ISOLATION AND MOLECULAR STUDY OF NONPATHOGENIC *MYCOPLASMA* SPP. FROM BROILER CHICKENS

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ABSTRACT

The present investigation was aimed to detect the most nonpathogenic *Mycoplasmas* prevalent in broiler chickens diagnosed by molecular amplification of 16S rRNA gene from respiratory diseased broiler chickens of Al-Qadisiyah province/Iraq. A total of 150 samples of trachea, air sac and choanal cleft cotton swabs and tissue parts were aseptically collected for traditionally culturing and detection of *Mycoplasma* spp. through PCR techniques. On artificial media growth developed as raised small circular colonies exhibited a fried egg shapes with or without dark centers and were positive for Dienes stain and some biochemical tests. DNA extraction was performed from seemingly characteristic morphological colony cultures and from air sac, trachea and choanal cleft pieces by utilizing commercial bacterial and tissue DNA extraction kit, respectively. Molecular assay was performed using general primers,the size product of amplification of the 16S rRNA gene was 1500 bp length designated for many *prokaryotes* spp. The sequence analysis and phylogenetic tree indicated that detected *Mycoplasmas* were as *Mycoplasma* sp., *Mycoplasma gallinaceum* and *Mycoplasma pullorum.*Total per cent of cultured *Mycoplasma* isolates were 5.33% while total per cent of positive clone analysis was 9.33%.The PCR products were subjected to sequence technique analysis, the three different strains, proved by NCBI blast analysis.

Key words: Molecular analysis, nonpathogenic *Mycoplasma* sp., 16S rRNA gene (1500bp), *Mycoplasma gallinaceum*

Introduction

One of the most important unique prokaryotes is genus *Mycoplasma*, belong to the class Mollicutes. *Mycoplasmas* are the tiniest known organism have ability to replicate independently, despite the fact that leaking of many genes of the genome that participate in many biosynthetic activities (Bradbury, 2005). *Mycoplasmas* respiratory infection in chickens and turkeys is produced by many virulence strains of avian *Mycoplasmas* with momentous effects on poultry sector profitability,pathogenic strains for poultry includes *M. synoviae,M. gallisepticum, M. meleagridis* and *M. iowae* (Nascimento *et al*., 2005; Forrester *et al*., 2011; Adeyemi *et al.*, 2018). While the less or nonpathogenic strains of avain Mycoplasmas include several species, *Mycoplasma lipofaciens* in both chickens and turkey birds and *Mycoplasma pullorum, Mycoplasma gallinaceum* and *Mycoplasma gallinarum,* in chicken birds (Stipkovits and Kempf, 1996). Poveda and co associates (1990) reported the most frequently identified species of nonpathogenic *Mycoplasmas* with different percentages including *M. gallinarum* (27.7%), *M. gallinaceum* (17.5%), *M. pullorum* (7.4%) in addition to other *Mycoplasmas* (Poveda *et al*.,1990)

Mycoplasma serotype C isolated from birds (Abolnik and Beylefeld, 2015) then after subsequently termed *Mycoplasma pullorum* (Jordan *et al*., 1982).

Similar as other *Mycoplasmas*, *M. pullorum* strains validated with genomic diversity (Lobo *et al*., 2004). It was isolated from certain bird species including chickens, quail, partridge, pheasant and turkey, it may have pathogenic effects on chicken and turkey embryos leading to decline in the embryonic hatchability (Moalic *et al*.,1997).

While, *M. gallinarum* believed to be worldwide in spreading and are considered non-pathogenic for chickens, it regarded as contaminants throughout efforts to isolate other virulent *Mycoplasmas* from adult chickens, it characterized by rapid growth on culture media in comparison with the growth of *M. gallisepticum (*Raviv and Ley, 2013), however there is one report related with air sacculitis in a broiler flocks but with concurrent Newcastle disease or infectious bronchitis vaccination (Koshimizu *et al*.,1982).

It has common features like other *Mycoplasmas* regarding colonial shape, deficiency of a cell wall, and the need for the presence of sterols,biochemical characteristics include negative glucose test, but positive totetrazolium, and arginine decarboxylase, and shows film and spots formation (Barber and Fabricant, 1971). RFLP technique demonstrate genetic heterogeneity among different strains/isolates (Dovc *et al.,* 1991) it mainly isolated from chickens, however, it found in turkeys (Jordan and Amin, 1980), forest fowl (Shah-Majid, 1987) and pigeons (Reece *et al*.,1986).

