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Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by using of cultural and PCR technique

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Abstract

Laboratory methods are essential for the diagnosis of Mycoplasmal infection. There are three laboratory approaches are essential for the diagnosis of Mycoplasmal infection in chicken including direct methods by culture method and polymerase chain reaction, and indirect methods by detection of Mycoplasmal antibodies by serological tests. This study aimed to detection of Mycoplasma by culture and PCR technique. Two hundred seventy-six samples were collected from infected adult boiler chicken in Salah Al-din province which suffering from respiratory signs and /or joint infection, 202 respiratory and 74 articular samples. According to the results of culture, Mycoplasma isolated in rate of 35.1% (36.6% from respiratory samples and 31.1% from articular samples). The sensitivity of culture was 100%, while the specificity of culture was 97.9% when comparing with PCR results. The current study concluded that the respiratory infection was more than articular infections, and *Mycoplasma gallisepticum* more distributed than *Mycoplasma synoviae* among chickens.

Keywords: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, PCR, Culture
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الكشف عن المفطورات المنتنة للدجاج والمفطورات المفصلية باستخدام طريقة الزرع وتفاعل البوليميرات المتسلسل

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فرع الأحياء المجهرية، كلية الطب البيطري، جامعة تكريت، تكريت، العرق

الخلاصة

يعتبر التشخيص المختبري مهم وضروري لتشخيص الاصابة بالمفطورات. هناك ثلاث طرق مختبرية لتشخيص الاصابة بالمفطورات وتتضمن الطريقة المباشرة عن طريق عزل وتشخيص الكائن المجهري بواسطة تقنية الزرع والكشف عن الدنا بتقنية تفاعل البلمرة المتسلسل والطريقة غير المباشرة عن طريق الكشف عن الاضداد المتخصصة بواسطة الطرق المصلية. هدفت الدراسة الحالية الى عزل وتشخيص المفطورات من دجاج اللحم البالغ في محافظة صلاح الدين. مائتان وست وسبعون عينة تم جمعها من الدجاج المصاب بعلامات تنفسية و/ أو إصابات مفصلية، وبواقع ٢٠٢ عينية من الجهاز التنفسي و ٧٤ عينة من المفاصل. نتائج الزرع الجرثومي أظهرت ان النسبة الكلية للعزل كانت ٣٥,١ % (٣٦,٦ % من عينات الجهاز التنفسي و ٣١,١ % من عينات المفاصل). حساسية طريقة الزرع كانت ١٠٠%، بينما كانت خصوصية نتائج الزرع ٩٧,٩% عند مقارنة نتائج الزرع مع نتائج تفاعل البلمرة المتسلسل. استنتجت الدراسة الحالية بان الإصابات التنفسية هي أكثر انتشارا من الإصابات المفصلية وان المفطورات المنتنة للدجاج أكثر انتشارا من المفطورات المفصلية.

Introduction

Avian Mycoplasmosis are highly contagious disease of poultry. The first description of the disease was in turkey in 1926 and then described in chicken in 1936 (1,2). Although there are more than 120 species, avian mycoplasmosis mainly caused by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). The disease called chronic

respiratory disease in chicken and infectious sinusitis in turkey (3,4). It causes a highly economic loss by decreasing in hatching rate, exclude of infected Chicks, decrease in eggs production, reduced growth rate, increased costs of eradication procedures which involve site cleaning and depopulation, and increased costs of medication and vaccination (5,6). Recurrent respiratory infection and bad environmental conditions considered as predisposing factors for Mycoplasmal infection (4,7). Avian Mycoplasmosis transmission occur horizontally through aerosols and vertically through the egg, leading to a rapid spread within the flock (8). The main clinical signs are: upper respiratory tract infection, sinusitis, coughing, sneezing, fluid secretion from nose and eye, bloody diarrhea, enlargement of joint and lameness (3,9). Laboratory methods are essential for the diagnosis of Mycoplasmal infection, since, clinical signs and pathological lesions cannot reliably diagnose the source of infection. Rapid and early diagnostic detection of Mycoplasmal infections is important to prevent the spread of infection and to limit economic losses in the poultry industry. There are three approaches to diagnose mycoplasma infection: isolation and identification of organism by culture techniques, detection of its DNA, and identification of specific antibodies by serological methods like ELISA, serum plate agglutination test, and hemagglutination inhibition test (10-12).

