Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

Sequencing Based Phylogenetic Analysis of Local *Mycoplasma Gallisepticum* of Broiler Chickens in Al-Dewaniyah Province / Iraq

Alaa AbdulAziz Abed1, Ali A. Al-Iedani2, Ahmed Jasim Neamah3

1: Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq.
2: Department of Microbiology, College of Veterinary Medicine, University of Basrah, Iraq.
3: Unit of Zoonotic Diseases, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq.
Corresponding author: Alaa AbdulAziz Abed
E-mail: alla.abed@qu.edu.iq

Abstract

Mycoplasma gallisepticum (*MG*) is regarded as finest bacteria can replicate independently and they one of the illustrious pathogens for chickens cause body retardation and losing weight thereby it considered as one of the costliest worldwide significant pathogens for poultry. *Mycoplasma* detection using traditional culture method is not adequate procedure because it has several strains also the difficulty of cultivation technique and the obstructs that faced, current study was considered culture method and conventional Pcr assay for direct diagnosis of *MG* using 16S rRNA and mgc1(GapA) gene.

Out of 20 commercial broiler farms at Al-dewaniyah province, Iraq, 150 tissue specimens were assembled wherever manifestation of respiratory infections was demonstrative.

Whereas 16 out of 150 samples (10.66 %) were recognized as positive via conventional cultivation based on morphology of growing colonies, Diene's staining and some biochemical, however confirmed by amplification of 16S rRNA gene of polymerase chain reaction technique of suspected colonies, while cloning of direct tissue samples by 16S rRNA amplification exhibit 36/150 (24%) corresponding to the genus of *Mycoplasma*, the 16S rRNA amplification product was 1500 base pair.

The inference of the findings of current study, is that, the PCR revealed greater sensitivity and viable reliability, quality and precision than bacterial culture techniques and therefore might be very suggestive for the supervision of flock's health imperviousness against *MG* and to enable application of effective preventative and control measures. all 16 cultured isolates that affirmative as *Mycoplasma* spp by 16S rRNA amplification were undergo by PCR analysis of GapA gene in addition to sequence analysis of 16S rRNA, the outcomes of 16 isolated *Mycoplasma* spp. colonies showed that 8 isolates were belongs to *Mycoplasma gallisepticum* out

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

of 150 total tested samples with percent of (5.33%), this result provided by virulence GapA (mgc1) gene analysis data, however positive tissue samples by cloning assay was 22/150 (14.66%) were belongs to *M. gallisepticum.* Based on results of 16S rRNA sequencing, we detect other *Mycoplasma spp.* and other unrelated bacteria not showed in the current article. The results of *Mycoplasma* isolates approval through amplification of the GapA gene showed a bands of 332 bp for *M. gallisepticum*, the16S rRNA gene was directed to Soul University (Korea) for sequence analysis. The available data are presented in Gene bank database of 16S rRNA for documentation.

Introduction

Mycoplasma genus are located in the Mollicutes class (**1,** Sasaki et al., 2002); which involved on more than 200 species *Mycoplasmas* have no cell wall and is categorized by observable characteristics i.e. phenotypes, and serology and genotype sequences including 16s rRNA, mgc1(GapA), mgc2, pMGA and pvpA and other related genes (**2,** Brown et al., 2007; **3,** Yasmin etal,2014; **4,** Yasmin etal,2018).

Avian Mycoplasmosis was initially distinguished in chickens in 1936 (**5,** Charlton etal., 1996). Among the most highly pathogenic avian *Mycoplasmas* and the most prominent member is the *MG*, it is the most well-known respiratory agent, a condition resulting from a *MG* infection is termed chronic respiratory disease and infectious sinusitis in chickens and turkeys respectively, the chronicity of *MG* infections demonstrates a failure of the host immune response to confronted effectively the *MG* infection (**6,** Levisohn and Kleven, 2000; **7,** Elbehiry etal.,2016), this is may be due to antigenic variation of surface proteins plus *Mycoplasma* disappeared or concealed within host cells (**8,** Papazisi et al., 2003).

Regarding a financial adverse impacts on of the poultry sector on multiple levels of apparent influences i.e. the level of hatchery involving the decline hatchability and demoted quality of newly hatching chicks, or at the farm level i.e. inflating the production cost in terms of an increase in the number of retarded bird and mortalities or as a result of the high cost of using medicines, vaccination programs and other control measures, or at the processing operations i.e. carcasses condemnation or rejection,(**9,** Liu, etal.,2001; **10,** Kaboli et al., 2013; **11,** Khalifa et al., 2013).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

Historically and on present time, as part of a long-established reliable practice tools,, identification of *MG* in the chickens was determined by serological screening such as serum plate agglutination (SPA) and enzyme-linked immunosorbent assay (ELISA) hemagglutination inhibition (HI), which are judged and detect subclinical *MG* colonization (**12,** Barua et al., 2006; **13,** Purswell et al., 2012; **10,** Kaboli et al., 2013); despite that many studies refers to the losing the both sensitivity and specificity (**14,** Carli, and Eyigor, 2003; **10,** Kaboli et al., 2013), there for current study regarded the traditional cultivation and molecular technique, because our expertise in culture technique, and because fast, highly specific and sensitive polymerase chain reaction (PCR) assay both are valuable for laboratory identification of MG infected flocks (**15,** Ehtisham-ul-Haque et al., 2015; **16,** Raviv and Kleven,2009).

Yet, some challenges are coupled with PCR, such as the presence of degrading materials in the specimens, or nonliving *Mycoplasmas*, the risk of contamination and costlier (**17,** Kempf,1998; **18,** Kleven, 1997; **14,** Carli and Eyigor, 2003; **19** Cremonesi etal.,2007; **3,** Yasmin etal,2014).

The purpose of our research was to study the accuracy and specificity of molecular diagnosis by using the 16S rRNA and GapA gene for identification compared to the traditional culture technique and the percentage of *Mycoplasma gallisepticum* dissemination, in addition there is inadequate data on molecular categorization; rapprochement degree and concerning the resemblance of Iraqi local strains/isolates with global strains, based on detailed examination of phylogenetic tree regarding 16S rRNA gene.

Materials and methods

collected Samples

A total of 150 tracheal, air sacs and choanal cleft samples were collected from twenty broiler chicken commercial farms suffering from clear respiratory signs like sneezing, coughing, respiratory voices and face swelling. All samples were collected during the period from February to May 2020 in Al-Dewaniyah province, Iraq.

Samples collected divided into two parts, first for traditional laboratory culture work and second for DNA extraction for submit to PCR diagnosis on isolated suspected colonies and direct

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

cloning assay for collected tissue samples.

All samples were brought to the Pathology and Poultry Diseases laboratory at the College of Veterinary Medicine/ University of Al-Qadisiyah.

