ISOLATION AND MOLECULAR CHARACTERIZATION OF *MYCOPLASMA SYNOVIAE* FROM INFECTED CHICKENS WITH RESPIRATORY SIGNS.

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ABSTRACT
The aim of this study was to investigate the presence of *Mycoplasma synoviae* in broiler and layer chickens infected with respiratory signs .A total of 80 samples were collected randomly from layer and broiler chickens with respiratory signs in Baghdad from the period between January to May 2017 from trachea, Lung, Air sac, oviduct, tracheal swabs, conjuctivia swabs, choanal swabs and nasal swabs and cultured in PPLO medium with supplements, then positive culture subjected to DNA extracted and Polymerase Chain Reaction assay (PCR) to detect *Mycoplasma* as a genus and *Mycoplasma* spp by using specific primers targeting16S rRNA gene. The results of culture revealed that the total rate of *Mycoplasma* isolates was19/80(23.75%). DNA was extracted from 19 positive isolates, all nineteen isolates were positive for Mycoplasma genus by conventional PCR assay, and a product of 270 bp was generated by amplification of 16SrRNA gene,while a 210 bp region of 16S rRNA gene was amplified for the *Mycoplasma synoviae* in 19 isolates.The products of amplification of *Mycoplasma synoviae*16SrRNA gene was sent to MACROGEM (Korea) for sequencing, then submitted in Gene bank database and have accession number:ID: MG846121.1. Sequencing alignment showed that local MS isolates had highly identical with standard references at gene bank, analysis the phylogenetic tree revealed the presence of 100% identity of the Iraqi isolate to USA: West Virginia, United Kingdom , Australia and Brazil, also had 99% identity to South Africa ,China , Sweden , USA and VitNamHatey. These result was concluded that circulation of the *Mycoplasma synoviae* among birds of the flock and caused respiratory signs in chickens.

Keywords:Molicutes,salpengitis,hens,respiratorysign,MS,primers

مجلة العلوم الزراعية العراقية - 2020 :51 (5): 1466- 1473 **علي وآخرون**

عزل وتوصيف جزيئي *Mycoplasma synoviae* من دجاج مصاب بعلامات تنفسية

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المستخلص
الهدف من هذه الدراسة التحري عن وجود *Mycoplasma synoviae*في دجاج لاحم والبياض المصاب باعراض تنفسيةحيث تم جمع 80عينة بصورة عشوائية من دجاج لحم وبياض مصاب بعلامات تنفسية في بغداد في الفترة من كانون الثاني الى حزيران 2017 لكل من الرغامي,الرئتين, الاكياس الهوائية,قناة البيض,مسحات الرغامي, مسحات الملتحمة, مسحات من شق الحنك ومسحات الانف وزرعها بالوسط الزرعيPPLO المدعوم بمكملات النمو وبتوفير الظروف الملائمة للنمو وبعدها تخضع عينات الزرع الموجبة لاستخلاص الDNA لغرض فحصها بطريقة تفاعل البلمرة المتسلسل الاعتيادي لتحديد جنس المايكوبلازماومايكوبلازماساينوفي باستخدام بادئات خاصة لتضخيم جين16S rRNA, ولتاكيدالمايكوبلازما تم اجراء سلسلة التتابع الجيني والشجرة التطورية. واظهرت النتائج نسبة عزل المايكوبلازما(19/80) (23.75%)/% وان اعلى نسبة عزل في الرغامي ولم تعزل من مسحات الانف والحنك. واجري استخلاص الدنا ل 19 عزلة موجبة. اظهرت نتائج تقنية سلسلة التفاعل المتبلمرة ان ال19 عزلة كانت موجبة للجين 16S rRNA270, زوج قاعدي لجنس المايكوبلازما و210زوج قاعدي ل *Mycoplasma synovia*. أظهرت نتائج التسلسل الجيني النتائج وجود تطابق مع السلالات المرجعية و سجلت ببنك الجينات العالمي وبرقم قبول MG846121.1. واظهرت نتائج التطور الشكلي تطابق العزلة العراقية 100٪مع كل من الولايات المتحدة, المملكة المتحدة, استراليا و البرازيل في حين كان التطابق 99٪مع سلالات جنوب افريقيا و الصين و السويد , الفيتنامية.نستنتج ان تشخيص *Mycoplasma synoviae* يؤكد انها موجودة بحقول الدجاج وتسبب اعراض تنفسية.

