for all isolated field positive MG strains and the reference MG vaccinal (F) strain. The antibiogram results cleared that the antimicrobials (pefloxacin followed by spiramycin, enrofloxacin and tylosin tartarate) were highly active in inhibition of MG growth *in vitro*, whereas doxycycline, streptomycin and tiamutin were less effective against the tested isolates.

INTRODUCTION

Mycoplasmas are the smallest prokaryotic cells belonging to class Mollicutes. They are fastidious and slow grower micro-organisms (Nicholas and Ayling, 2003).

Mycoplasma gallisepticum (MG) is the major and the most important pathogen of poultry world wide due to the great economic losses in poultry resulting from its infection. In broilers, MG causes chronic respiratory disease (CRD), poor feed conversion, low growth rate, increased mortalities, increased carcass condemnation at processing and increased vaccination and medication costs (Ley and Yoder, 1997). In commercial layer flocks, MG is involved in severe egg production losses (Domermuth and Gross, 1962; Mohamed et al., 1987; Kleven, 1998 and Levisohn and Kleven, 2000) also; it leads to deterioration of egg quality (Pruthi and

Kharole, 1981). Indirect losses caused by MG is associated with increased the susceptibility of the infected birds to other viral infections like infectious bronchitis, infectious laryngotracheitis and Newcastle disease or bacteria like *Escherichia coli* (**Carpenter et al., 1981 and Ley et al., 2003**).

There were several documents concerning the diagnosis of mycoplasmosis in different Egyptian poultry farms (**Sabry, 1962; El-Ebeedy, 1973; Metwalli, 1980; El-Ebeedy et al., 1984; Abd El-Rahman, 1986; El-Shater, 1986; Salem et al., 1986; Sokkar et al., 1986; Ahmed et al., 1988; El-Shater et al., 1990; Taha et al., 1991; El-Shater et al., 1995; Mansour, 1995; Shaker, 1995; Dardeer, 1996; Mohamed, 1997; Saif-Edin, 1997; Ahmed, 1998; Aly, 1998; El-Shabiny et al., 1999; Eissa et al., 2000; El-Shater et al., 2000; Mageed, 2000; Sharaf, 2000; El-Shater and Oraby, 2001; Hassan, 2001 ; Mohammed, 2001; Abou El-Makarem, 2003; El-Siefy 2004; Soliman, 2004; Sharaf, 2004; Abd El-Gawad, 2005; Serag 2005 and Usama, 2008**). This refers to the wide spread of mycoplasmas infections in the Egyptian Governorates which provides an urgent need for control of such infections.

The improvement of diagnostic tools for the direct detection of mycoplasmas has always been a subject for research as these tools play an important role in the disease eradication program. The diagnosis of MG infection in chickens has long depended upon serologic assays and/or the isolation and identification of the organism (**Anonymous, 2001**).

A serological procedure like serum plate agglutination (SPA) test is usually used to detect immunoglobulin M (IgM) antibodies from few days after infection up to 72 days (**Stipkovits, 1979**). As SPA test is quick, relatively inexpensive and sensitive, it has been widely used as a predictive initial screening test for flock monitoring and serodiagnosis of MG infections (**Yamamoto and Adler, 1958; Kuniyaso, 1969; Roberts, 1970; Kleven, 1975; Glisson et al., 1984; Talkington et al., 1985; Dardeer, 1996; Ewing et al., 1996; Kleven and Levisohn, 1996; Kleven et al., 1998 and Sharaf, 2004**). Although SPA test is useful for examining flocks, but it's hampered by non specific reactions and interspecies cross reactivity (**Olson et al., 1963; Bradbury and Jordan, 1973; Glisson et al., 1984; Avakian et al., 1988 and Yoder, 1989**).

Cultivation techniques for diagnosis of mycoplasmas infections are laborious, expensive and require a relatively long time. Problems experienced in the past with culture include chronic MG cases or infection with strains of low pathogenicity. The overgrowth of non pathogenic faster growing mycoplasma species or other organisms or even no growth in subculture may also

interfere with cultivation of MG from clinical sample in the laboratory (**Mallinson and Rosenstein, 1976 and Garcia et al., 1995**).