Isolation and identification of *MG*

The collected trachea, air sacs and choanal cleft were inoculated into 3ml of pleuropneumonia-like organisms(PPLOs) broth media and incubated at 37 ºC for 3 -5 days until color changed (**20,** Kleven, 2008), subsequently, 20 µl of the colored broth was streaked on PPLO agar plate and incubated at 37ºC in a candle jar under decreased O2 tension (**21,** Kizil and Ozdemir, 2006; **22,** Khalifa etal.,2014) for 21 days at maximum, 2 media plates inoculated from same broth tube at least.

By operating a microscope, the plates were then checked at 6, 11, 16 and 21 days of incubation for the presence of matching fried-egg colonies of *Mycoplasma* spp.

Suspected *Mycoplasma* isolates were approved by Pcr assay technique and documented at GenBank website.

Purification of the isolates

A one colony of characteristic fried egg appearance was selected and cut with an agar mass and transmitted into a PPLO broth to get a pure culture, sometimes this step is repeated for many times of several single colonies (**23,** Sabry, 1968; **7,** Elbehiry etal.,2016).

Mycoplasma colonies staining method:

For more confidence of isolation work and to examine the morphology of the supposed *Mycoplasma* colonies and ability to acquired pigment several plates were stained by Diene's stain according to (**24,** Razin et al., 1998; **25,** Quinn et al., 2002).

Biochemical tests:

Digitonin diffusion test were prepared according to (**26,** Boonyayatra etal., 2012). Glucose fermentation were done according to (**27,** Aluotto et al., 1970; **28,** Poveda,1998). Arginine hydrolysis test were achieved according to (**29,** Michael, 1983).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

DNA extraction and Molecular analysis of *MG*

Mycoplasma DNA Extraction from trachea, air sacs, and choanal cleft tissue of the sampled birds was conducted according to the formerly designated method (**30,** Santha et al., 1990; **31,** Khan,2002). DNA was isolated matching to manufacture instructions of tissue extraction kit (Anatolia, Turkey) primers based 16S ribosomal RNA gene (1500bp) were originated by (**32,** Gray etal.,2005; **33,** Raji etal.,2008) for conventional PCR assay was applied in this study for detection of Mycoplasma genus and other prokaryotes. while primers of GapA gene were has been approved by (**34,** Ferguson et al.,2005; **35,** Fujisawa etal.,2019).

The process for DNA extracting from tissue pieces was implemented by utilizing 95 °C in a water bath heating for10 min and -20°C for cooling / 10 min, then samples were put quickly in phosphate buffer saline(PBS) and centrifuged at 13 000 x g / 20 min, the eventual product i.e. resultant pellet then laved twice in PBS and the supernatant liquid was preserved in 1.5 ml eppendorf tubes for purpose of DNA amplification. DNA yields were estimated by a NanoDroop 2000 (ThermoScientific, Germany), the concentration was ranged between 30-100ng/µl. DNA was stored at deep freeze for future assay.

DNA amplification

Electrophoresis

The amplified PCR products were investigated by gel electrophoresis in 1.5% agarose gel involving ethidium bromide (**36,** Garcia et al., 2005) and the resultant bands were pictured with Gel documentation (Bio-Rad USA).

PCR Detection of MG 16s ribosomal RNA gene and GapA gene.

Of all positive cultures and all collected tissue organ samples the PCR assessment assay accomplished for the identification of *MG* 16S ribosomal RNA and GapA genes. The PCR reaction was achieved according to (**37,** Kleven etal.,2004; **38,** Rasoulinezhad etal.,2017) with some modifications.

Briefly: 50 µL reaction volume consisting of 25µL 2X PCR Mastermix, 3 µL of each primer (20 µM) *Mycoplasma spp.* 1500 bp 16sRNA 27F, 5'-AGA GTT TGA TCC TGG CTC AG- '3 and 1492R, 5'-GGT TAC CTT GTT ACG ACT T- '3 (**32,** Gray etal.,2005; **33,** Raji etal.,2008). *Mycoplasma gallisepticum* 332bpp, GapA 3F, 5'-TTC TAG CGC TTT AGC CCT

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

AAA CCC- ‘3 and 4R, 5’-CTT GTG GAA CAG CAA CGT ATT CGC- ‘3 (**34,** Ferguson et al.,2005; **35,** Fujisawa etal.,2019), 20 µL of deionized distilled water and 2 µL of template DNA (Abm, Canada).

The thermal cycler conditions involved four phases as follow: Initial denaturation (DNA separation) was carry out at 94 ˚C for 3 min., annealing primer to target sequence or binding to the complementary sequence a t 55 ˚C for 30sec and then extension at 72 ˚C for 60 secs were achieved, 35 cycles comprised three sections as denaturation at 94 ˚C for 30 secs for each, eventual extension was fulfilled at 72 ˚C for 5min. (Kleven etal.,2004).

The separation of PCR products were conducted electrophoretically on 1.5% agarose gel for 1 hr at 80 V and tinted with ethidium bromide then photographically documented.

Statistical analysis:

Microsoft SPSS program, 2010 (**39,** Leech *et al*., 2011).

RESULTS

Mycoplasma isolation, and PCR technique

The present findings of bacterial cultivation were determined on the basis of observation with the naked eye of single colonies with characteristic fried-egg appearance through observing agar plates under the 4× and 10× microscope and confirmed by PCR analysis.

Based on DNA band size around **1500** bp of 16srRNA gene (Fig.1), 8 positive *Mycoplasma spp*. samples of trachea out of 50 (16%), while in air sacs the positive samples were 7 out of 50 (14%) at last the positive samples of choanal cleft was 1 out of 50 samples (2%) all not differed statistically as showed in table 1, obviously the peak finding was presented in the trachea samples. While Detection of uncultured *Mycoplasma spp.* positive tissues by clone analysis that confirmed by16S rRNA gene showed different ranges as follows the positive tracheal samples were 18 out of 50 (36%), air sacs displayed a 15 (30%) positive samples out of 50 finally choanal cleft tissue samples revealed 3 (6%) positive samples out of 50 also results displayed that trachea was higher in detection of *Mycoplasma spp.* comparison to the findings of air sacs and choanal cleft (Table 2). The total isolation rate of *Mycoplasma spp.* positive colonies was 16/150 (10.66%) table.1 that confirmed by 16S rRNA gene analysis (Fig. 1),

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

although the total positive tissue samples submitted with PCR cloning assay of uncultured *Mycoplasma* spp. was 36/150 (24%) table.2 with significant difference among them.

With respect of specific GapA *MG* gene, DNA band size was 332 bp, PCR molecular product was detected *Mycoplasma gallisepticum* (Fig. 2) of cultured colonies with percent of (5.33%) while tissue clone analysis recorded 14.66% positive samples with significant difference (table 3).

Table. 1. Results of isolation rate from cotton swab samples on PPLO media of presumably *Mycoplasma spp.* colonies that confirmed by molecular assay of 16SrRNA gene analysis.