Regarding *Mycoplasma gallinaceum MGC* which also considered as nonpathogenic with no serious economic losses (Stipkovits and Kempf. 1996). *MGC* is often found as a contaminant throughout efforts to investigate of pathogenic *Mycoplasma gallisepticum* and/or *M. synoviae* in chickens, however it has the ability to exacerbate respiratory manifestations concurrent with IBV infection in chickens, also they proved that *MGC* improve replication of IBV in the infected chicken's trachea, despite of that *M. gallinaceum* usually have very low pathogenicity in chickens (Adeyemi *et al*., 2018), although, *MGC* is associated with respiratory disease of pheasants and partridges (Bradbury *et al*., 2001).

Throughout an evaluation screening study achieved in South Africa in 2014 and 2015, *Mycoplasma* spp.were obtained

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by bacterial culture from chicken flocks exhibiting characteristic respiratory findings of *Mycoplasma* infection, in addition to declined weight gain, feed convertibility ratio efficiency, moreover producing of egg in layer and breeder chicken flocks was decline, and was isolated from many cases approximately 23% of diseased cases as pure colonies on the cultured media (Adeyemi *et al*., 2018).

Mycoplasma gallinaceum reactively high-quality to glucose fermentation, poor to tetrazolium chloride, arginine or urea hydrolysis (Jordan *et al*.,1982), it colonized upper respiratory tract, eyes, sinuses, and reproductive tract (Bradbury *et al*.,2001; Wang *et al.*, 1990). The genome of *M. gallinaceum* strain B2096 8B was 845, 307 bp in length, with a GC content of 28.38% (Abolink and Beylefeld, 2018).

Materials and Methods

Clinical samples

One hundred and fifty cotton swabs and 150 tissue parts of trachea, air sacs and choanal cleft were collected from broiler chicken farms located at different parts of Al-Dewaniyah province/Iraq. Each of both types of samples were taken from the same bird.

Culture Technique

Swab samples were plated directly onto PPLO broth until color change with very light turbidity then streaked on PPLO agar provided with required supplements, the plates were incubated in 5% CO_2 atmosphere in candle jar and observed every 2 days under microscopeat 4x and 10x power (Bradbury *et al*., 1993; Kleven, 2008; Kizil and Ozdemir, 2006; Khalifa *et al*., 2013; Jalaladdini *et al*., 2014).

All media were considered as negative after 7 days of incubation (Razin and Vincent, 1983; Beylefeld *et al*., 2018), each fried egg colony was sub-cultured by cut out an block agar piece containing a separated colony then upturned it surface-down on the new agar plate superficies of PPLO media, fried egg colony was once more picked same way as above and inoculated in PPLO broth medium, if a color of broth media were changed the broth was cultured again on PPLO agar medium (Al-Azawi, 2012) for more purification an ascertain there is no other bacterial or fungal contamination.

Staining of *Mycoplasma* colonies method

To examine the morphology of the supposed *Mycoplasma* colonies they were stained by Dienes stain according to Razin *et al*. (1998) and Quinn *et al*. (2002).

Biochemical

Digitonin diffusion test was done according to Boonyayatra *et al*. (2012).

PCR clinical samples

Tissue samples from trachea, air sacs and choanal cleft from chickens showing respiratory signs and lesions were collected carefully in sterile capped tubes to avoid contamination as much as possible, all samples were transferred to the laboratory on ice box and handled within 24-48 hrs of arrival for DNA extraction (OIE, 2008; Islam *et al*., 2011; Rauf *et al*., 2013).

PCR assay by clone and positive cultures of *Mycoplasma*

Clone analysis or Direct detection achieved by submitting different collected tissues for molecular analysis (Tomar *et al*., 2017) and indirect analysis include any positive culture showed characteristic shape of *Mycoplasma* colonies, also positive for Dienes stain and digitonin test, would be submit to analysis by PCR technique for confirmation (Gharaibeh and Hailat, 2011).

Isolation of genomic DNA

The isolation of genomic DNA from cultured *Mycoplasma* colonies strains were conducted using the bacterial DNA extraction and tissue DNA extraction kits (Anatolia, turkey) according to the instructions of the manufacturer. The quality of the isolated genomic DNA was evaluated after running of the DNA on 1% agarose gel followed by ultra-violet (UV) visualization (UV-Transilluminator, Clever, UK). However, the quantity of the isolated genomic DNA was estimated by the aid of Nano-drop Spectrophotometer (Thermo, USA).

