



## Evaluation of Isolation and Polymerase Chain Reaction in Diagnosis of *Mycoplasma Gallisepticum* in Broiler Chickens in Kirkuk Governorate, Iraq

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**T**HE objective of this study was to evaluate isolation, and evaluate the polymerase chain reaction (PCR) technique to confirm diagnosis of *Mycoplasma gallisepticum* (MG) in broiler chickens. One of the finest independent organisms is MG, can be reproduced autonomously, the lack of a cell wall, allowed it to take on various shapes and sizes, and to resist cell-wall targeting antibiotics. When MG infect chickens it caused chronic respiratory disease (CRD), characterized by rales, sneezing, coughing, nasal discharges, dyspnea, conjunctivitis. Decreased feed intake, feed conversion, an increase in mortality, carcass damage and medication costs, causing high economic losses. Diagnosing the cause is the first step in treatment, for evaluation isolation and direct PCR a total of 180 tracheal swabs were collected from broiler chickens (28-40) days old who had symptoms of CRD, during the period (1/12/2022-28/2/2023). Prevalence of MG by, isolation and direct PCR was 30.5% (55/180) and 32.77% (57/180) respectively. The sensitivity and specificity of direct PCR were 100% and 96.8% respectively. When comparing culturing with PCR, the study found that the sensitivity and specificity were 93% and 100% respectively. The study concluded that culturing is still the golden standard test for MG detection for its high sensitivity and specificity but takes a long time, direct PCR is very fast and efficient.

**Keywords:** *Mycoplasma gallisepticum*, broiler, CRD, Isolation, PCR.

### Introduction

*Mycoplasma gallisepticum* is a member of *Mycoplasma* genus and belongs to the Mollicutes class, which means "soft skin" in Latina, the simplest self-proliferating microbe, phylogenetically related low G+C (Gram+) bacteria, they are cell wallless microorganisms with genome size ranging from 500-1500 kilobase pairs (kbp) [1,2]. Representatives of the Mollicutes class are immune to most antimicrobial drugs, including beta-lactam antibiotics, glycopeptides and fosfomycin, since they lack cell walls [3,4]. MG is capable of taking on variety of forms. Because of this, it is challenging to recognize them, even with electron microscope [5]. *Mycoplasma* infection can spread horizontally or vertically [6] and can affect turkeys and chickens at all ages, but young birds are more susceptible [7],

The causative agent of 'CRD' in poultry is MG. Infected birds might exhibit rales, coughing, sneezing, nasal discharge, and bulging infraorbital sinuses as clinical signs. Reduce of; egg production, hatchability, feed efficiency, and weight. Rise in; mortality, healthcare cost [8, 9], bird carcasses with low-quality [10]. *Mycoplasma* costs the poultry industry a lot of money because it causes a decrease in production by 10-20% and an increase in embryo mortality by 5-10%, add to that control and prevention costs [9, 11]. Among the pathogenic mycoplasmas in poultry, the International Office of Animal Epidemiology (OIE) considered MG as a must-notifiable pathogen due to its dangerous [12]. Therefore, early detection and control of disease outbreaks can be greatly aided by the rapid and effective detection of avian pathogenic mycoplasmas [13]. Because mycoplasmas infect the respiratory

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tract, it causes typical symptoms including nasal discharge, coughing, and lesions in the air sac, however in some cases, no clinical signs appear [14], also a variety of respiratory illness (including Newcastle disease, infectious bronchitis, *Escherichia coli*) attack the respiratory organs and often create same symptoms to CRD, so when diagnosing MG, clinical signs cannot be relied upon [47]. Therefore, in most cases, conventional bacteriological culture, serology, and molecular methods are used to diagnose MG infection [13]. Difficulties with the serologic tests used in diagnosing mycoplasmas lie in the defects associated with interpreting the results [15]. PCR is a very sensitive technology capable of producing billions of copies of a specific segment of DNA for cloning, sequencing, and analysis [16]. It has high specificity and sensitivity facilitating MG detection even in clinical samples taken from animals that are asymptomatic, or receiving antibiotic therapy [17], but it requires specialized laboratory facilities, an experienced workforce, and the costly nature of detecting and screening for pathogen, which limits its use in conventional laboratories, especially in the developing world [18]. The gold standard test for MG diagnosis is isolation [19], but it is expensive, laborious, and time-consuming, other bacteria including non-pathogenic *Mycoplasma* may contaminate it, and often *Mycoplasma* does not grow on the ordinary media [20]. Therefore, this research was done to evaluate the bacterial isolation test using the PPLO culture medium, and compare with the use of direct PCR test for the detection of MG in chickens in Kirkuk Governorate in Iraq.