الكلمات المفتاحية:رقيقة الجلد,علامات تنفسية,التهاب قناة البيض ,MS.البرايمرات

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INTRODUCTION

Mycoplasma synoviae(MS) is one of the important Mycoplasma species that infect avian species, has been listed as a notifiable Mycoplasma by World Organization for Animal Health(28). MS infection causing symptoms like sinusitis, airsacculitis,synovitis,eggshellapexabnormalities and result in reduction in egg production and meat quality,causingeconomic losses in the poultry industry worldwide (16).MS cause subclinical upper respiratory tract infection and infectious synovitis in turkey(29) It may cause air sac lesions when combined with Newcastle disease (ND), infectious bronchitis (IB), or both(42).The clinical signs of MS infection could not be differentiated easily from those caused by other avian pathogens, such as avian reovirus, *Mycoplasma gallisepticum* (MG), *Staphylococcus aureus*, *Escherichia coli*, *Pasteurella multocida*, and "*Salmonella* spp., which in poultry species could cause symptoms similar to MS infection (17).Transmission of MS is accomplished laterally via direct contact and respiratory aerosols, and vertically within eggs(27). Like other pathogenic *Mycoplasmas*MS growth and persist in the host (24;11;1) The morbidity of about 5 - 15% in chicken and 1-20% in turkey has been associated directly with MS infection alone and indirectly by co-infection with other pathogens (32;17). The methods recommended by "OIE "for MS dignosis are isolation (culture), serological assays and polymerase chain reaction (PCR) (26;2; 6). DNA sequence analysis of the" 16S rRNA gene" has proven to be useful for Mycoplasma identification and in phylogenetic studies(40). The molecular characterization of MS strains may assist in epidemiological studies to determine the source of infections and relationships among strains isolated from neighbouring or related flocks Kursa (18;15) Because of the above causes and possibility of contributing of MS in distributing the respiratory disease in Iraq the current study is designed to detect the presence of MS in the broiler and layer chickens infected with respiratory signs and study the molecular characterization ,sequencing , phylogenetic tree of in Iraq.

MATERIALS AND METHODS

For isolating *Mycoplasma synoviae* different samples were collected during the period of 3 months .A total of 80 samples (organs and swabs) including trachea,lung,air sac, oviduct, tracheal swabs ,conjuctivia swabs,air sac swabs and choanal cleft swabs taken from layer and broiler poultry flocks in Baghdad were suffering from respiratory signs. were collected at necrobsy,samples were transported to the laboratory labore in refrigerated container(25,27) and culture in PPLO broth and PPLO agar which was prepared by using 6.37gand 8.87gm respectively /250ml distal water then added sterile supplementary growth(horse serum 10%,yeast extract 10% and ampicillin 500 or 1000mg)and inhibitory solutions(thalium acetate 1%)were added under restricted sterile environment(27).Small pieces of organ or swab were placed in 1–2 ml of PPLO broth with supplement growth and incubated aerobicaly at 37 for three days and then streaked on PPLO agar in an inverted position in candle jar at 37 increased humidity and CO2 tension for 6 days.All plates were examined for *Mycoplasma* growth at 3days intervals under dissecting microscope (43) DNA extraction was performed on positive *Mycoplasma* colony suspected, that grow on pplo agar were sub culture overnight in pplo broth and used for DNA extraction according to(21) DNA was extracted according to manufacture of DNEasy blood and tissue extraction kit (QIAGEN, USA)conventional PCR assay was used in this study for detection of Mycoplasma.The positive samples of Mycoplasma genus subjected to PCR for detection of *Mycoplasma synoviae* so for detection of *Mycoplasma* genus by using primers based 16Sribosomal RNA gene were designed by Botes *et al*. (5) primers based 16S ribosomal RNA gene were designed by(27) for detection of *Mycoplasma synoviae*,amplification*Mycoplasma synoviae* strain obtained from conventional PCR was sent by Macrogen company to Korea. (NICEM) company for sequencing (sanger method), Nucleotides sets were used to obtain the identity score of our isolate strain with the other world references strains by the Mega6+NCBI program according to(39) .