Detection of Cultured Suspected Mycoplasma spp. colonies that confirmed by 16S rRNA gene		
samples	*No.*	*%*
Trachea	**8/50**	**16**
Air sacs	**7/50**	**14**
Choanal cleft	**1 /50**	**2**
total	**16/150**	**12**
X^2	**5.77**	
P value	**0.056(NS)**	

NS: No significant difference (P>0.05)

Table.2. Results PCR analysis by direct tissue clone analysis of uncultured *Mycoplasma spp.* using 16SrRNA gene analysis.

Detection of uncultured Mycoplasma spp. positive tissues clone analysis confirmed by16S rRNA gene		
Tissue samples	*No.*	*%*
Trachea	18/50	36
Air sacs	15/50	30
Choanal cleft	3/50	6
total	36/150	24
X^2	13.81	
P value	0.001(HS)	

HS: Highly significant difference (P<0.01)

Table 3. Represent comparable results of detected cultured *Mycoplasma gallisepticum* colonies and detection of uncultured *Mycoplasma gallisepticum* by cloning assay of examined

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

tissues using GapA genes

Diagnosis of MG cultured colonies using GapA gene			uncultured MG positive tissues clone analysis using GapA gene	
MG	**8/ 150	5.33%	**22/ 150	14.66%
X^2	7.259			
P value	0.007(S)			

S: significant difference(P>0.05)

**** the other identified organism data not shown**

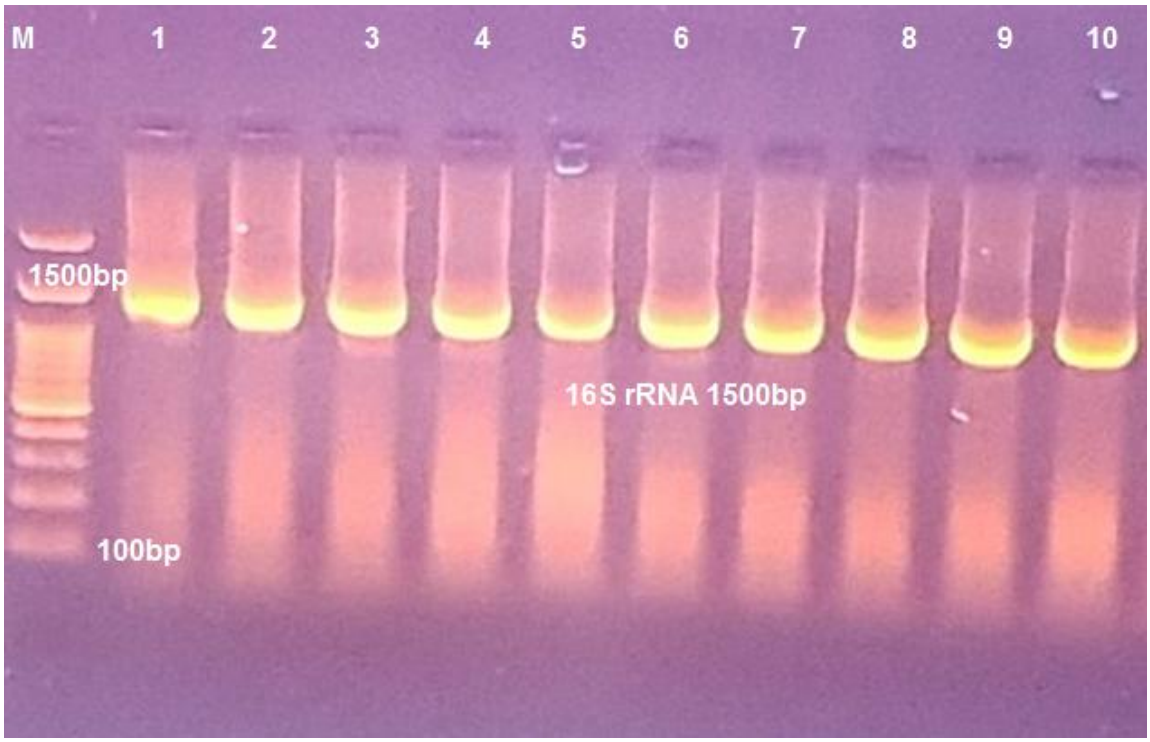

Figure 1: Agarose gel electrophoresis of PCR products of *Mycoplasma spp.* Lane M: DNA Marker; lanes 1,2,3,4,5,6,7,8,9, and 10: positive *Mycoplasma spp.* samples for 16S rRNA gene.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

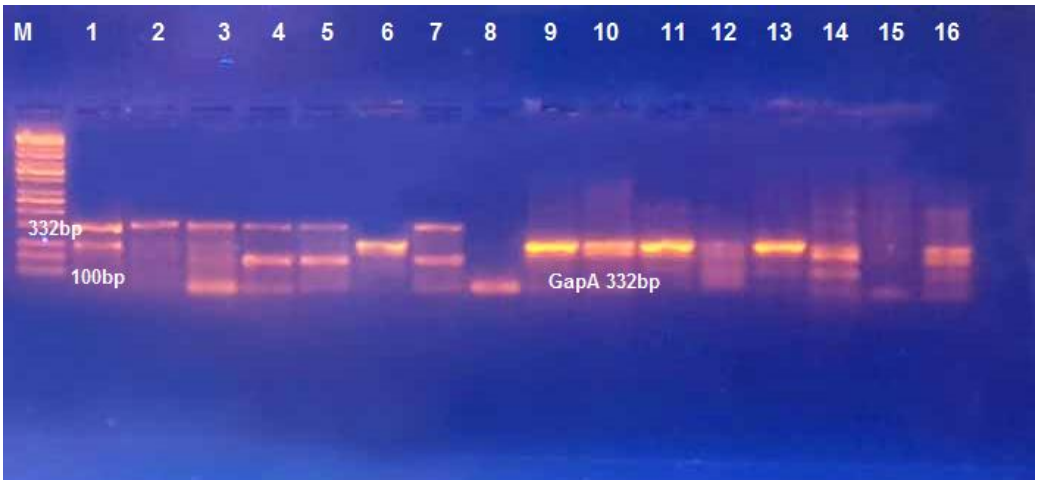

Figure 2: Agarose gel electrophoresis of PCR products of *Mycoplasma gallisepticum* (*MG*). Lane M: DNA Marker; lanes 1, 6, 9, 10,11 and 13 were positive MG for GapA gene, the other bands are nonspecific reaction.