Molecular Identification

Amplification of 16S rRNA gene was amplified from the genomic DNA by PCR using universal primers F8-27 (5-AGAGTTTGATCCTGGCTCAG-3) and R1510-1492(5-GGTTACCTTGTTACGACTT-3) of *Escherichia coli* 16SrRNA gene (Edan *et al.,*1991; Gray *et al*., 2005; Raji *et al*., 2008). The reaction mixture (50 μL) contained 4 μL (25 ng genomic DNA), 25 μL of Dream Taq Green Master Mix 2X (ABM Co., Canada), 1.5 μL (15 pmol) of each forward and reverse primer, and 18 μL of nuclease free water. Thermocycler primus 25 (IDT, Canada) was used in this study. PCR conditions consisted of an initial denaturation at 95°C for 5 min, 30 cycles: each cycle consisted of denaturation at 94°C for 30s, annealing at 55°C for 45s,extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. The amplified PCR product was analyzed *via* agarosegel electrophoresis. After that, the PCR product was purified using Wizard SV Gel and PCR Clean-Up kit, ABM Co., Canada.Then, the purified PCR product was sequenced directly as sequencing templates using the same primers. Sequencing was carried out at Eurofin DNA Co., Luxembourg.

The obtained nucleotide sequence was edited and analyzed by finch TV version 1.5.A partial nucleotide sequence ranged between 900-1300 bp was utilized to explore the international nucleotide databases (*e.g*., GenBank, EMBL, DDBJ, etc.) through BLASTN (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information) to determine the relative phylogenetic position of the bacterial isolate of inquiry. Moreover, the phylogenetic tree depicting the phylogenetic affiliation of the isolate of inquiry was constructed *via* Mega x software.

Results and Discussion

Current clinical investigation focused on broiler flocks that showed any respiratory signs such as rales, nasal discharge face swelling with increased mortality.

Our laboratory work technique for *Mycoplasma* isolation showed several constraints beginning from special growth requirements, expecting other bacterial and fungus contaminations, and prolonged periods of stressful work with uncertain growth results.

Isolation of *Mycoplasmas*

In this study, different clinical samples including, cotton swab and tissues samples of tracheal and air sac and choanal

cleft were collected from randomly selected broiler chickens of several flocks that clinically suffered from respiratory signs at different areas in Al-Dewaniyah province for detecting *Mycoplasmas*.

The culture media revealed a typical fried eggs colony, although colonies with less or no centers were found also as illustrated in Fig.1, this feature was also found by Nicholas *et al.* (2008), Khalifa *et al*. (2013) and Ali and Ali (2019).

Mycoplasma staining technique exhibit characteristic stained blue colonies with dark blue centers under microscope presented in Fig. 2. and give positive for digitonin tests.

The traditional laboratory work and after many sub culturing all suspected colonies subjected to PCR technology for species identification, results showed that the positive nonpathogenic *Mycoplasma* isolates were 8 out of 150 cotton swab samples with rate of 5.33%, while the clone PCR analysis revealed that the total positive nonpathogenic *Mycoplasmas* were 14 out of 150 (9.33%) of tested different tissue samples,the total percentage of detection of *Mycoplasmas* by indirect method (culturing) and direct tissue samples (clone analysis) are not equal, however only nonpathogenic *Mycoplasmas* presented in this paper while other detected *Mycoplasma* isolates are not presented here.

Present study was able to isolate pathogenic (data not presented in this paper) and nonpathogenic *Mycoplasma* sp. including; *Mycoplasma gallineceum, M. pullorum* and *M.* spp. whereas *Mycoplasma gallineceum* and *M. pullorum* are considers as non-significant economic losses agents and least infectious Mycoplasmosis for chicken industry also they are most prevalent and most frequently isolated species on a chicken farm and other domesticated birds as formerly several studies proved this facts (Shimizu *et al*., 1979; Jordan,1983; Bencina *et al*., 1987a,b; Poveda *et al*.,1990; Raviv and Ley, 2013), although several available researches have been submitted evidences in the collaboration between nonpathogenic *Mycoplasmas* mixed infections with respiratory viral diseases or even during traditional vaccination against viral diseases that can advance air sacculitis (Leigh and Evans, 2009), with pathological lesions in the tracheal epithelium (Shah-Majid,1987).