Although polymerase chain reaction (PCR) can't replace entirely conventional methods for isolation, it can be used for rapid diagnosis of clinical infection of MG if it supported by the clinical picture, the serological profile of the flock and finally with culture (**Kempf, 1998**). Developments of the diagnostic PCR technology have significantly overcome the problems with time, specificity and sample size. PCR represents a rapid, sensitive and specific diagnostic method which is able to provide accurate results in the presence of mixed mycoplasma infections and bacterial contamination or inhibition of the growth by antibiotics or by other host factors (**Kempf et al., 1993 and Fan et al., 1995**). PCR is used to detect the presence of MG specific DNA sequences as it is able to determine as low as 10^{-6} pictogram of MG DNA a fraction from a total chromosomal contents of one cell (**Hyman et al., 1989; Nascimento et al., 1991; Slavik et al., 1993; Salisch et al., 1998 and Charlton et al., 1999a,b**). This may be a useful method in the diagnosis of MG infection at the early and the later stage of the disease (**Kempf et al., 1993**), in which conventional cultural and serological tests are unsuccessful (**Nascimento et al., 1991**).

The persistence of MG infections in the commercial poultry farms suggested that efforts for eradication were not successful; limiting the losses is the primary target (**Gary, 2004**). The use of antimicrobials remains the most common and effective means for controlling of mycoplasmas infections in poultry farms. To reach a successful treatment using antimicrobials, it is necessary to assess the sensitivity of mycoplasmas present in the flock (**Burch and Stipkovits, 1994**). Decrease the efficacy of antibiotics against avian mycoplasmosis is a phenomenon frequently observed in the field which was attributed to development of resistance (**Fahey, 1957**). Different strains of mycoplasmas showed considerable variations in sensitivity to a given antibiotic, probably due to extended or improperly administered therapy (**Osborn and Pomeroy, 1958; Osborn et al., 1960 and Newnham and Chu, 1965**). *in vitro* tests to assess the sensitivity of various isolates of MG to a wide spectrum of chemotherapeutic agents particularly tylosin and quinolones have been carried out (**Kleven and Anderson, 1971; Levisohn, 1981; Whithear et al., 1983; Jordan and Knigh, 1984; Lin, 1987; Jordan et al., 1989; Kempf et al., 1989; Tanner and Wu, 1992; Bradbury et al., 1994; Jordan and Horrocks, 1996; Jordan et al., 1998 and Pakpinyo and Sasipreeyajan, 2007**).

Herein, this work tried to through the light on the following points:

1. Serological detection of antibodies to MG infections (using SPA test) in broiler and layer breeder, day-old and broiler chicken flocks at different Egyptian Governorates.
2. Isolation and identification of MG from the swabs and organs collected from broiler and layer breeder, day-old and broiler chicken flocks using conventional methods for detection.
3. Molecular characterization (using PCR) of the identified field strains of MG in comparison with the reference vaccinal (F) strain.
4. Testing the antibiogram of certain antimicrobials against some MG strains *in vitro*.

MATERIAL AND METHODS

1. Samples:

Serum samples as well as swabs and organ samples were collected from different breeds of broiler and layer breeder chicken flocks at ages (20, 26, 30, 35, 46, 50 and 55 weeks) and also from different breeds of day-old and 4 week-old broiler chicken flocks. These flocks were located in Giza, Cairo and Alexandria Governorates and were not vaccinated against MG infection.

A. Serum samples:

A total number of 305 blood samples were collected from broiler and layer breeder chickens, also 70 blood samples were taken from day-old chicks and broiler chickens. These blood samples were taken from the wing veins or from sacrificed birds and the sera were separated by centrifugation of the clotted blood at 3000 r.p.m for 10-15 minutes. Each serum sample was collected in labeled sterilized Epindorff tube and kept in -20°C till testing.

B. Tracheal and nasal swabs:

A total numbers of 345 tracheal and nasal swabs were taken from broiler and layer chicken breeder chicken flocks; out of which, 60 tracheal swabs were collected from day-old and broiler chicken flocks. The collected swabs were taken from MG serologically positive birds and freshly dead ones. Swabs were immediately immersed into suitable mycoplasma growth medium containing bacterial inhibitors (thallium acetate and penicillin) and were preserved in ice container and transported to the laboratory.

C. Organs:

Organs including lungs and air-sacs with a total numbers of 165 were collected from broiler and layer breeder chickens, while a total of 75 lungs and air-sacs samples were taken

from day-old and broiler chickens. Respiratory organs were collected from MG serologically positive and freshly dead birds suffering from respiratory manifestation (coughing, rale, sinusitis and conjunctivitis) and stunted growth; also ovaries were collected from broiler and layer chicken breeders with drop in egg production (5%). The collected lungs showed lesions varied from congestion to severe pneumonia, the air-sacs were turbid or containing caseous material while the ovaries were atrophied and discoloured. Samples of each bird were aseptically collected and immersed into vials containing mycoplasma broth medium with double dose of bacterial inhibitors.

