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The Recovery and Molecular Diagnosis of *Mycoplasma gallisepticum* Infection in Commercial Poultry Flocks in Egypt

Mahmoud Hossam[1], Armanious Wagih[1], Elenbawy Mona[1], Elhariri Mahmoud[1], Elhelw Rehab[1]* and El-Din Taher Salah[2]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt; hossameldinmahmoud@cu.edu.eg, warmanious@yahoo.com, dr_mona_1@hotmail.com, mahmoud_elhariri@cu.edu.eg, rehab.elhelw@cu.edu.eg
[2]Nanotechnology Center, Regional Center of Feed and Food, Agriculture Research Center

Abstract

Objectives: The aim of this study to investigate *MG* in field cases of chronic respiratory disease (CRD) and its tissue distribution in positive cases**. Methods**: Polymerase Chain Reaction by different approaches was used in comparison to the culture method. The study included 385 birds, from which (1,155) samples of trachea, lung tissue and air sacs were collected from different farms of Layers, broilers and Breeders flocks located in seven governorates. **Findings**: Three detection methods of *MG* revealed that, the highest recovery rate of *MG* was achieved by PCR on 48 hours incubation PPLO broth (70.9%), followed by direct PCR on infected tissue (65.45%). The lowest recovery rate was for conventional cultivation method (17.66%) indicating that PCR is more sensitive. On the other hand tissue distribution pattern of *MG* showed higher percentage from trachea followed by air sacs then lung. **Application:** The PCR is superior to culture as it is rapid and sensitive for diagnosis of *MG*, which is compatible with surveillance and control programs of *MG*. Regarding tissue distribution pattern the trachea has a more privilege during diagnosis of *MG* infection.

Keywords: Egypt, Molecular Diagnosis, Mycoplasma gallisepticum, Poultry, Recovery

1. Introduction

Mycoplasma gallisepticum (*MG*) infections are commonly known as "chronic respiratory disease" (CRD) of chickens. *M. gallisepticum* diseases are characterized by respiratory rales, coughing, nasal discharge, and Conjunctivitis and it is the most pathogenic and economically significant mycoplasmal pathogen of poultry. Airsacculitis in chickens resulting from *MG* infections, with or without complicating pathogens, causes increased condemnations at processing[1].

Economic losses from condemnations or downgrading of carcasses, chick mortality, poor feed conversion, drop of egg production, increased medication costs, expensive controlling and investigation program and rapid transmission through eggs or by air borne droplets, along with arthritis, salpingitis, conjunctivitis and fatal encephalopathy. All are the criteria behind the fact that make this disease, one of the costliest disease problems facing commercial poultry production worldwide[2-7].

The gold standard investigation for *MG* diagnosis is isolation and identification of the organism through cultivation followed by morphological, biochemical, serological and molecular confirmation[8]. However, Cultivation techniques are laborious, high coast and require sterile condition and chances of false positive and false negative results are possible while confirming the isolates[9,10]. As *MG* is a slow-growing organism in culture test, the detection of *MG* requires one or more weeks for culture growth and diagnosis in chicken[11]. As well, for

*Author for correspondence

serological testing it needs at least of 1 week after infection for antibodies production[12]. Disadvantage of which fall in false positive and false negative results due to non-specific reactions[10,13,14]. While PCR test can be carried out on clinical samples without the need of cultivation, therefore, depending on PCR for direct diagnosis from tissue may replace the conventional method. It is more accurate, rapid and efficient for early identification[15]. Its high sensitivity and rapidity making it the most frequently used technique to monitor *MG* infection[16].

The aim of this study was focusing in comparing different approaches of PCR for detection of *MG* in comparison to culture method for *MG* diagnosis.