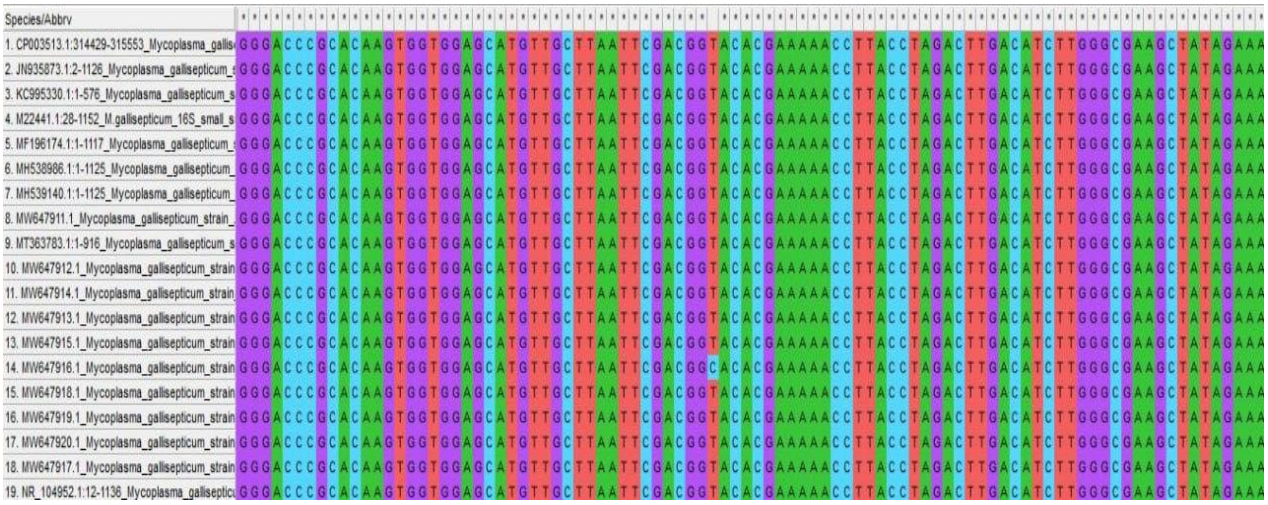

Fig.3. Analysis of Multiple sequence alignment of the incomplete 16S rRNA gene, partial sequence of indigenous *Mycoplasma* spp. 16S rRNA gene, partial sequence isolates with for NCBI-Genbank *Mycoplasma spp.* based on analysis by ClustalW alignment by using (MEGA. X, multiple alignment assay tool). The multiple alignment analysis resemblance (*) and variances in 16S rRNA gene, partial sequence nucleotide sequences.

The results of local *Mycoplasma gallisepticum* by 16srRNA genomic partial sequences were showed ten sequenced isolates 1125 bp (strain AAAQB1), 1,272 bp(strain AAAQB2), 1,185 bp (strain AAAQB3), 1,048 bp (strain AAAQB4), 1,122 bp (strain AAAQB5), 1,045 bp (strain AAAQB6), 1,039 bp (strain AAAQB7), 1,052 bp (strain AAAQB8), 1,070 bp (strain

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

AAAQB9) and 1,043 bp (strain AAAQB10) under accession number of MW647911, MW647912, MW647913, MW647914, MW647915, MW647916, MW647917, MW647918, MW647919 and MW647920, respectively and were correlated with several global strains as illustrated in Fig. 3 and table 4.

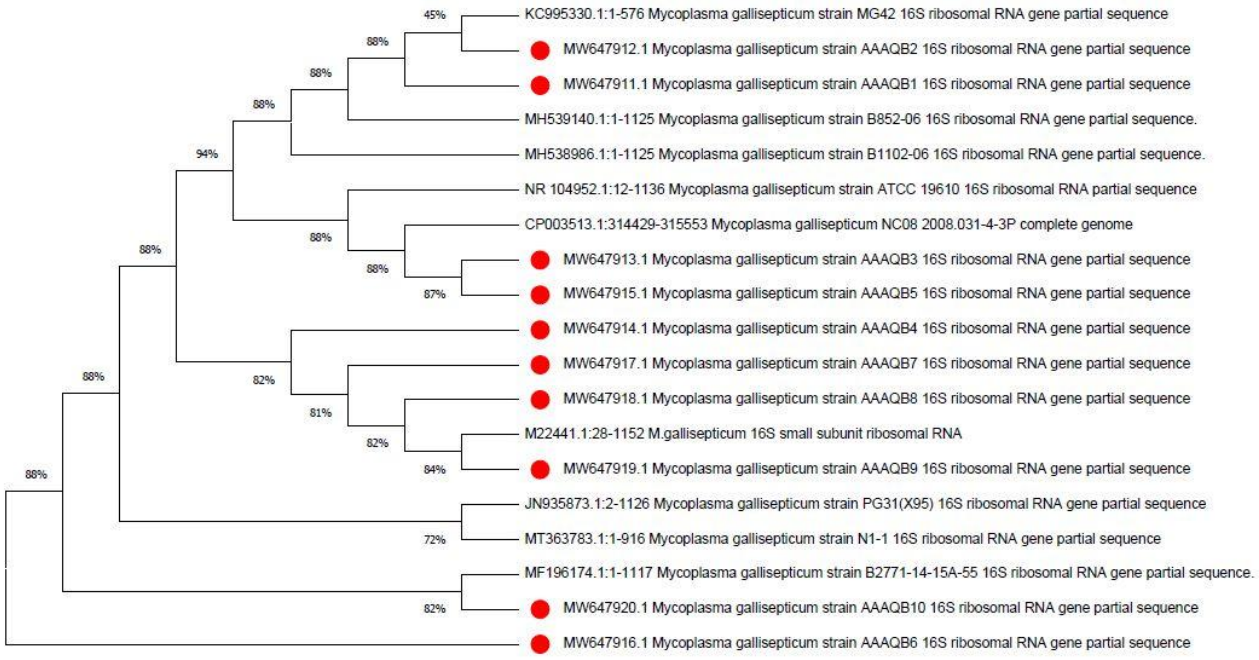

Fig.4. Analysis of Phylogenetic tree according to the 16S ribosomal RNA gene partial sequence that utilized for *Mycoplasma* sp. detection of local *Mycoplasma gallisepticum*. isolates. applying test maximum parsimony tree in (MEGA X. version).

With respect to the results of *MG* local strains the analysis of phylogenetic tree of the 16S rRNA gene nucleotides, the amplicons were correlated with world *MG* strains of Canada, USA and south Africa as showed in Fig.4, elevated matching with the 16S rRNA gene sequence of local *MG* strain compared to another globe strains deposited in the GenBank.