2. Serum plate agglutination (SPA) test:

The collected serum samples were tested to detect the presence of MG antibodies using serum plate agglutination (SPA) test according to the methods of **Kleven and Yoder (1989) and Kempf et al., (1997)**. The already prepared stained MG antigen (Intervet International B. V. Boxmeer, Holland) was tested with known positive and negative MG control sera. The serum samples were diluted (1:10) with phosphate buffer saline (PBS) to eliminate high incidence of non specific reactors. One drop of each diluted serum sample was pipetted on a square of the plate. Equal volume (one drop) of undiluted whole cell stained MG antigen was added to one drop of the diluted sera and then thoroughly mixed. Another one drop of saline was mixed with the antigen as control. The result was read within 1-2 minutes at room temperature. A positive reaction of SPA was indicated by a definite clumping (agglutination), while the negative one was indicated by the homogenous mixture without appearance of flocculation.

3. Isolation and identification of MG isolates:

Each of the collected tracheal and nasal swabs and organs of lungs, air-sacs and ovaries were cultured onto sterile pleuropneumonia like organism (PPLO) broth and agar media with inhibitors and indicators for mycoplasmas isolation according to **Razin (1978)** and propagated as described by **Razin and Tully (1983)**. The purification of the mycoplasma isolates were done as mentioned by **Tully (1983)**. Maintenance of isolates was performed after **Leach (1983)**. Differentiation between mycoplasma and acholeplasma isolates (genus determination) using digitonin inhibition test was conducted as described by **Freundt et al., (1973)**. The isolates were microscopically identified using stereomicroscope to detect the characteristic fried egg appearance of mycoplasma colonies (**Quinn et al., 2002**). Mycoplasma isolates were biochemically characterized by using glucose fermentation test (**Erno and Stipkovitis, 1973**)

and by using arginine hydrolysis (deamination) test (**Frenske and Kenny, 1976**). Serological identification was done using growth inhibition test as described by **Clyde (1964)**. The locally prepared specific antisera were kindly supplied by Dr. El-Mostafa El-Metwalli (Late lecturer, Microbiology Department, Faculty of Veterinary Medicine, Cairo University)

4. Reference MG vaccinal strain:

Lyophilized live vaccine of MG (F) strain (F-vax MG[®], Shering-Plough, USA) was used as a reference strain in polymerase chain reaction (PCR).

5. Polymerase chain reaction (PCR):

The PCR assay was prepared according to the previously mentioned method of **Kempf et al., (1993)**.

A. Testing of field MG strains and the reference (F) vaccinal strain:

Eight of the serologically identified MG strains (5 strains representing tracheal swabs, nasal swabs, lungs, air-sacs and ovaries of breeder chickens and 3 representing tracheal swabs, lungs and air-sacs of broiler chickens), also the reference (F) strain were tested. Both of the field and reference strains were cultured in modified Frey's liquid media and agar without addition of inhibitors and then examined for typical MG colonies using dissecting microscope. Each culture was plated on 8% sheep blood agar medium and on Sabouraud dextrose agar medium to prove the purity of the strains from any bacterial or fungal contamination, respectively.

B. Sample preparation prior to DNA amplification:

Mycoplasma isolates as well as the reference strain were prepared for extraction of the chromosomal DNA as described by **Blanchard et al., (1998)**. The samples were stored at 4°C till amplification step.

C. Amplification of DNA:

The PCR primer (Kempf et al., 1993).

Small subunit rDNA of MG was amplified using a forward primer RANG1 which corresponding to nucleotide positions 173-195 (5'-TAA CTA TCG CAT GAG AAT AAC-3') and a reverse primer RANG2 which corresponding to the complement of positions 502-481 (5' – GTT ACT TAT TCA AAT GGT ACA G-3').

The PCR assay was done with a thermostable DNA taq polymerase in an automated DNA thermal cycler. The PCR was performed at an initial incubation temperature of 90°C for 1 minute; thermal cycling (40 cycles) proceeded with each segment of one cycle being:

denaturation at 95°C for 15 second, annealing at 60°C for 20 second and extension at 75°C for 15 second. At final step, an additional cycle (95°C for 15 second, 60°C for 45 second and 75°C for 5 minutes) was included.