2. Materials and Methods

Table 1. Number of collected samples and its distribution according to governorates and flocks production type

Governorate	Layer	Broiler	Breeder	Total
Qalyubia	10	30	10	50
Sharqia	15	25	15	55
Gharbia	15	35	10	60
Monufia	20	30	15	65
Giza	20	35	15	70
Faiyum	10	25	10	45
Minya	10	20	10	40
Total	100	200	85	385

2. 1 Sampling and Sample Preparation

Samples were collected from broiler, layer and breeder flocks of different age ranges (from 5 to 51 weeks). These flocks were with history of chronic respiratory disease. The health status was evaluated depending on clinical symptom and necropsy, the diseased flocks showed a drop in egg production, low hatchability, low performance and increased mortality, accompanied with tracheitis, mild airsacculitis and salpingitis were seen on necropsy.

The samples were collected from birds after post mortem examination and detection of *MG* gross lesions **Figure 1**. Trachea, air sacs and lung were collected and divided into two parts, one for immediate inoculation on PPLO broth. The other was preserved for DNA extraction and directs PCR. Samples were transported to laboratory of Microbiology department, Faculty of veterinary medicine, Cairo University in a refrigerated ice tank. Samples (n=385 birds) were collected from different commercial farms in seven Egyptian governorates shown in Table 1.

2.2 Culture Method

Tubes of PPLO broth inoculated with samples were incubated in humid and microaerophilic condition at 37°C for 24-48 hr. Positive tubes were subcultured on PPLO agar plates, incubated and examined for up to 21 days under the same condition from temperature and Oxygen[17]. Plates were examined under inverted microscope for characteristic fried egg colony. Suspected *MG* isolates were processed for DNA extraction and PCR.

2.3 DNA extraction

DNA extraction was carried out from both positive suspected fried egg colonies and from 48 h incubation PPLO Broth. From suspected plate, block of agar was transferred onto PPLO broth overnight. from PPLO broth one ml of cultures was transferred into microtubes and was centrifuged at 14000 rpm 20 min. supernatants were discarded but precipitate were collected. 100 ml lysis buffer was added to per microtubes and mixed. This suspension incubated at 56°C for 4 hours. Microtubes were centrifuged at 13000 rpm for 15 min. Following centrifuge, two phases was observed in microtubes. Upper phase was included total DNA. This phase was transferred into new microtube. Equal volume of Phenol/chloroform were added to microtubes, voterxed and centrifuged at 13000 rpm for 15 min. upper phase was transferred into new microtube, added chloroform and centrifuged for 5 min. 0.1 time of upper phase was added Sodium acetate 3 M. after mix, cold ethanol was added 2 time of volume in microtube. Microtubes were incubated at -20°C for 20 min and centrifuged at 13000 rpm for 15 min. washing step was performed on precipitate. For ensuring the success of DNA extraction process, the DNA suspension was run through agarose gel electrophoresis to visualize the presence or absence of any DNA bands. Total DNA of isolates were stored in -70°C after drying in 56°C.

2. 4 Direct PCR on Tissue

Direct PCR on Trachea, air sacs and Lung was performed using (**Phire Animal Tissue direct PCR Kit**) as per manufacturer instruction. Briefly puncher was used to obtain uniform and small (0.4mm) sample. Then samples were place directly into the PCR reaction (50 µL of volume). Tissue samples were placed into 20 µL of Dilution Buffer. Tube was mixed by briefly vortexing and spun down

the solution. Reaction was incubated for 2–5 minutes at room temperature and then placed into the preheated (98 °C) block for 2 minutes. The remaining tissue was spun down and 1 µL the supernatant was sufficient for a 20 µL PCR reaction. The reaction components were as the following, per 50 µL volume, 2.5 µL from the sample, 1 µL forward primer, 1 µL reverse primer, 12.5 µL Phire animal tissue PCR buffer and distilled water up to 50 µL. The amplification condition initiated with initial denaturation at 98 for 5 minute for one cycle, followed by 40 cycles of cyclic denaturation, annealing and extension (98/5 sec and 72C/20 sec), followed by on cycle form final extension at (72 C/ 1 minute).