Table 4. Summarized the symmetrical sequence identity of local *Mycoplasma gallisepticum.* by NCBI-Blast based on 16S rRNA gene

Name of isolate	*Genbank accession number*	*NCBI BLAST identity isolate*	*accession number*	*Identity %*	*Query cover %*	*Total score*	*Country*
M.G. AAAQB1	MW647911	*M.G.* strain MG42	KC995330	100	51	1064	South Africa
M.G. AAAQB2	MW647912	*M.G.* strain B852	MH539140	99.76	100	2335	South Africa

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

M.G. AAAQB3	MW647913	*M.G.* strain B1102	MH538986	99.41	99	2150	South Africa
M.G. AAAQB4	MW647914	*M.G.* NC08	CP003513	99.43	100	3798	USA
M.G. AAAQB5	MW647915	*M.G.* strainPG31(X95)	JN935873	99.91	100	2067	USA
M.G. AAAQB6	MW647916	*M.G.* strain N1-1	MT363783	**99.02**	87	1637	Iraq
M.G. AAAQB7	MW647917	*M.G.* strain B2771	MF196174	99.23	100	1879	South Africa
M.G. AAAQB8	MW647918	*M.G.* strain ATCC 19610	NR_104952	99.43	100	1912	USA
M.G. AAAQB9	MW647919	*M.G. strain A5969*	M22441	99.53	100	1951	Canada
M.G. AAAQB10	MW647920	*M.G.* strain B2771	MF196174	99.24	100	1886	South Africa

Table 4. also showed the percent of similarity of the query sequence to the target sequences, the greater the ratio identity is the more considerable the resemblance, also displayed exactly how long the sequences are close to each other i.e. query cover.

Discussion:

Current study investigation focused on broiler flocks that showed any respiratory signs such as rales, nasal discharge face swelling, while necropsy technique revealed, air sacculitis with cloudy appearance to yellow cheesy materials in the thoracic and abdominal air sacs, increased mucus with different degrees of congestions in the tracheas with increased mortality.

The total detection was 10.66% of cultured colonies of isolated suspected *Mycoplasma* spp. using traditional technique of bacterial isolation and confirmed by 16S rRNA gene analysis, while the uncultured *Mycoplasmas* spp. was 24% of positive tissues clone analysis using 16S rRNA gene, however diagnosis of *MG* cultured colonies using GapA gene detection was 5.33% although uncultured *MG* positive tissues clone analysis using GapA gene was 14.66% this is indicating the high sensitivity of PCR of uncultured *Mycoplasma* than culture technique due to the fastidious nature of *Mycoplasma* a to grow on artificial media of cultivation method and this is supported by several workers (**40,**Yilmaz etal., 2011; **41,** Rauf etal., 2013) they suggest that

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

recognition of *MG* by PCR technique had significant difference than traditional culture isolation methods, also Yilmaz and collaborators they proved that some examined samples were positive with PCR and negative for culture media, suggesting a mild sensitivity of cultivation than PCR technique (**40,** Yilmaz etal., 2011) as showed by current study results.

Several reports indicate that *MG* have influences on the commercial poultry production internationally (**42,** Raviv and Ley,2013). In Iraq, according to the **43,** Ali and Ali, (2019) and **44,** Al-Mahmoudi etal., (2020) *MG* infections are reflected as one of the most common spreading bacterial pathogen among poultry farms with significant impacts on performance of poultry and owner's profitability, although local studies concerning this organism and related issues is still modest and less than ambition and need more investigations.

A study conducted in Baghdad/Iraq, Ali and Ali reported that out of 200 samples the total rate of *MG* was (10%) of examined positive cultures and out of twenty positive *Mycoplasma* spp isolates only 10% were found to be corresponding to *MG* by Pcr assay using Mgc1 gene (**43,** Ali and Ali,2019). Pointing the gene of *16S rRNA* partial gene sequencing (643bp), very high percentage recorded by Al-Mahmoudi and coworkers they detected *M. gallisepticum* in 90% of collected tested samples of chickens (**44,** Al-Mahmoudi etal.,2020), *MG* incidence of 23.2% in broiler chickens has been recorded by (**45,** Seifi and Shirzad.2012), however, Rasoulinezhad and co associates has detected *M. gallisepticum in* 48.38% of total collected samples from Iran using Mgc2 gene (**38,** Rasoulinezhad et al., 2017). In the current study GapA gene was chosen because it is supported by several previous studies and it was formerly proved that the GapA gene is more preserved than mgc2 (**36,** Garcia et al., 2005).

Our findings are not identical with the previous studies, that the *MG* prevalence was 5.33**%** according to the positive cultures but **14.66%** according to the Pcr results, these results may refer to the management and control programs need review the procedures pursued in the poultry farms in a standard and regular manner in the Al-Dewaniyah province and related regions. The need to establish compulsory strategies to be applied by these poultry producers and under the auspices and control of the official authorities has become a necessary issue nowadays also main suppliers must have guaranteed their breeder flocks are free of *Mycoplasmas* in this context Buim and co-workers recorded the decline for *MG* in Layers and breeders, they attributed the reason to the powerful control and vaccination practice against *MG* (**46,** Buim

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

etal.,2009).

In the current study, the diagnosis of *MG* isolated from the trachea utilizing PCR was greater **36%,** than in the air sac and choanal cleft tissues, at **30%** and **6%**, respectively these results are corresponding with other studies (**14,** Carli and Eyigor, 2003; **47,** Abd El-Ghany, 2008). related studies were performed by **7,** Elbehiry et al. (2016), they observed 70-75% approval value of *MG* recognition between culture and PCR results for several respiratory related organs assembled from diseased chickens, the positive culture was 55.8% from the trachea, along with 20.7% and 16.9% from the air sac and the lungs, respectively, while, PCR findings of trachea, air sac, and lungs exhibit different percentages as follows 79.4%, 28.3%, and 22.6%, respectively, these results are in agreement with present findings.

Yasmin and co-authors reported that there is elevated prevalence of *MG* infection/colonization of examined poultry farms in Malaysia, as the majority of the investigated farms were reactively positive, the prevalence rate in layer breeders was 45% while, in broiler chickens was 33% by using specific primer for GapA (**3,** Yasmin etal.,2014).

The results of present study showed that the PCR assay of 16S rRNA provide very good amplification of *MG* DNA (also for other prokaryotes, data not shown) this finding is supported by Kahya and co- partners which they demonstrated that the use of 16S rRNA gene sequencing assay for detection of cultured or uncultured bacterial prokaryotes was sensitive and specific (**48,** Kahya etal.,2015). However, there is obvious symmetric of the 16S rRNA gene of *MG* and *M. imitans* (**17,** Kempf,1998; **49,** Markham etal.,1999), and the primers that are seek out the 16S rRNA gene amplify both species also other prokaryotes. however, the molecular close relation between the two *Mycoplasma* species are unimportant for investigative studies, and diagnostic procedures, because *M. imitans* is commonly habitats water birds while has not been found in different industrial poultry farms (**36,** Garcia etal.,2005),

The specific amplification of *MG* GapA gene generated a specific PCR yield of 332 bp. Nevertheless, our molecular analysis recorded a nonspecific PCR product band of 200 bp. However, is not confused our results of the specific GapA because it simply differentiated by size also can be discriminated by nucleotide sequence (**36,** Garcia etal.,2005), also they proved that the Blast sequence assessment of the 200-bp**,** exhibit no considerable identical with the DNA

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

genome of *MG* (**36,** Garcia etal.,2005), however, the explanation of this nonspecific product was considered as naturalistic interior govern for the GapA gene amplification as stated by Garcia and coworkers.