D. Identification of PCR amplified product:

It was done according to **Sambrook et al., (1989)**. The amplified products (10 µl) were subjected to electrophoresis through 2% agarose gel and stained by ethidium bromid. Then DNA was visible under UV lamp and the bands were sized by DNA ladder molecular marker.

6. The antibiogram:

Eight characterized strains of MG using PCR (five from breeder and three from broiler chickens) were *in vitro* tested using different members of antimicrobial compounds that commonly used in the Egyptian poultry field. The method was conducted previously by **Sharma et al., (1997)**. Each mycoplasma strain was cultured in PPLO broth medium for two days, then ten fold serial dilutions was carried out and a 10⁵ dilution was chosen for achievement of disc susceptibility test. A 30 µl of each dilution was pipitted onto PPLO agar plates, after dryness of the plates the antimicrobial discs were discarded. The plates were incubated at 37°C under reduced oxygen tension for two days. The inhibitory zone around each disc was measured and interpreted as described by **Bauer et al., (1966); McAllister (1990) and Quinn et al., (1994)**. The used antibiotic discs, their concentrations and the producing companies were listed in table (1).

RESULTS AND DISCUSSION

As a result of severe and significant economic losses caused by mycoplasma infections (stunted growth, mortalities, drop in egg production and carcasses rejection) in poultry flocks, together with the costs of medication and vaccination for controlling of such infections (**Evans and Hafez, 1992; Fiorentin et al., 1997 and Parker et al., 2003**). Therefore diagnostic (serological and bacteriological) investigations of mycoplasmas especially MG infection in our breeder and broiler chicken farms were done. Also, the sensitivity of the isolated field MG strains to the different antimicrobial agents *in vitro* was investigated.

Concerning the results of SPA test, table (2) revealed that, out of 305 serum sample collected from non MG vaccinated breeder flocks at different ages (20, 26, 30, 35, 46, 50 and 55 weeks) only 253 (82.95%) was positive (showed clumps) for MG. We could notice that high percentage of MG positive sera were found in older ages (50 and 55 week-old) and that could be

explained as mycoplasmas have the ability to resist the host immune defenses and selective antibiotic therapy, establishing chronic infection (**Winner et al., 2000**). Moreover, mycoplasmas adsorb host protein in their surface so they can not recognize for a while as an antigen (**Stipkovits, 2001**), therefore, the immune response to mycoplasmas was delayed.

However, out of 70 serum samples collected from day-old and non MG vaccinated 4 week-old broiler flocks, 30 (42.85%) was positive for MG (table 3). This result is nearly similar to that of **Shukla et al., (1985)** who detected 88/195 (42.5%) seropositive reactors for MG, **Ulgen and Kahraman (1993)** who found MG positive broilers in 242/449 (48.5%), and **Mohamed (1997)** who detected positive reactors to MG infection in 33 day-old chickens in 44% of the birds. On the other hands, our result is considered to be lower than that recorded by **Saif-Edin (1997)** who recorded MG positive reactors in 66% of broiler flocks, **Soliman (2004)** revealed presence of antibodies to MG in 100% of the all examined birds; also, **Usama (2008)** proved that 277/325 of the examined 4 week-old chickens were positive to MG with a percentage of 85.2%.

Dilution of avian serum using PBS before applying SPA test is recommended by **Ross et al., (1990)** and **Kleven et al., (1998)** to significantly decrease the possibility of non specific reaction. This reaction may occur due to presence of different types of mycoplasmas or application of Gumboro vaccine (**Salisch et al., 1999**).

The results of primary cultural isolation of mycoplasma collected from 20, 26, 30, 35, 46, 50 and 55 week-old broiler and layer breeder flocks were seen in (table 4). From a total 510 samples, the isolation yielded 405 mycoplasma spp. isolates (79.41%). About 145/180 (80.65%) tracheal swabs, 130/165 (78.78%) nasal swabs, 59/70 (84.28%) lungs, 43/55 (78.18%) air-sacs and 28/40 (70%) ovaries were suspected microscopically to be mycoplasma. Figure (1) showed the characteristic fried egg shape colonies (tiny, smooth circular, translucent mass with a dense raised central area) (**Quinn et al., 2002**). Furthermore, table (5) showed the results of digitonin sensitivity test for differentiation between mycoplasma and acholeplasma species collected from different breeder flocks. The positive mycoplasma species cultures showed inhibition zone around the digitonin impregnated discs. The total recovery rate was 317/405 (78.27%) representing 105/145 (72.41%) tracheal swabs, 110/130 (84.61%) nasal swabs, 45/59 (76.27%) lungs, 38/43 (88.37%) air-sacs and 19/28 (67.85%) ovaries were positive digitonine test (mycoplasma not acholeplasma cultures). Nearly similar results were obtained by **Salem et al.,**

(1986); Saif-Edin (1997) and Mageed (2000) who concluded that the isolation rate of MG from different flocks in upper Egypt was ranged from 20-100% and that revealed high prevalence of MG infections in Egyptian farms. In addition, **Mansour (1995) and Serag (2005)** isolated MG with percentages 58% and 23.2%; respectively from chicken's respiratory and reproductive samples.