## **Material and Methods**

### **Ethical approval and sampling**

Chicken handling and sample collection were carried out after the approval of the field owners, and the utmost safety measures were taken to prevent any possible complications while being careful to follow the instructions of the Animal Care Committee in Kirkuk Governorate. To ensure that the birds are not exposed to stress. The study was conducted on 180 of 9000 broiler chickens, type Ross 308, in 3 small fields (60 sample of each) west of Kirkuk Governorate, during the period of three months from December 2022 to February 2023, the chicken ages between (28-40) days. In a non-random manner birds were selected who suffered from respiratory symptoms and were suspected to be infected by *Mycoplasma gallisepticum*. Tracheal swab sample

dipped in 4 ml of sterile *Mycoplasma*(PPLO) broth and transported to the Laboratory of veterinary medicine college, Tikrit University, incubated at 37°C ,5% CO<sub>2</sub> for 14 days (after 1 day of incubation to ensure *Mycoplasma* activation, 1ml of each culture tube was transferred to an Eppendorf tube bearing the same sample number and kept at -20°C until PCR was performed on it)[22].

### **Identification by Isolation**

*Mycoplasma* broth medium (PPLO broth) was prepared according to [21] and the instructions of the manufacturer (Solarbio/China), and poured 4 ml into a transport tube. The samples were inoculated in PPLO broth tubes and incubated at 37°C under (5-10)% of CO<sub>2</sub> for 14 days. When the color of the medium changes from red to yellow or orange, or turbidity formed, the sample is considered positive for *Mycoplasma* growth, while the samples are considered negative if they did not suffer a color change within a maximum period of 14 days, if the color changed before the fourth day it was discarded of contamination. The samples were stored after completing the culture test under freezing. (-20 °C) if there is a need to conduct other tests. *Mycoplasma* agar medium (PPLO agar) was prepared according to [21] and the manufacturer's instructions (Himedia/India). Seventy ml of the agar medium was poured into a Petri dish with a diameter of 8 cm, 25 microliters of positive culture sample were transferred to the solid medium using a sterile pipette. Culture dishes were placed inside the candle jar with a source of moisture and anaerobic conditions (the candle was fired), then the cover was closed tightly, and the dishes were checked daily after 3rd day, to ensure the growth of *Mycoplasma* using an anatomical microscope with 25X magnification to note the growth of *Mycoplasma* in colonies with the distinctive *Mycoplasma* colonies are very small, rounded, smooth and translucent having a "fried egg" appearance with a thick center mass, at the fifth day colony diameter is less than 0.3mm, after 21 days the samples are considered negative if no growth of *Mycoplasma* colonies appeared on them [22, 23]. Diene's stain is used to make colonies more clear.

### **Identification by PCR**

#### **DNA extraction**

Following the instructions of the American company, QIAGEN, which manufactures extraction kits- Tracheal swab samples were taken out of the

freezer to thaw and take room temperature- The samples were placed in a microcentrifuge and rotated at a speed of 7500 r/min for 10 minutes.- The supernatant was discarded and 180 microliters ( $\mu$ l) of ATL buffer was added to the sediment.- Twenty  $\mu$ l of proteinase K was added to the tube and the mixture was mixed for 15 seconds by vortex. - The samples were placed in a water bath (56 °C) for half an hour, with the samples being stirred every 5 minutes.- The tubes were transferred to a fine centrifuge and rotated lightly until there was no residue left on the cover.- Two hundred  $\mu$ l of AL buffer was added to each sample and mixed intermittently by vortex for 15 seconds, then transferred to the water bath (70 °C) for half an hour.- Two hundred  $\mu$ l of alcohol (96-100)% were added, and after intermittent mixing by vortex, the samples were lightly rotated by a microcentrifuge so that no materials attached on the cover.- The mixture was emptied into a QIAamp Mini spin column (installed on a 2ml collection tube), then closed the filter tube tightly and expelled at a speed of 8000 r/min for one minute.- The filter tube was extracted and fixed in a new collection tube and the old collection tube was discarded, then the cover of the filter tube was opened and 500  $\mu$ l of AW1 (washing buffer 1) was added to it, the filter tube cover was closed tightly and then expelled at a speed of 8000 r/min for one minute- The filter tube was removed and mounted on a new collection tube; the old one was discarded, and 500  $\mu$ l of AW2 (washing buffer 2) was then added to it, the cover was closed and rotated at a speed of 14000 r/min for 3 minutes.- The filter tube was transferred to a new collection tube, then rotated at a speed of 14,000 r/min for one minute, to ensure complete disposal of the washing buffers.- The filter tube was removed and fixed on an Eppendorf tube with a capacity of 1.5 ml, 200  $\mu$ l of elution buffer AE (elution buffer) was added to it, then closed and left for a minute at room temperature, then spun for a minute at a speed of 8000 r/min- After getting rid of the filter tube, the purity and concentration of the DNA were measured in the Eppendorf tubes using the NanoDrop spectrophotometer, then the samples were stored at -20 °C until use.