The remarkable rate of sequence resemblance (strongly associated alignment) was declared between Iraqi isolate with Canada, USA and south Africa isolates, suggesting the epidemiological correlation of Iraqi *MG* and other globe strains, indicating inefficient or feeble measures of biological security policies (**38,** Rasoulinezhad etal.,2017). The everywhere travelling birds of the world may have a great role in *MG* distribution from region to another, and this was confirmed in Eastern North America in which passerines have been observed to carry and spread *MG* (**50,** Dhondt et al., 2008; **501,** Staley etal.,2018). PCR technique able enough to recognize both uncultured *Mycoplasma* colonized tissues and growing *Mycoplasma* colonies in culture media and other bacterial species, on other hand 16S rRNA can distinguished between *Mycoplasma* species relying on the sequence information (**34** Ferguson etal.,2005; **52** Ghaniei, 2016; **53** Ponnusamy etal.,2018).

The sequencing of DNA provides nearly 1000 - 1300 nucleotides of 16S rRNA gene (NCBI website), NCBI BALSTN analysis, multiple sequence alignment MSA alongside phylogenetic tree (Fig. 3 and 4) affirmed that the local *Mycoplasma spp*. adjusted with each other, these findings might propose they came from same predecessors as they may have created and transmitted from certain zones of the globe; however , undistinguishable few isolates/strains to another isolates/strains from the specific districts of world may have adjusted in different branches of the phylogenetic tree may demonstrate a mutated process driving to development in to some degree variable strain and this may be interpreted due to occurring of numerous partial duplicates in conjunction with changing of zone that encode the protein ends, this changeability made the capacity to deliver tens or indeed hundreds of thousands of varied isolates (**54** Browning etal.,2010),so diverted strain of *Mycoplasma* may play a vital part at certain phases in evolutionary tree (**55,** Woese etal.,1980).

The capacity to adjust sequencing of 16S rRNA to appraise of the similitudes or variations between *Mycoplasmas* phylogenetic affiliations is undeniable (**56**, Olsen, and Woese. 1993), moreover can help in genus classification (**57,** Pettersson etal.,1994)

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

As *Mycoplasmas* have elevated change rates, it could be proposed that they are in a condition of fast evolution (**58,** Rogers etal.,1985), and as proved by Delaney and his collaborators their results suggest that evolutionary progression of *Mycoplasma gallisepticum* genome might be very fast, they proved high variety and whole change of CRISPRs in chickens *MG* strains preceding the disastrous transmission to the wild birds concurrent with ongoing loss of diversity CRISPR repeats and may be go to loss the functional process of CRISPRs (**59,** Delaney etal.,2012)

The achievement of *MG* control strategies be contingent on correct and quick detected methods, where current results indicating the *Mycoplasma gallisepticum* is prevalent and spread in broilers was proved either by culturing or molecular examination, this dissemination may be due to lacking of biosecurity also suggesting that the source of chicks is clinically or sub clinically infected, on this basis, it requires exerting great efforts for the purpose of implementing the strategies of biological protection of poultry farms.

Conclusion

Current results specify that the PCR assay realized as a dependable tool for early, preliminary and even final discovery of field pathogens. On the basis of the analysis of phylogenetic tree of 16S rRNA incomplete nucleotide sequences of *MG* Iraqi strains/isolates, and international strains, it be able to be presumed that Iraqi strains have high identical similarity, as of the global strains.

Based on current data It should be essential for more studies carried out on other important *MG* genes for more recognition and description the local *MG* field strains. This information it will help in the developing and choosing the type of vaccine to be applied as it considered as a significant part in the biosecurity.

also the study advised that firmly practical prevention and control programs against avian Mycoplasmosis and the use of PCR on a large scale to help disease extermination programs to reduce economic damages in fowl farms.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

References:

1- Sasaki, Y., Ishikawa, J., Yamashita, A., Oshima, K., Kenri, T., Furuya, K., Yoshino, C., Horino, A., Shiba, T., Sasaki, T., & Hattori, M. (2002). The complete genomic sequence of Mycoplasma penetrans, an intracellular bacterial pathogen in humans. Nucleic acids research, 30(23), 5293–5300. https://doi.org/10.1093/nar/gkf667.
2- Brown, D.R., R.F. Whitcomb, and J.M. Bradbury. 2007. Revised minimal standards for description of new species of the class Mollicutes (division Tenericutes). Int J Syst Evol Microbiol. 57:2703-2719.
3- Yasmin, F., Ideris, A., Omar, AR., Hair-Bejo, M., Tan, SW., Tan, CG., Ahmad, K., 2014. Molecular detection of Mycoplasma gallisepticum by real time PCR. Jurnal Veterinar Malaysia, 26(1), 1–7.
4- Yasmin F., Aini Ideris, Abdul Rahman Omar, Mohd Hair Bejo, Rakibul Islam, Tan Sheau Wei, Tan Ching Giap and Kartini Ahmad (2018). Molecular characterization of feld strains of Mycoplasma gallisepticum in Malaysia throughpMGA and pVPA genes sequencing. Cogent Biology, 4: 1456738. doi.org/10.1080/23312025.2018.1456738.
5- Charlton, B.R., Bermudez, A.J., Boulianne, M., Eckroade, R.J., Jeffrey, J.S., et al. Avian Disease Manual. In: Charlton, B.R. (Ed.), American Association of Avian Pathologists, Kennett Square, Pennsylvania: USA; 1996, p. 115–125.
6- Levisohn S, and Kleven SH. 2000. Avian mycoplasmosis (M. gallisepticum). Rev Sci Tech. 19(2):425–442.
7- Elbehiry, Ayman; M. Al-Dubaib and E. Marzouk. (2016). Serological, Rapid Molecular Characterization and Antibiotic Resistance for Field Isolates of Mycoplasma Gallisepticum in Chicken in Saudi Arabia. Alexandria Journal of Veterinary Sciences 49 (2): 70-79. doi: 10.5455/ajvs. 224786.
8- Papazisi, L., Gorton, T.S., Kutish, G., Markham, P.F., Browning, G.F., Nguyen, D.K., Swartzell, S., Madan, A., Mahairas, G., Geary, S.J. 2003. The complete genome sequence of the avian pathogen Mycoplasma gallisepticum strain R(low). Microbiology, 149: 2307– 2316.
9- Liu, T., Garcia, M., Levisohn, S., Yogev, D., Kleven, S.H., 2001. Molecular variability of the adhesinencoding gene pvpA among Mycoplasma gallisepticum strains and its application in diagnosis. Journal of Clinical Microbiology 39, 1882-1888.
10- Kaboli, K. P.; Bijanzad, A. R.; Moggadam, J.; Shahbazi, M. and Hosseini, H. (2013). Evaluation of Mycoplasma gallisepticum infection diagnosis in rural poultry by 16SrRNA PCR methods. European Journal of Zoological Research, 2 (4):63-66.
11- Khalifa, K.A., Abdelrahim, E.S., Badwi, M., Mohamed, A.M. 2013. Isolation and Molecular Characterization of Mycoplasma gallisepticum and Mycoplasma synoviae in Chicken in Sudan. J. Vet. Med. 2013: 1–4.
12- Barua, S.R., Prodhan, A.M., Chowdhury, S. 2006. Study on Mycoplasma gallisepticum in Chicken in selected areas of Bangladesh. Banglad. J. Vet. Med. 4: 141– 142.
13- Purswell, J.L., Evans, J.D., Leigh, S.A., Collier, S.D., Olanrewaju, H.A., Kim, E.J., Pharr, G.T., Peebles, E.D., Branton, S.L. 2012. Mycoplasma gallisepticum transmission: Comparison of commercial F-strain vaccine versus layer complex-derived field strains in a tunnel ventilated house. Poult. Sci. 91: 3072–3079.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