The aim of the current study was to determine mycoplasma prevalence ratio in suspected chicken and evaluation of mycoplasma diagnostic methods used in poultry.

Materials and methods

Samples collection

276 samples (202 tracheal or air sac samples and 74 articular samples) collected from infected adult boiler chicken suffering from respiratory signs and /or joint infection. The samples which were taken in the present study include; tracheal samples: collected by using of sterile cotton swabs, or part of trachea after postmortem, articular samples: collected by either synovial fluid by using of sterile syringes or whole joint after postmortem, air sac samples: collected by taking part of air sac after postmortem.

Culture methods

All samples were cultured in Freyś broth medium with additives which included 150 ml horse serum collected from jugular vein, then centrifuged and filtrated by using 0.22 µm mellipore, 100 ml of yeast extract prepared according to Kleven (13), 5 ml of cysteine hydrochloride (Himedia- India), 5 ml of NAD (Himedia- India (0.1g:5ml), 20 ml of Thallium acetate (1g:100ml), 5 ml of penicillin solution (1,000,000 I.U.) (Segmi), 10 ml dextrose (50 gm:100 ml), and 10 ml phenol red (1g:100ml). The final pH adjusted to 8.9, then the broth incubated with 5-10% CO_2 at 37C for 2 weeks. Presence of turbidity or color conversion from red to yellow refer to Mycoplasma growing (13). 100 µl of Freyś broth medium were cultured on two Freyś agar media (Oxoid, England) with the same additive expect phenol red. One agar with NAD (for isolation of MS) and other without NAD (for isolation of MG), then incubated with 5-10% CO_2 at 37C^o and examined after 3 days. The appearing of fried eggs colony under dissecting microscope refer to positive result (13).

Genetic methods

DNA extraction: DNA template was prepared by thermal methods and according to (11).

A single colony was taken and dissolved in 100µm l of distilled water, then centrifuged to discard the remaining culture media, supernatant was discarded then 100 µl of distilled water were re-added and heated at 100 C^o for 10 mints, then centrifuged (1400g/M) for five minutes, supernatant was taken which contain DNA and kept at -20 ^oC.

Reaction mixture consist of 0.5 µl of Primer F (20 p mole/µl), 0.5 µl Primer R (20 p mole/µl), 0.25 µl of Taq Polymerase, 1 µl dNTP, 2 µl of MgCl2, 5 µl of 10x PCR Buffer and 35.75 µl of distilled water

Thermocycler program: include 35 cycles, cycle steps temperature was as follows: 94^{o}C for 30 seconds in Denaturation step, 55^{o}C for 30 seconds for Primer-annealing, and 55^{o}C for 1 mint. for DNA extension.

Table 1: Primers used in the current study

Primer		Primer sequence	bp	Reference
Mycoplasma gene	F	5-GGGAGCAAACAGGATTAGATACCCT3	270	9
	R	5-TGCACCATCTGTCACTCTGTTAACCTC-3		
MS	F	5’-GAGAAGCAAAATAGTGATATC-3’	207	10
	R	5’-CAGTCGTCTCCGTTAACAA-3’		
MG	F	5’-AAC ACC AGA GGC GAA GGC GAG G-3’	530	11
	R	5’-ACG GAT TTGCAACTG TTT GTATTGG-3’		

Interpretation of the results: Positive and negative agreement, sensitivity and specificity, and positive and negative predictive value for each method were calculated according to the following equations:

$$\text{Positive agreement} = \frac{\text{No. of samples gave positive results in first test}}{\text{No. of samples gave positive results in second test}} \text{ X } 100$$

$$\text{Negative agreement} = \frac{\text{No. of samples gave negative results in first test}}{\text{No. of samples gave negative results in second test}} \text{ X } 100$$