2. 5 DNA Amplification

The DNA was used as template in PCR assay. For detection of *MG*, specific primer pair was used to amplify 16S rRNA gene (185 bp.), Forward primer*MG*-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3' Reverse *MG*-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'. Using a set of dedicated pipetters the reaction mixture was prepared in a separate clean area. For one 50 µl PCR reaction the mixture was as follows: H_2O Ultra-pure 35.75 µl 10 × PCR Buffer 5.00 µl dNTP (l0 mM) 1.00 µl F Primer (20 pmole/µl) 0.50 µl R Primer (20 pmole/µl) 0.50 µl Taq (5 U/µl) 0.25 µl *MG* Cl_2 (50 mM) 2.00 µl A 45 µl volume of the reaction mixture was dispensed into PCR tubes in the thermocyler (Esco-swift minipro) which is equipped with a heated lid. The tubes were then taken to another clean area where the appropriate DNA sample (5 µl) was added to each tube. In each run positive and negative controls should be used. The tubes were then placed in a thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and then soaked at 4°C.

2. 6 Electrophoresis

PCR products are detected by 1.2 % Agarose gel electrophoresis, incorporating marker. Electrophoretic tank containing 1X TAE running buffer, after 40 minutes at 100 volts, Gels were observed under UV light and image was taken by gel documentation system. The PCR product for *MG* is 185 bp.

3. Results

Post-Mortem Examination

There was a serious involvement of trachea, lungs, air sacs, also heart and liver Catarrhal exudates present in nasal passages and frothy exudates. The trachea showed the evidence of congestion and haemorrhages Figure 1.

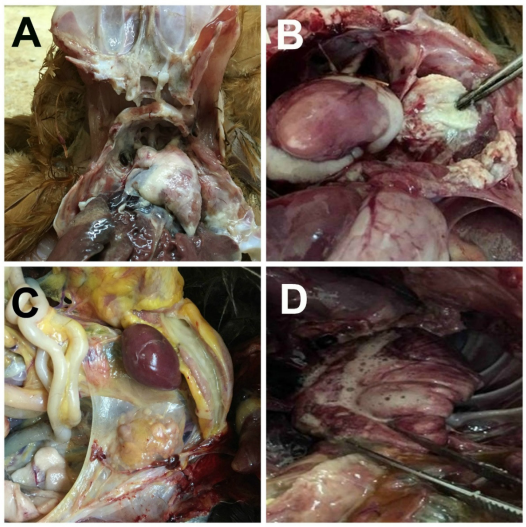

Figure 1. Heart covered with fibrinopurulent covering, while liver was congested and perihepatitis. B and C: Caseousmaterial were found in the bronchi and pneumonic areas in the lungs were observed. Air sacculitis was observed in air sacs which are covered with caseous exudates complicated infections. D: Lungs were dark red color appearance and showed congestion, Frothy exudate was present in some cases.

Table 2. The recovery percentage of *MG* from examined samples from different flocks according culture method

Flocks Type	Samples No.	Before PCR confirmation		After PCR confirmation	
		Number	%	Number	%
Layers	100	46	46	21	21
Broilers	200	125	62.5	26	13
Breeders	85	49	57.6	21	24.7
Total	285	220	57.14	68	17.66

Table 3. The recovery percentage of *MG* from examined samples from different flocks according to PCR

Flocks Type	**Samples No.**	**PCR from 48-hrs incubation PPLO broth**		**Direct PCRon infected tissue**	
		Number	**%**	**Number**	**%**
Layers	100	67	67	57	57
Broilers	200	140	70	137	68.5

Breeders	85	66	77.6	58	68.24
Total	385	273	70.9	252	65.45

Table 4. Tissue distribution pattern by culturing of *MG* from different tissues samples confirmed by PCR

	Trachea	Air sacs	Lung
Total	28	17	11
Percentage out of 86 isolates obtained by culture method	41.18	25	16.17