14- Carli, T., Eyigor, A. 2003. Real-time polymerase chain reaction for Mycoplasma gallisepticum in chicken trachea. Avian Dis. 47: 712–717.
15- Ehtisham-ul-Haque, S., Rahman, S.U., Khan, M.I., Younus, M., Awais, M.M., Nasir, A. 2015. A simplified duplex real-time PCR incorporating TaqMan minor groove binder (MGB) probes and an exogenous internal positive control for the simultaneous detection of Mycoplasma gallisepticum and Mycoplasma synoviae cultures. J. Vet. Med. 60: 268–273.
16- Raviv, Z., Kleven, S.H. 2009. The development of diagnostic real-time TaqMan PCRs for the four pathogenic avian mycoplasmas. Avian Dis. 53: 103– 107.
17- Kempf, I. 1998. DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis. Avian Pathol. 27: 7–14.
18- Kleven, S.H. 1997. Changing expectations in the control of Mycoplasma gallisepticum. Acta Vet. Hung. 45: 299-305.
19- Cremonesi, P., Vimercati, C., Pisoni, G., Perez, G., Miranda Ribera, A., Castiglioni, B., Luzzana, M., Ruffo, G.and Moroni, P., 2007. Development of DNA extraction and PCR amplification protocols for detection of Mycoplasma bovis directly from milk samples. Veterinary Research Communications, 31(Suppl. 1), 225–227.
20- Kleven, S.H. 2008.Mycoplasmosis. In: A laboratory manual for the isolation, identification and characterization of avian pathogens, 5th ed. L. Dufour-Zavala, D. E. Swayne, J. R. Glisson, J. E. Pearson, W. M. Reed, M. W. Jackwood, and P. R. Woolcock, ed. American Association of Avian Pathologists, Athens, GA. Jacksonville, Florida. pp 59-64.
21- Kizil, O., & Ozdemir, H. (2006). Clinical, haematological and biochemical studies in goats naturally infected with Mycoplasma agalactiae. Bull Vet Inst Pulawy 50, 325-328.
22- Khalifa, R., Eissa, S., El-Hariri, M., Refai, M., 2014. Sequencing analysis of M. gallisepticum wild strains in vaccinated chicken breeder flocks. J Mol Microbiol Biotechnol 24, 98-104.
23- Sabry, M.Z. 1968. Characterization and classification of avian Mycoplasmas. Ph.D. Thesis, Cornell University USA; P. 244.
24- Razin, S., D. Yogev, and Y. Naot. (1998). Molecular biology and pathogenicity of mycoplasmas. Microl Molec Biol Rev 62:1094—1156.
25- Quinn, P. J., Carter, M. E., Markey, B., and Carter, G. R. (2002). The Mycoplasmas, In: Clinical Veterinary Microbiology, Mosby, Virginia Tech, and Blacksburg, USA. Pp. 320-326.
26- Boonyayatra, Sukolrat; Lawrence K. Fox, John M. Gay; Ashish Sawant; Thomas E. Besser .(2012). Discrimination between Mycoplasma and Acholeplasma species of bovine origin using digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase chain reaction. Journal of Veterinary Diagnostic Investigation 24(1) 7–13.
27- Aluotto, B. B., Ruth G. Wittler, Carol, O. Williams and John E. Faber. (1970). Standardized bacteriologic techniques for the characterization of Mycoplasma Species. International Journal of Systematic Bacteriology, 20:1, 35-58.
28- Poveda, J.B. (1998). Biochemical characteristics in Mycoplasma identification,

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

Mycoplasma protocol; In: Methods in Molecular Biology, Vol.104, edit. R.J. Miles and R.A.J. Nicholas, Humana Press Inc. Totowa, NJ.

29- Michael, F. B. (1983). Biochemical and enzymatic test in Mycoplasma identification. Vol 1. ed.by Razin, S.; and Tully, G. Academic Press. 345-347.

30- Santha, I. M., K. K. Koundal and S. L. Mmekta (1990). Biochemical and bio-physiological techniques used in recombinant DNA work. In genetic engineering and biotechnology concepts, Methods and application (EDS). Calcutta, India, 9-12.

31- Khan, M. 2002, Multiplex Pcr of avian pathogenic Mycoplasmas. In: Methods in Molecular Biology, vol.216: PCR Detection of microbial pathogens: Methods and Protocols, edited by: K. Sachse and J. Frey, Humana Press Inc., Totowa, NJ.

32- Gray, Larry D., Kerry L. Ketring, and Yi-Wei Tang. (2005). Clinical Use of 16S rRNA Gene Sequencing to Identify Mycoplasma felis and M. gateae Associated with Feline Ulcerative Keratitis. J. Clin. Microbiol., 43 (7):3431-3434.

33- Raji, A. I.; C. Moller; D. Litthauer, E. van Heerden and L. A. Piater. (2008). Bacterial diversity of biofilm samples from deep mines in South Africa. Biokemistri 20(2):53-62.

34- Ferguson, N.M., Hepp, D., Sun, S., Ikuta, N., Levisohn, S., Kleven, S.H., Garcia, M., (2005). Use of molecular diversity of Mycoplasma gallisepticum by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies. Microbiology 151, 1883-1893.

35- Fujisawa, S., Shiro Murata1 , Masaki Takehara, Ken Katakura, Myint Myint Hmoon, Shwe Yee Win and Kazuhiko Ohashi.(2019). Molecular detection and genetic characterization of Mycoplasma gallisepticum, Mycoplama synoviae, and infectious bronchitis virus in poultry in Myanmar. . BMC Veterinary Research ,15:261-268.