#### DNA amplification

With PCR technique For MG diagnosis, and based on the 16S rRNA sequence (Macrogen/Korea), *Mycoplasma* genus primer pair (F-5' GGGAGCAAACACGATAGATACCCT 3', R-5' TGCACCATCTGTCCTCTGTTACCCTC 3')(285

bp) [24] and MG specific primer pair (F-5'GAGCTAATCTGTAAAGTTGGTC 3', R-5'GCTTCCTTGGGGTTAGCAAC 3') (580bp) [25], the efficacy of these primers was confirmed by [26, 27]. The work was carried out according to the manufacturer's guidelines and by following [21]. After taking the necessary laboratory and work safety measures and preparing the materials necessary for the reaction, the reaction tubes (PCR Premix tube) with a capacity of 20  $\mu$ l manufactured by the Korean company Bioneer, containing a mixture, were numbered. For the reaction (DNA polymerase, dNTPs, reaction buffer), 1  $\mu$ l of the forward primer added and the same amount of the reverse primer, then 8  $\mu$ l of the DNA sample (distilled water was placed instead in the negative control tube) , the mixture volume was completed to 20  $\mu$ l by adding pure water Dnase free. After the tubes were closed tightly, the reaction materials were mixed using Vortex. Then it was transferred to the Thermocycler after programming it. See Tables (1&2). Ten  $\mu$ l of the amplified DNA was placed in special holes made in a 2% Agarose gel for electrophoresis and the results were photographed under a UV Transilluminator.

**TABLE 1. PCR Thermocycler program for *Mycoplasma***

Stage	°C	Time	Cycles No
Initial denaturation	95	5 min	1
Denaturation	95	20 sec	
Primer-annealing	59	30 sec	35
Extension	72	30 sec	
Final extension	72	5 min	1

**TABLE 2. PCR Thermocycler program for MG**

Stage	°C	Time	Cycles No
Initial denaturation	95	5 min	1
Denaturation	95	20 sec	
Primer-annealing	53	30 sec	35
Extension	72	30 sec	
Final extension	72	5 min	1

#### Statistical analysis

Microsoft Excel 2019 was used to calculate the percentages of prevalence, Sensitivity, specificity and agreement values of the two tests, isolation and direct PCR, according to the equations [28-30]

$$Prevalence = \text{Positive cases} / \text{total population} \times 100$$

$Sensitivity = \frac{True\ positive}{True\ positive + False\ negative} \times 100$

$Specificity = \frac{True\ negative}{True\ negative + False\ positive} \times 100$

$Agreement = \frac{(Positive\ cases\ in\ both\ tests + Negative\ cases\ in\ both\ tests)}{total\ population} \times 100$