In fact, there is little attention about less or nonpathogenic *Mycoplasma* in chickens and turkeys but there is an issue recently recorded by Beylefeld *et al*. (2018) which is the problem of drug resistance of these organisms and it seems to be a matter of concern because they proved the presence of more antibacterial resistance in these species comparably than MG and MS. Moreover, many strains of *M. gallinaceum* was distinguished by its possession a multiple antimicrobial resistant trait, this would have momentous consequences or even dreadful results if these species, are proficient or possess the power or ability to transmit their antibacterial genomic resistance to different *Mycoplasmas* or even to other bacteria (Beylefeld *et al*., 2018). This trait augments their undesirable effects such as possible drug resistance transferring to the pathogenic *Mycoplasma gallisepticum* MG and *Mycoplasma synoviae* MS in chickens and turkey, they recorded strong connection of macrolide resistance in MG and MS, and in *M. gallinaceum* MGC and *M. gallinarum* MG (Beylefeld *et al*., 2018), however advance research is essential to ascertain the mechanism of pick up resistance of these *Mycoplasmas*.

The positive cultures were 8 positive isolates out of 150 cotton swab samples with rate of 5.33%, confirmed by PCR technology, whereas the clone PCR analysis showed that the total positive samples for nonpathogenic *Mycoplasma* was 14/150 (9.33%), although statistically not significant; X2=1.766; P value = 0.184 (NS) but still there is variations in the ratios indicating that PCR is more sensitive.

While gene sequences displayed three species of nonpathogenic *Mycoplasmas*, although the isolation rate of current study may differ from other workers (Poveda *et al*.,1990; Bradbury *et al*., 2001; Adeyemi *et al*., 2018).

The results of DNA 16s rRNA sequence analysis revealed theses isolated strains are three different types with different rates as showed in Fig. 3 and Fig. 4.

The diagnosed cultured isolates include the following species, *Mycoplasma sp., M. pullorum Mp* and *M. gallinaceum MGC*, counted 2, 2, 4, out of 8 isolates with ratio of 1.33%,

Fig.1: Mycoplasma colonies with fried egg shaped on PPLO agar

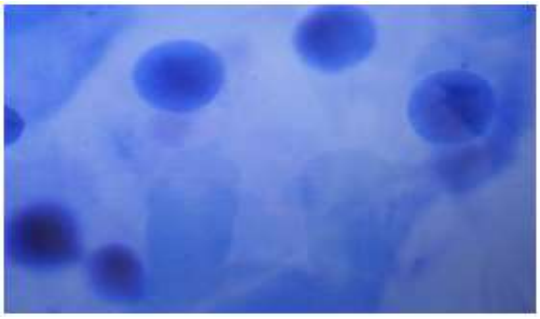

Fig. 2: *Mycoplasma* fried egg colonies on PPLO agar (Dienesstain)

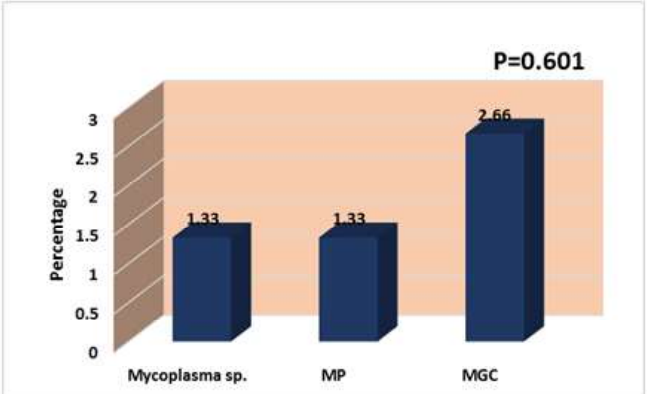

Fig. 3: Results of diagnosis of *Mycoplasma* spp. by PCR technique of isolated suspected colonies, No significant difference (P>0.05)X2=1.018.

1.33% and 2.66%, respectively as showed in Fig. 3, while clone analysis and sequencing of examined respiratory tissues displayed same cultured non pathogenic *Mycoplasmas,* in addition to other detected bacteria and *Mycoplasma* species (data not showed) but apparently with different per cent as showed in Fig. 4. *Mycoplasma sp., M. pullorum Mp* and *M. gallinaceum* MGC, they were 4,3,7 out of 14 positive samples with percentage of 2.66%, 2%, 4.66%, respectively, although statistically there is no important differences but there is numerical variations, according to these results the higher detected *Mycoplasma* was MGC.