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + false negative}} \text{ X } 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative + false positive}} \text{ X } 100$$

$$\text{Positive predictive values} = \frac{\text{True positive}}{\text{True positive + false positive}} \text{ X } 100$$

$$\text{Negative predictive values} = \frac{\text{True negative}}{\text{True negative + false negative}} \text{ X } 100$$

Results

Mycoplasma isolation

The result of respiratory samples culture for Mycoplasma on Freyś agar showed that Mycoplasma isolated in rate of 36.6%. High isolation rate recorded from tracheal samples which was 40.4% compared with air sac sample which was 23.9% (Table 2). The isolation rate of Mycoplasma from articular samples was about 31.1% (Tables 3).

Table 2: Mycoplasma isolation ratio from respiratory samples

Types of samples	No. of samples	No. of positive cases	Rate
Tracheal samples	156	63	40.4%
Air sac sample	46	11	23.9%
Total	202	74	36.6%

Table 3: Mycoplasma isolation ratio from articular samples

Types of samples	No. of samples	No. of positive cases	Rate
Total	74	23	31.1%

Classification of Mycoplasma isolates into MG and MS

The Diagnosis of isolated Mycoplasma into species was done according to the DNA content of the isolated microorganisms by using of PCR. The results showed that the 97 isolates grown on Freyś agar (Figure 1) were belong to Mycoplasma genus, from those, 58 (59.8%) were belong to MG and 39 (40.2) diagnosed as MS (Table 4). Also, from table 4 it is clear that all 23 (100%) articulate samples diagnosed as MS, while respiratory samples showed that 58 (78.4%) from total 74 respiratory isolates diagnosed as MG and 16 (21.6%) were belong to MS species (Figures 2-4).

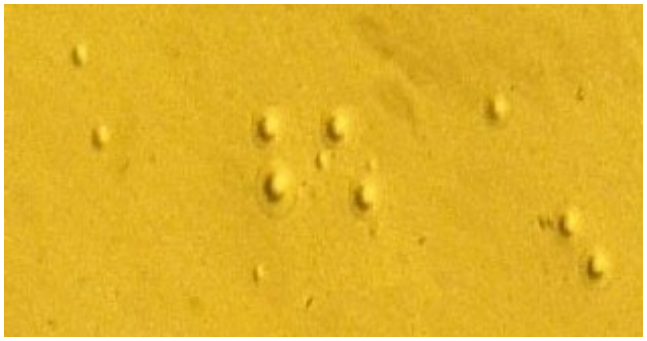

Figure 1: The colony of Mycoplasma spp. on Freyś agar.

Table 4: Classification of Mycoplasma isolates using PCR

Types of samples	No	MG		MS	
		No	Rate	No	Rate
Respiratory isolates	74	58	78.4%	16	21.6%
Articular isolates	23	0	0%	23	100%
Total	97	58	59.8%	39	40.2%

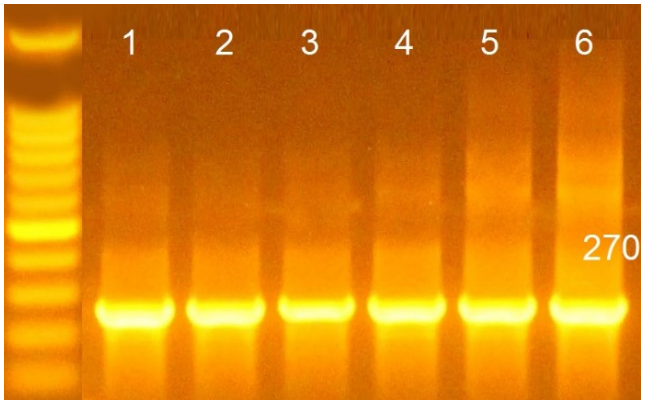

Figure 2: Electrophoresis on 2 % agarose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker, wells 1-6 positive samples of Mycoplasma genus with band size 270 bp.

Comparison between the results of culture method and PCR for MG

Table 5 showed that the sensitivity, specificity, positive predictive values and negative predictive values were: 100%, 89.7%, 72.4% and 100% respectively.