Table 5. Tissue distribution pattern by PCR from 48-hrs incubation PPLO broth

	Trachea	Air sacs	Lung
Total	222	168	129
Percentage out of 273 positive result obtained by PCR on 48 h incubation PPLO	81.32	61.54	47.25

Three hundred eighty five flocks with history of chronic respiratory disease (CRD) were examined for *MG* infection; it's comprised 85 breeders, 100 layers and 200 broilers flock were distributed over seven Egyptian governorates Table 1. Different approaches were used for detection and diagnosis of *MG* infection. Depending on culture methods; 220 mycoplasma suspect colonies were detected. Overall the recovery rate of *MG* by culture was varied depending on PCR confirmation. The *16SrRNA* PCR method is commonly used for confirmation of *MG*. The recovery rate determined through PCR was 13% in broilers, 24.7% in breeders and 21% in layers Table 2. These percentages of recovery were 46, 62.5, 57.6, and 57.6% for layer, broiler and breeder respectively before confirmation by PCR on 220 *MG* suspected colonies Table2.

The Polymerase chain reaction test was functionalized into two approaches either by direct PCR on tissues or on 48-hrs incubation PPLO broth. The percentages from 48-hrs incubation PPLO were 67, 70, 77.64 % while from direct PCR; the results were 57, 68.24, and 68.24 for layer, broiler and breeder respectively Table 3.

The tissue distribution for *MG* to some extent varied between different respiratory tissues. The highest percentage of isolation were from trachea followed by air sacs, then lowest percentage for lung Table 4&5.

4. Discusion

Sensitive and accurate detection of the pathogenic *MG* strains plays an important role in the control of one of the major causes of CRD and CCRD outbreaks in poultry floks. Different techniques of detection and diagnosis may be used, including: isolation and cultivation, serological, and molecular assays.

Furthermore, serological methods for *MG* diagnosis depend in the first place on immune responses to antigens and the subsequent detection of the produced antibodies. Since seroconversion lags behind infection, it takes a minimum of 1 week after infection before detectable antibodies are produced to give a result in agglutination test. While it can take up to 3 weeks to conduct the hemagglutination inhibition test[17]. So, serological tests cannot be used for the detection of early infections.

PCR is a serviceable tool in the accurate diagnosis of *Mycoplasma* infections, not only for its sensitivity but also for its high specificity[17]. This technique overcome culture method as it depends on the direct detection of the microrganism's DNA without the need for cultivation. PCR has allowed the study of microbial genes, directly amplified from samples. It is a sensitive, easy, rapid and inexpensive technique, and the most important advantage is eliminating the need for isolation of *MG*. In this study the difference in recovery percentages between conventional *MG* cultivation and molecular methods was obvious.

There is a significant difference between the positive results obtained by isolation than that obtained by PCR[18].

The percentages of recovery by cultivation methods before PCR confirmation were 46, 62.5 and 57.6 % for layer, broiler and breeder respectively with a total percentage 57.14% (220/385) Table 2. However these results were greatly reduced after confirmation by PCR to 21% (21/100), 13% (26/200) and 24.7 (21/85) respectively, with a total occurrence of 17.66% (68/385)[19], showed a result of 27.3 % overall. As the cultivation techniques in the present study was completely dependent upon on fried egg colonies with lack of differentiation of other colony characteristics, there was a great chance for confusion between *Mycoplasma gallisepticum* and other species from one side and L form bacteria from other side[20]. PCR could easily pick up *Mycoplasma gallisepticum* through targeting 16SrRNA specific sequence. Due to the drawbacks of conventional cultivation techniques, the study applied PCR on two different condition, the first con-