RESULTS AND DISCUSSION

In the present study, a total of 80 samples were collected randomly from layer and broiler chickens with respiratory signs (10) from trachea, (10) Lungs, (10) Air sacs, (10) oviducts, (10) tracheal swabs, (10) conjunctiva swabs, (10) choanal swabs and (10) nasal swabs . percentage of positive samples revealed by culture was 23.75% as show in table (1), positive samples revealed growing typical fried egg colonies figure(1).Isolation by culture was the gold-standard method for MS detection(8) isolation may also be compromised by rivalry from other pathogens" especially in the cases of chronically infected animals" which in general have relatively low MS loads (33). In the present study the isolation of Mycplasma was done by PPLO broth and agar medium with supplements which was found suitable for isolation of Mycoplasma with characteristic morphology obtained on PPLO agar was small, smooth, circular, having fried egg appearance with central dense and outer translucent area, similar characteristics of colonies of Mycoplasma spp. have been reported by several studies (16;19) the result showed the highest occurrence of Mycoplasma spp. were in the tracheal 5/10(6.25%) following by air sac swabs 4/10(5%) , tracheal swabs and oviduct 3/10(3.8%),Conjunctival swabs and lung 2/10(2.5%) these result was similar to other studies by (12) ; Rauf *et al.(* 32) and Gondal *et al.(*13) whom found that the highest number of samples collected from trachea of birds were positive for Mycoplasma through culture technique ,there was no isolate from the choanal and nasal swabs this may be due to using of dry swabs reducing the viability of microorganisms (44) while the result of Mycoplasma isolation from oviduct in this study was similar to the result that obtained by Mansour(24) and Serag (40) whom isolated Mycoplasma from chicken's respiratory and reproductive systems this related to the air sacs and surface of the ovaries "which are in close proximity to the abdominal air sacs", as well as the mucosal surface of the oviduct (35)or that Mycoplasma organism can spread from the respiratory tract via blood stream to the ovaries causing pathological conditions and that indicated the tissue proclivity of Mycoplasma (29). Nineteen positive Mycoplasma samples were tested by conventional PCR technique with primers a" 270 bp region of 16S rRNA gene" was amplified for the Mycoplasma genus the result show all isolates were scored to be positive Mycoplasma spp. fig. (2).While a 210 bp region of 16S rRNA gene was amplified for the *Mycoplasma synoviae* also all isolates positive to MS as shown in fig. (3). The results of the 16Sr RNA gene revealed that MS primers of the16S rRNA gene had successfully targeted the respective gene and shown the single bands of the16S RNA gene of MS at 1500 bp in the isolate of MS as showed in figure (4) It was stated that a combination of culture and PCR is the most recommended method because of Mycoplasma are having very small genome size, they fail to show the many biochemical pathways as compared to bacteria to identify at species level (7;43) These results are in line with results obtained by Bagheri *et al.(*3); Zahraa*et al*.(45); 14 ; 38) whom reported that 16SrRNA gene was able to identify all the examined *avian* Mycoplasma and routine PCR test in conjunction with conventional identification methods could be effective in providing a more accurate profile of the prevalence of Mycoplasma in poultry flocks. Although several researchers have described the use of conventional PCR in detection Mycoplasma directly from clinical samples such as nasal swab, trachea and air sac (30;4;32;41).Due to lack of differentiation in colony characteristics of different Mycoplasma species, PCR could easily pick up *M.synoviae* through targeting specific primers for diagnosis of MS by PCR amplification of the 16S rRNA gene using MS specific primers (26;31;36) the increase in MS happen might be due to two causes ; the subclinical form known in most MS infection cases *as well* as the implemented control strategies ,many national control programs focus on MG "as more clinically relevant avian Mycoplasma″ with less strict strategies towards MS control(20).The high prevalence of MS infection in present study was agreement with(9;10;37).whom recorded in above 60 weeks of age .Sequencing of 16SrRNA gene of *Mycoplasma synoviae*was performed to isolates, the nucleotide sequence

of chicken *Mycoplasma synoviae* 16SrRNA gene were submitted in gene bank database and have accession number: ID, the phylogenic of one Iraqi field isolate of MS that was deposited in NCBI under the accession number MG846121.1. Based on the nucleotide phylogenetic tree of 16Sribosomal RNA gene found that MG846121.1Iraqi isolate was 100% similar to USA: West Virginia CP011096.1, United Kingdom: LS991953.1, Australia: CP021129.1 and BrazilAE017245.1, also had 99% similar to South Africa MH539137.1, China MF319540.1 , Sweden U04645.1 , USA NR_044811.1 and VitNamHatey : AM073015.1 figure (5).The phylogenic analysis of one Iraqi field isolate that was deposited in NCBI under the accession number , based on the nucleotide phylogenetic tree of 16Sribosomal RNA gene we found that Iraqi isolate was 100% similar to USA: West Virginia, United Kingdom: , Australia: and Brazil, also had 99% similar to South Africa, China, Sweden , USA and VitNamHatey isolate indicating the geographical distribution of MS strains between countries due to weak biosecurity strategy ,also implicating the epidemio geographical relation-ship between these isolates(23) . The current study was consider first report in Iraq that involvement isolation of *Mycoplasma synoviae* by culturing and confirmation by PCR test.