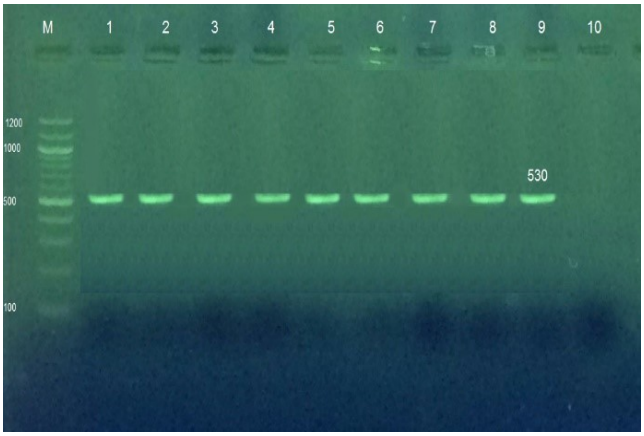

Figure 3: Electrophoresis on 2 % agarose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker, wells 1-9 positive samples of *Mycoplasma gallisepticum* (MG), with band size 530 bp.

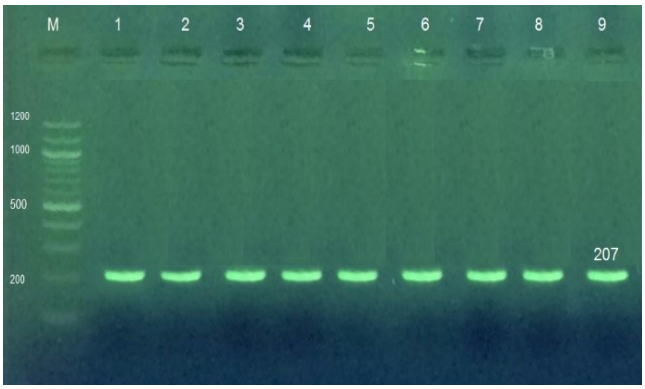

Figure 4: Electrophoresis on 2 % agarose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker, wells 1-9 positive samples of *Mycoplasma synoviae* (MS) with band size 207 bp.

Table 5: Comparison between cultural and PCR method for the diagnosis of MG

Culture Result	Result of PCR test				Total
	Positive		Negative		
	True positive	42	False positive	16	58
	False negative	0	True negative	218	218
Total		42		234	276

Comparison between the results of culture method and PCR for MS

From table 6 it is obvious that the sensitivity, specificity, positive predictive values and negative predictive values were: 100%, 97.9%, 87.1% and 100%.

Table 6: Comparison between cultural and PCR method for the diagnosis of MS

Culture Result	Result of PCR test				Total
	Positive		Negative		
	True positive	34	False positive	5	39
	False negative	0	True negative	237	237
Total		34		242	276

Discussion

The direct diagnosis of the Mycoplasma can be carried out by either culturing the microorganism or detection of its DNA using PCR procedures (4). Detection of causative agent in Mycoplasma considered as gold stander in diagnosis because of that serological test unable to detect the subclinical and early infection (3,4). Therefore, in the current study the culture and PCR methods were used as a basis for the diagnosis of Mycoplasma.

Mycoplasma isolated in rate of 35.1%. This ratio is more than that recorded by (15) which was 25.7%. That's may be due to difference in location of the study and different type of medium that was used and in additives. In regard to Mycoplasma species, the ratio of MG isolation was more than that of MS. Since, MG is the main cause of CRD and main important economically (4). The same results were recorded by other studies (3,16). According to MS, in addition to its isolation from chickens suffering from articular signs, it was also isolated from respiratory infection cases. Therefore, it may cause sub-clinical infection, air saculitis and secondary respiratory infection (13).

When comparing between culture results and PCR test, the result showed that the sensitivity of culture was 100%, while the specificity of the culture was 97.9%. That is mean that some of the pathogen which were diagnosed as MG or MS were misdiagnosed and maybe belonging to other Mycoplasma species.

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