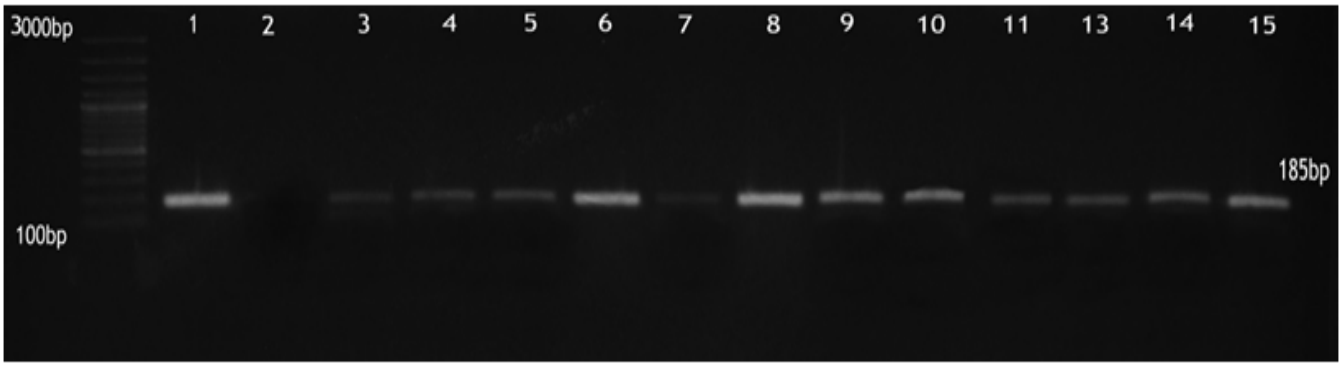

Figure 2. The electorophoretic profile of positive MG samples by 16srRNA PCR showing amplicon of 185 bp. Lane 1: DNA Ladder; Lane 2: Positive control (F strain) lane 3: negative control: E. coli. Lane 4,5 are positive from broiler farms. Lane 6,7,8,9 are positive from layer farms. 10,11,12,13,14,15 are positive from breeder farms.

dition was directly on the tissue while the other was on tissue inoculated PPLO broth after 48 hours incubation. The results obtained by direct PCR were 57% (57/100), 68.5% (137/200) and 68.24% (58/85) in layer, broiler and breeder respectively with a total rate of 65.45% (252/385). *MG* and other avian pathogenic *Mycoplasma* species are fastidious and slow-growing microrganisms that require one or more weeks for growth and bacterial identification[21]. Their isolation is often impaired by the overgrowth of non-pathogenic *Mycoplasma* species or other faster growing bacteria and fungi[21].

While[19], obtained a result of 49.74 % overall. Although Culturing of *Mycoplasma* is a gold standard technique but it could not isolate organism from chronic cases and medicated birds as *MG* concentration low in those conditions[22]. The difference between conventional cultivation technique and direct PCR which was shown in the current study is due to many reasons. **Firstly**, the sample size, during the acute stages of infection (generally in the first 4–8 weeks post-infection), the number of *MG* in the upper respiratory tract and the infection prevalence in the flock are high[23-25]. Therefore, swabbing tracheas or choanal clefts of 10–20 live birds is usually sufficient to recover the organism, whereas 30–100 cultures may be required at later stages. [21]**Secondly**, the stage of infection as tracheal or choanal numbers of organisms in chronically infected birds, as in commercial egg layers or backyard poultry, may be so low that *MG* organism may not be detected by sampling and culture methods. [18]**Thirdly**, the antimicrobial therapy, to optimize the possibility of isolation, flocks should be sampled for *MG* culture prior to initiation of antimicrobial therapy[26]. **Fourthly**, Ammonium chloride and perhaps other drinking water treatments may hinder the isolation of *MG* from infected birds[27]. **Fifthly**, overgrowth by other bacteria in the sample and the presence of non-cultivable or non-viable pathogens[28]. Viable organism needed for success in isolation, alternatively, PCR detect the nucleic acid of *MG* even from medicated birds and frequent than culture, higher percentage of positive samples is obtained by PCR (60.6 %)[29]. The result obtained by applying PCR on 48 hours incubation PPLO broth was of a little bit higher rate. They were 67% (67/100), 70% (140/200) and 77.64% (66/85) for layer, broiler and breeder respectively, with a collective rate of 70.9% (273/385). These results contradict with the study which declared that Direct PCR on tissue permits the detection of femtogram amounts of *Mycoplasma* DNA, corresponding to one bacterial cell[30]. So according to them, it is supposed that this step – PCR on PPLO broth - showed no difference. But this might be due to some multiplication occurred in the *MG* present which improved the detection limit of PCR, Moreover, more reduction of PCR inhibitor from PPLO broth than from tissue. Some studies recorded result of isolation from different production system in a rate of 31.3% of broiler, 38.1 of layer and 14.3% of breeder[16]. While other studies showed a greatly different isolation rate of 4.9% of broilers 33.3 per cent of layers, and 30.5 per cent of broiler breeder[28]. PCR is preferred for early detection of Mycoplasma from infected broiler[31]. Also the presence of *MG* was confirmed along with other respiratory pathogen through multiplex PCR[32]. The molecular diagnostic technique was more rapid and efficient for early detection of *MG* that conventional cultivation techniques[15]. The other face of this study is to show the tissue distribution pattern of *MG* in chicken. This was performed through conventional cultivation technique and from 48 hours incubation PPLO broth. The pattern from conventional cultivation