Table 1. Results of Mycoplasma isolation from specimens by culturing.

Samples (Organs and swabs)	No. of examined samples	No. and percentage of positive samples
Tracheal	**10**	**5(6.25%)**
Lung	**10**	**2(2.5%)**
Air sac	**10**	**4(5%)**
oviduct	**10**	**3(3.8%)**
Tracheal swabs	**10**	**3(3.8%)**
Choanal cleft swabs	**10**	**0(0.00%)**
Conjunctival swabs	**10**	**2(2.5%)**
Nasal swabs	**10**	**0(0.00%)**
Total count / percentage of isolate	**80**	**(19)/(23.75%)**

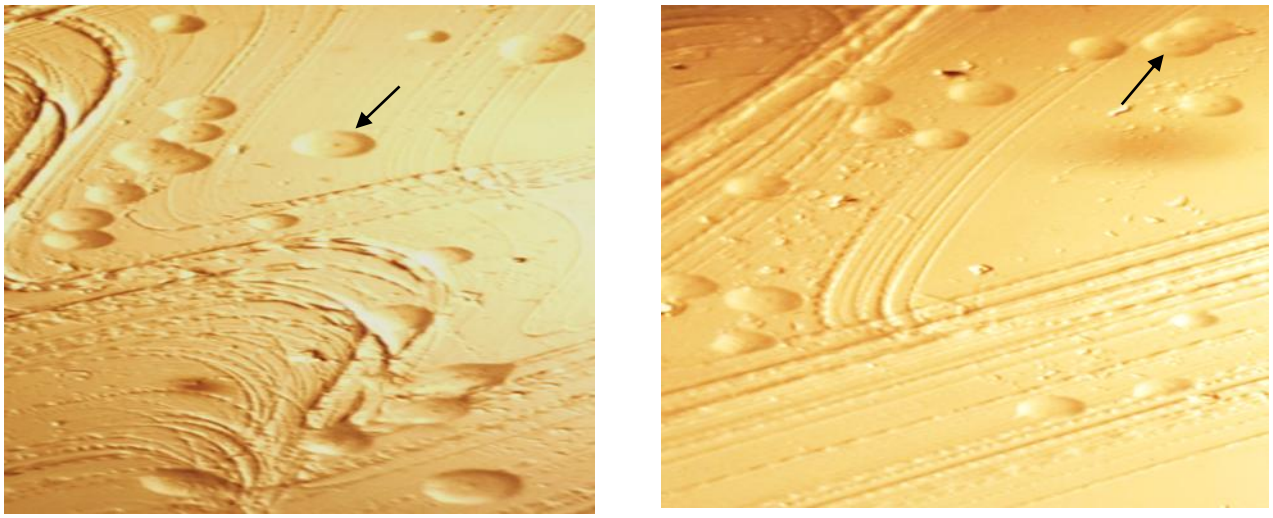

Figure 1.shows fried egg colony of Mycoplasma

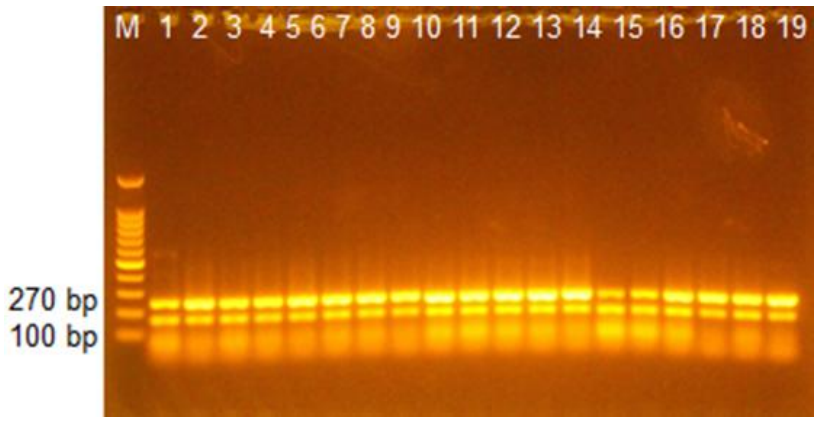

Figure 2. Agarose gel electrophoresis image that show the PCR product analysis of 16S rRNA gene in Mycoplasma genus positive isolates. Where M: marker (1500-100bp), lane (1-19) positive Mycoplasma genus at (270bp) PCR product