True positive: positive for bacterial culture and PCR

True negative: negative for bacterial culture and PCR

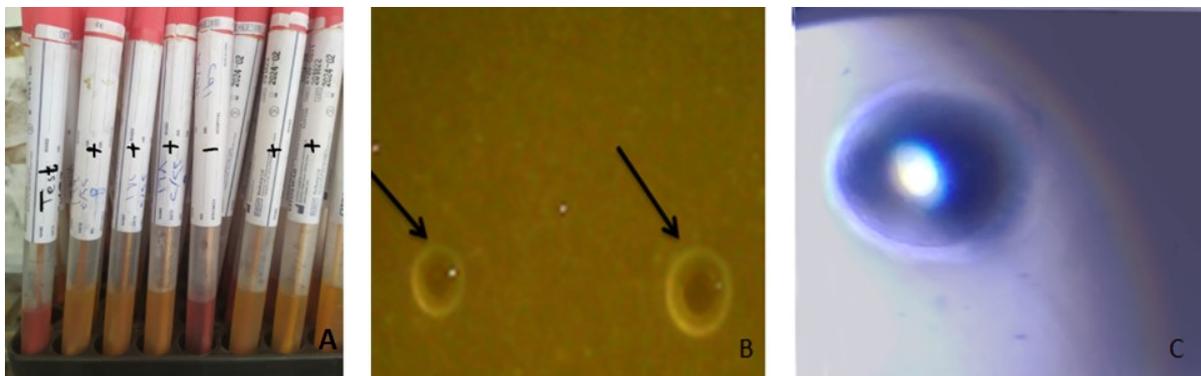
False positive: negative for bacterial culture and positive for PCR

False negative: positive for bacterial culture and negative for PCR

## Results

### Results of MG isolation

Of 180 tracheal swab samples, only 55 (30.5%) were isolates positive for *Mycoplasma* (Table 3). All the positive samples showed a change in the color of PPLO broth from red to yellow (Figure 1A). The results were confirmed by cultivating 20  $\mu$ l of each positive sample on *Mycoplasma* agar (PPLO agar), in which small colonies of *Mycoplasma* grown in all of them with the distinctive fried egg shape under a stereomicroscope. (Magnification  $\times 25$ ) (Figure 1B). When Diene's dye was applied, the colonies' centers showed up as dark blue, their peripheries as light blue, and the agar as light violet, this pigmentation lasts for a long time (Figure 1C).

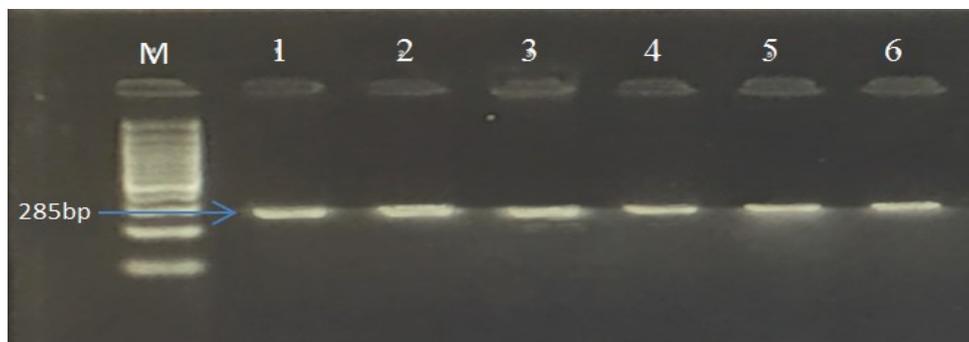


**Fig. 1. *M. gallisepticum* (A) PPLO broth color change. (B) Fried egg colony shape on PPLO agar 25X. (C) Diene's stain, colony center is dark blue, periphery is light blue 25X.**

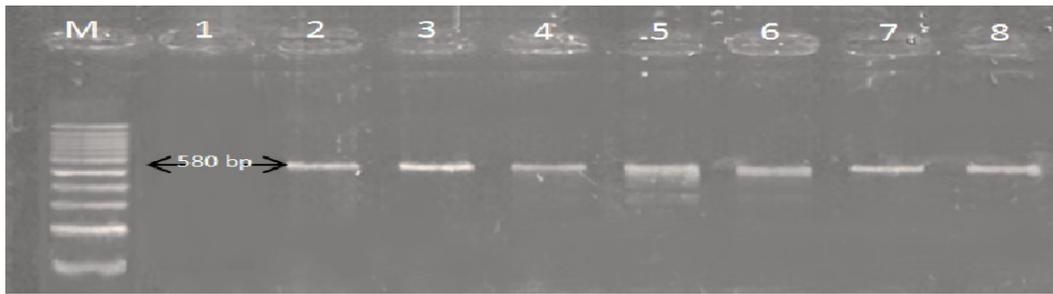
### Result of PCR Assay

Out of 180 samples, only 73 (40.5%) were positive at the PCR test with *Mycoplasma* genus primers (Figure 2). While it gave positive results in (59/180) samples for PCR using primers specific to the *Mycoplasma gallisepticum* (Figure 3), at a rate of 32.77%, of the fifty five samples that tested positive

for *Mycoplasma* isolation all tested positive for MG PCR, while out of 125 samples that had negative findings for *Mycoplasma* isolation test, 4 of them had positive PCR result and 121 had negative results. PCR sensitivity was 100%, specificity was 96.8% and agreement was 97.77% (Table 3).