From the above mentioned results, it could be observed that mycoplasma organisms not only isolated from the respiratory organs, but also from the ovaries. These results agree with those of **Pruthi and Kharole (1981) and Peebles et al., (2004)** who mentioned that MG organism can spread from the respiratory tract via blood stream to the ovaries causing pathological conditions and that indicated the tissue proclivity of MG.

Regarding the results of primary cultural isolation of mycoplasma from day-old and 4 week-old broiler flocks, the results revealed that, out of 135 samples representing, 60 tracheal swabs, 45 lungs and 30 air-sacs, only 105 samples (77.77%) representing 45 (75%), 35 (77.77%) and 25 (83.33%), respectively were microscopically suspected to be mycoplasma (fried egg shape colonies) (table 6 and fig. 1). In addition, out of a total 105 suspected cultures, the digitonin sensitivity test showed positive inhibition zones (mycoplasma not acholeplasma species) (**Freundt, 1983**) in 85 ones (80.95%). The above samples represented tracheal swabs (35 out of 45, 77.8%), lungs (31 out of 35, 88.57%) and air-sacs (19 out of 25, 76%) (table 7). This incidence of recovery is higher than that reported by **Metwalli (1980)** (50%); **Ulgen and Kahraman (1993)** (15.3%); **Dardeer (1996)** (19.2%); **Mohammed (2001)** (21.2%). On the other hand, this incidence consider to be lower than that recorded by **Saif-Edin (1997)** (100%) and **Usama (2008)** (89%).

Table (8) revealed the biochemical and serological identification of the isolated mycoplasma strains taken from broiler and layer breeder chicken flocks. Positive MG cultures gave positive glucose fermentation test (change in colour of the inoculated broth to orange or yellow), negative arginine hydrolysis tests (no change in colour of the inoculated broth) and positive growth inhibition test (inhibitory zones). From the table, it could be concluded that MG strains were found in 43 out of 105 tracheal swabs cultures (40.95%), in 52 out of 110 nasal swab cultures (47.27%), in 21 out of 45 lung cultures (46.66%), in 20 out of 38 air-sac cultures (52.63%) and in 8 out of 19 ovarian cultures (42.10%). The previous results are parallel to some

extent with that recorded by **Godoy et al., (2001)** who studied the epidemiology of mycoplasmosis in 25 layer chicken farms and found that the isolation rate of MG was up to 49%.

Furthermore, un-typed mycoplasma species were detected in 62 out of 105 tracheal swabs (59.0%), 58 out of 110 nasal swabs (52.72%), 24 out of 45 lungs (53.33%), 18 out of 38 air-sacs (47.36%) and 11 out of 19 ovaries (57.89%). Presence of un-typed mycoplasma species may refer to the synergistic situation between the field strains of MG and other types of class Mollicutes. The existence of field MG strains that colonize the upper respiratory tract leading to deciliation of trachea (**Lam, 2003**) and immunosuppression (**Gaunson et al., 2000 and Ganapathy and Bradbury, 2003**), consequently produced favorable media that favor the growth of other types of mycoplasmas.

The results of biochemical and serological identification of the isolated mycoplasma strains collected from broiler chicken flocks were tabulated in table (9). It could be observed that MG strains were isolated in percentages of (57.14%) 20/35, (58.0%) 18/31 and (52.63%) 10/19 from tracheal swabs, lungs and air-sacs; respectively. Similar results were proved by **Usama (2008)** who isolated MG from 148 out of 255 (58%) from tracheal swabs of 4 week-old broiler chickens. **El-Shater (1986)** also reported on isolation of MG from the respiratory organs with percentage of (16.7%), while **Sokkar et al., (1986)** revealed that 84% of the examined trachea, lungs and air-sacs of broilers were MG. Furthermore, **Eissa et al., (2000) and Dardeer et al., (2006)** recovered MG in percentages of 66.6% and 63.49%; respectively from the examined chicken's respiratory organs.