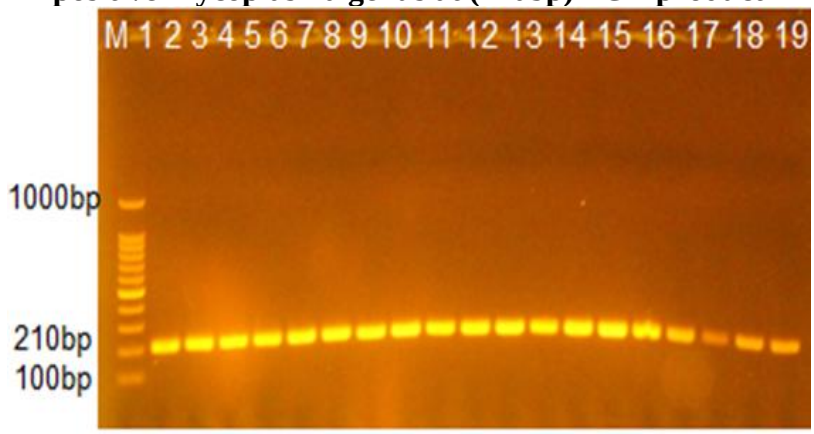

Figure 3.Agarose gel electrophoresis image that show the PCR product analysis of 16S rRNA gene in *Mycoplasma synoviae* positive isolates. Where M: marker (100-1000bp), lane (1-19) positive *Mycoplasma synoviae* at (210bp) PCR product

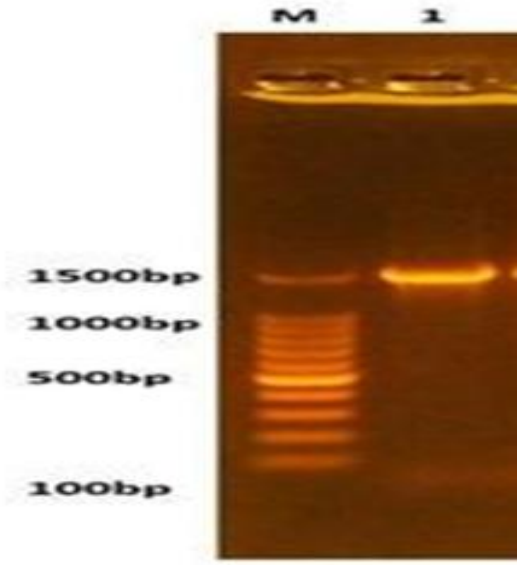

Figure 4.Electrophoresis of amplicon PCR products of field isolates of MS had a single band at size1500bp represent 16sRNA gene,(M: marker (1500bp)

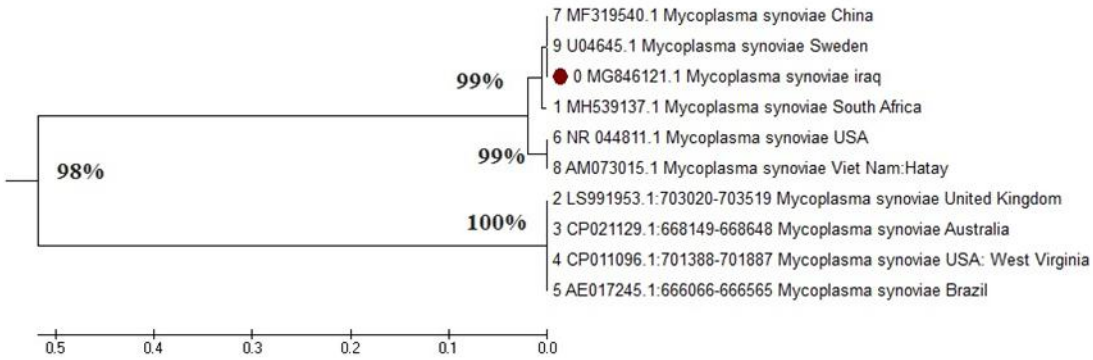

Figure 5.show phylogenic tree of one isolate using Mega6+NCBI

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