**Fig. 2. The DNA of the samples was amplified using genus-specific primers *Mycoplasma* at a molecular weight of 285 base pairs and the electrophoresis on Agarose gel 2%. M is loader. 1,2,3,4,5,6 are positive**



**Fig. 3.** The DNA of the samples was amplified using species-specific primers MG at a molecular weight of 580 base pairs and the electrophoresis on Agarose gel 2%. M is loader. 1 is negative. 2,3,4,5,6,7,8 are positive

**TABLE 3.** Prevalence, sensitivity, specificity & agreement between tests used in diagnosing of MG

	Prevalence %	Sensitivity %	Specificity%	Agreement%
Isolation	30.5	93.2 (to PCR)	100 (to PCR)	97.7
PCR	32.77	100	96.8	97.7

### Discussion

According to culture and isolation the infection rate with MG in the study fields was 30.5%, It is less than what was recorded by Jafar and Noomi,(2019) in Tikrit governorate, amounting to 40.4%, [31] and this may be due to their use of Frey's media, which some researchers indicate it is more efficient in growing *mycoplasma* than PPLO medium. While the infection rate was 10.66% in the study conducted by Abed et al., (2021) on broiler chickens in Al-Dewaniyah Province[32], in Baghdad Ali and Ali, (2019) recorded an isolation rate of 12% using tracheal swabs [48]. The reason for these low percentages may be due to the frequent use of antibiotics in those fields to treat the disease, while the current study was conducted in fields that did not use antibiotics, rather they used onions and garlic as a way to raise the immunity of birds and disease resistance, according to what the field breeders reported. In Saudi Arabia, Elbehiry *et al.*, recorded an isolation rate of 55.88% [33]. The researchers Gharaibeh and Al Roussan, (2008) in Jordan indicated that the isolation method gave positive results that were 31.3% in broilers, but they used the fields as samples, so each field is a single sample [34]. The shape of fried eggs of colonies was identical to what was described in previous studies by Kleven and Ferguson-Noel,(2008)[35]. The dark blue colony center and its surroundings for the light blue after staining with Diene's stain were identical to what was recorded by some investigators [5,36].

Microbes and fungi growth on *Mycoplasma* media was prevented by antibiotics, and broth color change and colony appears as fried egg on the agar were very clear [37] *Mycoplasma* isolation gives us the possibility to study their chemical characteristics, such as the fermentation of sugars [38], their biological characteristics, such as pathogenic characteristics [39], as well as their molecular characteristics, such as the PCR test, and thus their species can be determined [40]. Also the antibiotic susceptibility test done using cultural method [41], for these reasons, isolation is considered as the gold standard for *Mycoplasma* detection [19]. Disadvantages of bacterial isolation are that it is difficult to satisfy and slow, requiring more than 21 days to determine its result [42], It also requires the experience of the researcher [43] and requires other procedures to determine the species, as it only determines the genus of the *Mycoplasma*, not its species, the culture can also be contaminated, Add to that. It's kinda expensive [36,44].The results of direct PCR that conducted on the study samples indicated a prevalence of mycoplasmosis in chickens of 40.5% (73/180) for *Mycoplasma* genus in general, while the prevalence of infection with MG in chickens was 32.77% (59/180). In Tikrit Jafar and Noomi,(2019) found that 58 samples were diagnosed as MG out of 156 samples (tracheal swab), 37%[31]. In Mosul Mahmmoud *et al.*, recorded 85.9%, but for tissue samples[26], in Al-Dewaniyah, prevalence rate was 24%[32], in Jordan Gharaibeh and Al Roussan,

recorded 31.3% [34]. In Egypt Marouf *et al.*, indicated 85% as a prevalence of *mycoplasma* in broiler flocks [20], in Kuwait Qasem *et al.*, (2015) recorded a rate of 58%[40]. Direct PCR in broiler is a suitable tool for MG detection It is highly sensitive, specific, rapid, quantifies MG load, can differentiate among strains, and can detect early infection [44]. When comparing PCR results with culture results, it is more expensive and complex, as it requires special technical devices and high, Collecting and preserving samples in incorrect ways leads to false negative results, and also, when using primers that do not match the circulating strain. False positive results occur after using the live vaccine or when samples are contaminated, Another disadvantage is that it cannot differentiate between the active stage and the recovering stage of infection [16, 20, 21].