The growth inhibition test showed positive parallel results with the biochemical test and yielded un-typed mycoplasma species in 15/35 (42.85%), 13/31 (41.93%) and 9/19 (47.36%) cultures collected from tracheal swabs, lungs and air-sacs, respectively. This result is in agreement with that reported by **Mansour (1995)** who isolated other types of mycoplasma and un-typed ones from the respiratory tract of broiler chickens from different Egyptian Governorates.

The results of isolation, biochemical and serological identification indicated that MG is the predominant isolate in both breeder and broiler chicken flocks.

The difference between the recovery rate and the serodiagnosis results of MG could be explained from the fact that the rate of infection and its spreading is different from season to season,

the farm density and also differ from farm to another (construction and management). Those three items were differing also from locality to another as described by **Ley et al., (1997)**.

In practice, control programmes for MG infections have been adopted and are mainly based on serological and/or bacteriological testing. However, confirmation of infection by conventional culture procedures is time consuming (require 2 weeks), laborious, expensive and required sterile conditions and personal skills (**Hirsh and Zee, 1999**) and confusion surrounding serological tests has been encountered (**Perrin and Bennejean, 1982**). Therefore, the amplification of DNA of MG in the laboratory using PCR has been performed as very sensitive, specific and rapid method (requires less than 24 h) for detection and identification of the organism (**Khan and Kleven, 1993; Marios et al., 2002 and Mcauliffe et al., 2003**).

The molecular characterization using (PCR) of the biochemically and serologically identified MG strains of the breeder and broiler chicken flocks was shown in figure (2). The electrophoresis of the PCR products of amplified 16 SrRNA gene of MG from the tested positive cultures revealed products with a characteristic and specific band at 183 base pair (bp) for all isolated field positive strains and the reference vaccinal (F) strain.

The results of PCR examination of the positive culture isolates revealed the agreement percentage (100%) between the PCR results and the conventional cultural methods for detection of MG in the breeders and broiler chicken flocks. Detection of MG using PCR was primarily developed by **Nascimento et al., (1991 and 1993)**, then it was accepted world wide for rapid detection of all avian mycoplasma species (**Lauerman, 1998 and Nascimento et al., 1998**). In addition, **Marios et al., (2001); Hong et al., (2005); Mettifogo et al., (2006) and Pakpinyo and Sasipreeyajan (2007)** concluded that interspecies identification (DNA fingerprinting) of pathogenic avian mycoplasmas is a powerful tool for epidemiological studies and monitoring of strain identity.

Live (F) strain of MG was used extensively in the field as a vaccinal strain to provide some protection against the losses caused by MG like drop in egg production (**Branton, 1988**) and air-sacculitis (**Levisohn and Dykstra, 1987**). Nevertheless, some disadvantages were appear as a result of using of such vaccine as it could be transmitted through the egg from the infected hens (**Evans and Hafez, 1992**), also the (F) strain vaccinated flocks maintained the mycoplasma organisms in the upper respiratory tract for the life of the flock (**Kleven, 1981**). So, the intensity used of the vaccination in layer and breeder flock emerging the use of test helpful in diagnosis of

avian mycoplasmosis and differentiation between field infection with MG and vaccinal strains. The common serological tests like SPA, haemagglutination inhibition (HI) test and enzyme linked immunosorbent assay (ELISA) can't differentiate between the field and the vaccinal MG strains (**Geary et al., 1994**).

Although 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.

Table (10) elucidated the inhibitory zones of the tested antibiotics against ten different MG field strains. Pefloxacin was the most effective antibiotic (53.1 mm), followed by spiramycin (50.4 mm), enrofloxacin (45.3 mm) and tylosin tartarate (40.6 mm), while doxycycline (36.8 mm), streptomycin (34.5 mm) and tiamutin (30.9 mm) were less effective. This result revealed that pefloxacin followed by spiramycin, enrofloxacin and tylosin tartarate were highly active *in vitro* for inhibition of MG growth. Flouroquinolones group was established and possessed strong anti-mycoplasma activity than other groups (**Hannan et al., 1989; Arai et al., 1992; Megaki et al., 1993; Ortiz et al., 1995; Kempf et al., 1998 and Stanley et al., 2001**), consequently treatment of MG infection in birds using this group is recommendable. In addition, the highest *in vitro* sensitivity of MG isolates to ofloxacin, spiramycin and tylosin was reported by **Levisohn (1981) and Lin (2006)**. On the other hand, the decreased activity of doxycycline, streptomycin and tiamutin against MG isolates may denote the irrational application of these antibiotics for long periods which lead to establishment of MG resistant strains. **Zanella et al., (1998) and Gautier-Bouchardon et al., (2002)** recorded that frequent administration of antibiotics (2-6 passages) leading to development of new MG resistant strains.