36- Garcia, M., Ikut, N., Levisohn, S., Kleven, S.H. 2005.Evaluation and comparison of various PCR methods for detection of Mycoplasma gallisepticum infection in chicken. Avian Dis. 49: 125–132.

37- Kleven, S.H., R.M. Fulton, M. Garcia, V.N. Ikuta, V.A. Leiting, T. Liu, D.H. Ley, K.N. Opengart, G.N. Rowland, and E. Wallner-Pendleton. 2004. Molecular characterization of M. gallisepticum isolates from turkeys. Avian Dis. 48:562-569.

38- Rasoulinezhad Saeed, Mohammad Hassan Bozorgmehrifard, Hossein Hosseini, Nariman Sheikhi, Saeed Charkhkar.2017. Molecular detection and phylogenetic analysis of Mycoplasma gallisepticum from backyard and commercial turkey flocks in Iran. Veterinary Research Forum. 8 (4) 293 – 298.

39- Leech, R.; Kamourieh, S.; Beckmann, C.F.and Sharp, D.J. (2011). Fractionating the default mode network: distinct contributions of the ventral and dorsal posterior cingulate cortex to cognitive control. J Neurosci., 31:3217–3224.

40- Yilmaz, F.; Timurkaan., N.; Kilic., H.; Kalender., and Kilinc, U. (2011) detection of Mycoplasma synoviae and Mycoplasma gallisepticum in chickens by immunohistochemical, PCR and culture. Revue Med. Vet. 162(2) 79-86.

41- Rauf, M.; Chaudhary Z. I.; Younas M.; Anjum, A. A.; Ali, M. A.; Ahmad, A. N. and Khan, M. U. R., (2013). Identification of Mycoplasma gallisepticum by polymerase chain reaction and conventional diagnostics from white leghorn layer flocks. J. Anim. Plant Sci. 23(2): 393-397.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

42- Raviv, Z., and D.H. Ley. 2013. Mycoplasma gallisepticum infection. In: Diseases of Poultry. D.E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez and V.L. Nair, eds. Wiley-Blackwell, Ames, Iowa. pp 877-893.
43- Ali, E. J.; B. H. Ali. (2019). Isolation, Identification And Sequencing Of Mycoplasma Galisepticum By Culture And PCR In Baghdad City, Iraq. Indian Journal of Public Health Research & Development, 10, (8): 936-941.
44- Al-Mahmoudi, A. H. J.; H. A. Hammadi,; H. N. Ayyez, I. N. A. Al-Ibadi, H.M. Mutter and A. J.n Neamah. (2020). *Mycoplasma gallisepticum* based molecular and phylogenetic studies of infected chicken farms in Iraq. Plant Archives, 20(2), 4279-4282.
45- Seifi S, Shirzad MR. 2012. Risk factors and seroprevalence of Mycoplasma gallisepticum infection in broiler breeder farms in Mazandaran province, north of Iran. Revue Med Vet; 163(5): 215-218.
46- Buim, M. R., E. Mettifogo, J. Timenetsky, S. Kleven, and A.J.P. Ferreira. 2009. Epidemiological survey on Mycoplasma gallisepticum and M. synoviae by multiplex PCR in commercial poultry. Pesq. Vet. Bras. 29(7):552-556.
47- Abd El-Ghany, W.A., 2008. Diagnostic investigation on M. gallisepticum infections in different Egyptian breeder and broiler chicken flocks. J. Egyp. Vet. Med. Assoc., 68: 29-45
48- Kahya, S; Yılmaz O, Eyigor A, Temelli S, Carlı KT. (2015). Detection of Mycoplasma gallisepticum and Mycoplasma synoviae by Real-Time PCRs and Mycoplasma gallisepticum-antibody detection by an ELISA in chicken breeder flocks. Kafkas Universitesi Veteriner Fakültesi Dergisi.,21(3):361-366.
49- Markham, P. F., M. F. Duffy, M. D. Glew, and G. F. Browning. 1999. A gene family in Mycoplasma imitans closely related to the pMGA family of Mycoplasma gallisepticum. Microbiology 145:2095-2103.
50- Dhondt, A. A.; Dhondt, K. V.; and McCleery,B. V.(2008). Comparative infectiousness of three passerine bird species after experimental inoculation with Mycoplasma gallisepticum. Avian Pathology ,37(6), 635-640.
51- Staley, M., C. Bonneaud, K. McGraw, C.M. Vleck, and G.E. Hill, 2018. Detection of Mycoplasma gallisepticum in house fnches (Haemorhous mexicanus) from Arizona. Avian Diseases 62(1) 14-17. doi: 10.1637/11610-021317.
52- Ghaniei , A. (2016). Molecular characterization of Mycoplasma synoviae isolated from broiler chickens of West Azarbaijan province by PCR of vlhA gene. Veterinary Research Forum.,7 (3) 197 – 202.
53- Ponnusamy, P.; T. Lurthu Reetha, B.S.M. Ronald, B. Puvarajan and R. Manicakm. (2018) Detection of Mycoplasma gallinaceum by PCR amplification of the 16S rRNA gene from respiratory disease in village chickens. Indian J. Anim. Res. B-3604 (1-4) doi: 10.18805/ijar.B-3604.
54- Browning, G. F. M. S. Marenda , P. F. Markham , A. H. Noormohammadi , and K. G. Whithear. (2010). In; Pathogenesis of Bacterial Infections in Animals Fourth Edition, Edited by Carlton L. Gyles, John F. Prescott, J. Glenn Songer, and Charles O. Thoen, Blackwell Publishing, Pp549-565. USA.
55- Woese, C. R., Maniloff, J. & Zablen, L. B. (1980). Phylogenetic analysis of the

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 2719 - 2738
Received 15 April 2021; Accepted 05 May 2021.

mycoplasmas Proc. Natl. Acad. Sci. USA 77, 494-498.

56- Olsen, G. J., and C. R. Woese. 1993. Ribosomal RNA: a key to phylogeny. FASEB J. 7:113-123.

57- Pettersson, B.; Karl-Erik Johansson; and M. Uhlen. (1994). Sequence Analysis of 16S rRNA from Mycoplasmas by Direct Solid-Phase DNA Sequencing. Applied and Environmental Microbiology, 60(7) 2456-2461.

58- Rogers, M. J.; J. Simmons; R. T. Walker; W. G. Weisburg; C. R. Woese; R. S. Tanner; I. M. Robinson; D. A. Stahl; G. Olsen; R. H. Leach; and J. Maniloffi.(1985). Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. Proc. Natl. Acad. Sci. USA 82, 1160-1164.

59- Delaney, N.F., Balenger, S., Bonneaud, C., Marx, C.J., Hill, G.E., Ferguson-Noel, N., Tsai, P., Rodrigo, A., Edwards, S.V., (2012). Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, Mycoplasma gallisepticum. PLoS Genet 8, e1002511.