Our findings differed from those of other researchers for a number of reasons, including the management style and the degree to which biosecurity protocols are implemented, as well as the provision of environmental factors that are favorable to birds, such as food, water, air, and suitable antibiotics and vaccination schedules, all of which boost bird immunity to illness and slow its spread. The state of the birds in general. In contrast to weak birds, where diseases spread quickly and are readily killed, healthy birds are more resistant to illness and it is harder for the disease to spread in them. How effectively the strain transmits the illness, what stage the sickness was at when the samples were obtained, how many and what kind of samples there are, and how to treat and keep them [14, 43, 45, 46]

According to this study, isolation showed a reasonably high sensitivity of 93 % and perfect specificity of 100 % this suggests that the isolation method is excellent at correctly identifying birds with MG infection (few Falls negative) and high specificity in not incorrectly identifying uninfected birds. The highest specificity indicates a low rate of false positives, which is Crucial for preventing unnecessary culling or treatment. The isolation method is reliable for confirming *Mycoplasma* infection, but it take longer than molecular methods like PCR. In our test PCR demonstrated excellent sensitivity at 100%, which means it rarely misses true positives, it has very good specificity at 97% which indicates a low false positive rate. The high agreement of 98% between isolation and PCR refers to PCR as a dependable diagnostic method for MG detection.

## **Conclusions**

The investigation we carried out to identify MG using the direct PCR test and the culture and isolation method revealed that both tests were highly sensitive and specific, with only minor variations between them. The culture test lost its diagnostic value due to its extreme slowness, making the PCR test the most effective due to its rapidity in MG diagnosis. Particularly in the areas where broilers are reared, where they typically spend 45 days. The results of the study also showed the spread of MG in broilers in Kirkuk Governorate, so appropriate biosecurity measures must be taken to remove this disease.

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## *Conflicts of interest*

The authors don't have any conflicts of interest

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## تقييم العزل الجرثومي وتفاعل البلمرة المتسلسل في تشخيص الميكوبلازما جاليسيبيتيكوم في فروج اللحم في محافظة كركوك (العراق)

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هدفت هذه الدراسة الى تقييم اختبار العزل الجرثومي و تفاعل البوليميراز المتسلسل (PCR) في تشخيص الميكوبلازما جاليسيبيتيكوم (MG) في الفروج. تعد MG واحدة من أصغر الكائنات حرة المعيشة، وتتكاثر بشكل مستقل. ان عدم وجود جدار خلوي سمح لها باتخاذ أشكال وأحجام مختلفة و اتاح لها مقاومة المضادات الحيوية التي تستهدف جدار الخلية. عندما تصيب MG الدجاج فإنها تسبب المرض التنفسي المزمن (CRD)، الذي يتميز بالخرخرة والعطاس والسعال وإفرازات الأنف وضيق التنفس والتهاب الملتحمة. فقدان الشهية، وانخفاض كفاءة التحويل وزيادة معدل الوفيات، وتلف الذبائح وتكاليف الدواء، تسبب خسائر اقتصادية كبيرة. ان تشخيص المسبب يمثل الخطوة الاولى في العلاج ولتقييم الاختبارين تم جمع 180 مسحة من القصبة الهوائية من فروج اللحم بعمر (28-40) يوماً بدت عليها أعراض CRD خلال الفترة (2022/12/1-2023/2/28). كان معدل انتشار MG الذي سجل عن طريق العزل وتفاعل البوليميراز المباشر 30.5% (180/55) و 32.77% (180/57) على التوالي. وكانت حساسية ونوعية تفاعل البلمرة المباشر 100% و 96.8% على التوالي. عندما قارنا الاستزراع مع PCR وجدنا أن حساسيته ونوعيته كانت 93% و 100% على التوالي. لقد خلصت دراستنا إلى أن الاستزراع رغم عده الاختبار القياسي الذهبي للكشف عن MG لخصوصيته وفعاليته ولكنه يستغرق وقتاً طويلاً، فان PCR المباشر سريع وفعال للغاية.

**الكلمات الدالة:** الميكوبلازما جاليسيبيتيكوم، الفروج، المرض التنفسي المزمن، العزل الجرثومي، تفاعل البلمرة.