In conclusion, SPA test can be used as an initial screening test for serological diagnosis of MG infection. MG is the predominant mycoplasma species of chickens that cause respiratory problem in the different Egyptian Governorates (Giza, Cairo and Alexandria). Conventional cultural method is still remained the gold standard for diagnosis of MG although it is slow and time consuming. Although DNA fingerprinting (PCR) technique is more specific and more rapid than the traditional culture procedures, but it should be done parallel with the traditional method of isolation of MG. Periodic *in vitro* antibiotic sensitivity testing of mycoplasma field isolates is

required as a mean of monitoring the impact of mass medication programmes and as a guide to therapy.

Table (1): List of antimicrobials used in the antibiogram:

The active principle	Conc./disc	The produced company
Spiramycin	100 µg	Oxoid
Enrofloxacin	5 µg	Oxoid
Pefloxacin	10 µg	Oxoid
Tylosin tartarate	100 µg	Elanco
Doxycycline	30 µg	Pasteur lab
Sterptomycin	10 µg	Pasteur lab
Tiamutin	100 µg	Novartis

Table (2): Serological examination of serum samples collected from different broiler and layer breeder chicken flocks using serum plate agglutination test.

	No. of tested birds	Age/week	Serum plate agglutination	
			Positive MG	%
	30	20	16/30	53.33
	30	26	22/30	73.33
	35	30	29/35	82.85
	45	35	35/45	77.78
	50	46	46/50	92.0
	60	50	54/60	90.0
	55	55	51/55	92.72
Total	305		253/305	82.95

Table (3): Serological examination of serum samples collected from day-old and broiler chicken flocks using serum plate agglutination test.

	No. of tested birds	Age	Serum plate agglutination	
			Positive MG	%
	40	Day-old	4/40	10
	30	4 weeks	26/30	86.67
Total	70		30/70	42.85

Table (4): Primary cultural isolation of mycoplasma from different broiler and layer breeder chicken flocks.

Site of isolation	Age of birds/ weeks							Total No. of samples	Positive suspected isolates	% of positive
	20	26	30	35	46	50	55			
Trachealswabs	20	15	35	20	30	45	15	180	145/180	80.56
Nasal swabs	15	20	20	15	25	35	35	165	130/165	78.78
Lungs	9	7	5	10	12	17	10	70	59/70	84.28
Air-sacs	4	6	6	7	9	10	13	55	43/55	78.18
Ovaries	3	2	5	4	7	12	7	40	28/40	70
Total								510	405/510	79.41

Table (5): Differentiation between mycoplasmas and acholeplasmas isolates collected from broiler and layer breeder chicken flocks using digitonin test.

Site of isolation	No. of positive isolates	Digitonin sensitivity			
		Positive (Mycoplasmas)	%	Negative (Acholeplasmas)	%
Trachealswabs	145	105	72.41	40	27.58
Nasal swabs	130	110	84.61	20	15.38
Lungs	59	45	76.27	14	23.72
Air-sacs	43	38	88.37	5	11.62
Ovaries	28	19	67.85	9	32.14
Total	405	317/405	78.27	88/405	21.72

Table(6):Primary cultural isolation of mycoplasma from day-old and broilerchicken flocks.

Site of isolation	Age of birds		Total No. of samples	Positive suspected isolates	% of positive
	Day-old	4 weeks			
Trachealswabs	15	45	60	45/60	75
Lungs	10	35	45	35/45	77.77
Air-sacs	5	25	30	25/30	83.33
Total			135	105/135	77.77

Table (7): Differentiation between mycoplasmas and acholeplasmas isolates collected from broiler chicken flocks using digitonin test.

Site of isolation	No. of positive isolates	Digitonin sensitivity			
		Positive (Mycoplasmas)	%	Negative (Acholeplasmas)	%
Trachealswabs	45	35	77.8	10	22.2
Lungs	35	31	88.57	4	11.42
Air-sacs	25	19	76	6	24
Total	105	85/105	80.95	20/105	19.04

Table (8): Biochemical and serological identification of the isolated mycoplasma strains collected from different broiler and layer breeder chicken flocks.