technique was 41.18% (28/68) from trachea, 25% (17/68) from air sacs and 16.17% (11/68) from lung. These results are supported by the result of[15] which capture *MG* from trachea 39.28%, air sacs 27.34% and lung 15.92%. While[19] had results of 15.9% from trachea, 25% from air sacs and 27% from lung. Distribution pattern from 48 hours incubation PPO broth was 81.31% (222/273) from trachea, 61.54% (168/273) from air sacs and 47.25% (129/273). These results are substantiated with the result of[15] which got an isolation rate of 80.5% from trachea, 67.25% from air sacs and 54.86% from lung. While results illustrated with [19]were 42.47% from trachea, 50.5% from air sacs and 31.85% from lung. The highest rate from trachea might be due to that it is the first location along respiratory air ways which is prone to infectious agent[33]. But there was no great difference between percent occurrence of *MG* in different organ, and this in agreement with[15]. PCR based nucleic acid detection is considered as an alternative method to that of conventional isolation technique[34-39]. Eventually PCR confirmed 273 *MG* from 385 field samples collected during the study. PCR is a good technique for confirmation of *MG* from infected birds[39-47]. The PCR and other several pre-existing techniques, has contributed immensely in disease diagnosis[50]. PCR has been used widely in the clinical diagnostic and environmental monitoring[51]. Outbreaks continued to emerge despite the application of F-strain vaccination programs in breeder flocks. Moreover, outbreaks continued even with heightened biosecurity measures. As the costs of disease to the broiler industry are enormous[52] this flock surveillance for *MG* detection and rapid investigation into molecular detection of circulating *MG* strains[48]. It requires very small amount of material which is a major advantage when working with field collected material[53]. High prevalence of *MG* in these poultry flocks may be due to its horizontal transmission from the infected chickens, eggs, wild birds, vehicles or fomites to the healthy susceptible chicken flocks[49]. Moreover, inadequate ventilation, contaminated litter, frequent movement of rodents, wild birds, pets, professionals, visitors amounting to poor biosecurity.

The current study aimed to make a reliable comparison between three diagnostic ways toestablishthe most convenient, accurate and sensitive method for capturing the infected field cases measures. It is concluded that *MG* is prevailing in Egypt.

5. Conclusion

PCR assay for detection of *MG* infection in field is a good and rapid technique for diagnosis. The prevalence of *MG* is a real threat especially in the winter season, resulting in high economic losses. The tissue distribution pattern always including the trachea as the highest exposed organ for infection.

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