Site of isolation	Examined No.	Biochemical identification		Serological identification	%
		G	A H	G I	
Trachealswabs	105	43	-	43 MG	40.95 MG
				62 un-typed mycoplasma	59.0 un-typed mycoplasma
Nasal swabs	110	52	-	52 MG	47.27 MG
				58 un-typed mycoplasma	52.72 un-typed mycoplasma
Lungs	45	21	-	21 MG	46.66 MG
				24 un-typed mycoplasma	53.33 un-typed mycoplasma
Air-sacs	38	20	-	20 MG	52.63 MG
				18 un-typed mycoplasma	47.36 un-typed mycoplasma
Ovaries	19	8	-	8 MG	42.10 MG
				11 un-typed mycoplasma	57.89 un-typed mycoplasma

G: Glucose fermentation A H: Arginine hydrolysis G I: Growth inhibition -: Negative

Table (9): Biochemical and serological identification of the isolated mycoplasma strains collected from different broiler chicken flocks.

Site of isolation	Examined No.	Biochemical identification		Serological identification	%
		G	A H	G I	
Trachealswabs	35	20	-	20 MG	57.14 MG
				15 un-typed mycoplasma	42.85 un-typed mycoplasma
Lungs	31	18	-	18 MG	58.0 MG
				13 un-typed mycoplasma	41.93 un-typed mycoplasma
Air-sacs	19	10	-	10 MG	52.63 MG
				9 un-typed mycoplasma	47.36 un-typed mycoplasma

G: Glucose fermentation A H: Arginine hydrolysis G I: Growth inhibition -: Negative

Table (10): The inhibitory zones (in millimeters) of the tested antimicrobials against *Mycoplasma gallisepticum* field isolates (n=8)

The active principle	Inhibitory zone
Spiramycin	50.4
Enrofloxacin	45.3
Pefloxacin	53.1
Tylosin tartarate	40.6
Doxycycline	36.8
Streptomycin	34.5
Tiamutin	30.9

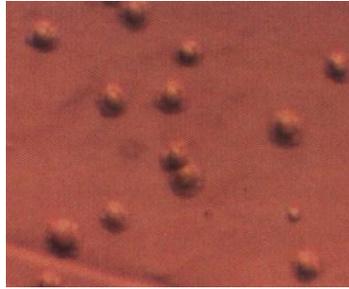


Fig. (1): The identified colonies of MG on PPLO agar showing fried egg colonies.

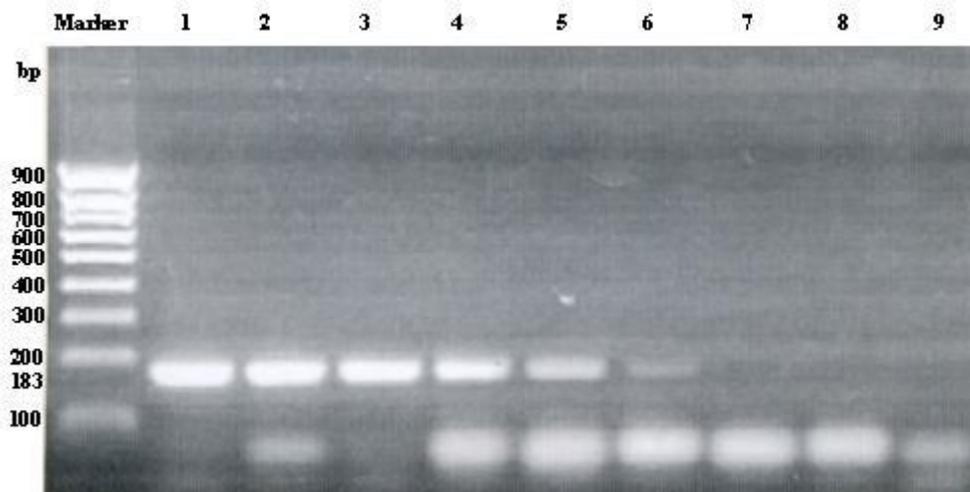


Fig. (2): The PCR product of the amplified fragment 16 SrRNA of isolated MG at (183) bp

Lane (1): MG vaccinal (F) reference strain.

Lane (2): MG strain from the tracheal swabs of breeder chickens.

Lane (3): MG strain from the nasal swabs of breeder chickens.

Lane (4): MG strain from the lungs of breeder chickens.

Lane (5): MG strain from the air-sacs of breeder chickens.

Lane (6): MG strain from the ovaries of breeder chickens.

Lane (7): MG strain from tracheal swabs of broiler chickens.

Lane (8): MG strain from lungs of broiler chickens.

Lane (9): MG strain from air-sacs of broiler chickens.

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