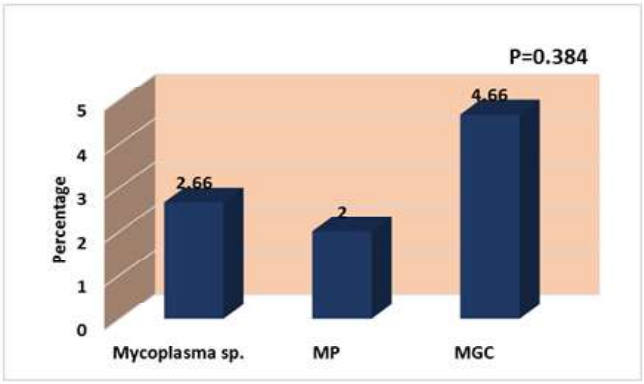

Fig.4: Results of diagnosis of *Mycoplasma* spp. PCR cloning assay, No significant difference (P>0.05) X2=1.917.

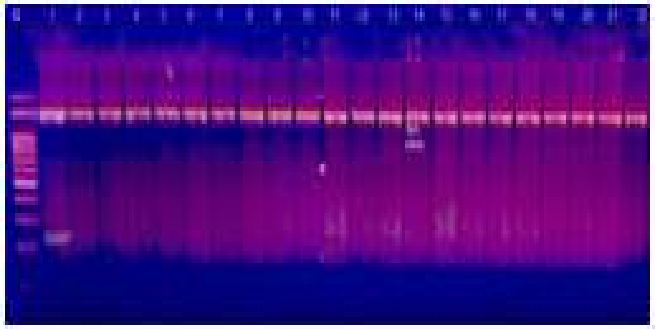

Fig. 5: Molecular Agarose gel electrophoresis pattern of *Mycoplasma*spp. that display the analysis of PCR product of 16S rRNA gene in *Mycoplasma* spp. positive isolates and tissues. Where M: marker (100-1500bp), lane (1-22) positive *Mycoplasma* spp. at (1500bp) PCR product.

PCR analysis, DNA amplification, 16S rRNA gene sequence alignment and phylogeny

An amplification of 16S rRNA from 150 sample was performed to confirm bacterial identification. Primers for conserved region of 16S rRNA were designed and used for amplification of DNA of *Mycoplasma* isolates by PCR then PCR products were separated on agarose gel Fig. 5. The result demonstrated that 22 isolates of nonpathogenic *Mycoplasma* had 16S rRNA gene band with 1500 bp. However, according to the current results identification of *Mycoplasma* by using 16S rRNA is more accurate than bacteriological and biochemical assays.

Pettersson *et al*. (1994), Rawadi (1998) and Kahya *et al.* (2015) demonstrated that 16S rRNA gene PCR was sensitive, specific, and used for diagnosis of prokaryotes which gave culture-negative bacteria,16S rRNA gene sequencing seems to be specifically well suitable as a clinical laboratory identification means and valuable method since usage of viable microorganisms is not essentially required (Gray *et al*., 2005), also it could be useful for identification of *Mycoplasma* that formerly treated with antibiotics during infection, as PCR can detect the dead bacteria as well as a live organisms (Young *et al*., 2007; Kobayashi *et al*., 2009) or detect alive *Mycoplasmas* that has antibiotic resistance as proved by Beylefeld *et al*. (2018).

The PCR products of isolate were submitted to Seoul University for sequencing. Sequences were edited using Finch TV sequences software and compared with sequences reported in Gene Bank (National Center for Biotechnology Information NCBI). The 22 local *Mycoplasma* isolates were showed 97-100% similarity to global *Mycoplasma.*

The construction of phylogenetic tree was carried out applying test maximum parsimony tree in MEGA X version.

The sequence was blasted in NCBI against standard strain of *Mycoplasma* genome. The identifying result showed 97-100%. The results of multiple sequence alignment MSA are taken into account a really necessary step within the current study for evolutionary development and diversification of a species investigation (phylogenetic analysis), which apparently the occurring of substitutions throughout relationships of biological evolutionary process between the various sequences sets (Fig.6).

The multiple sequence alignment was achieved by utilizating (clustal WMegaX) program on-line that is organized

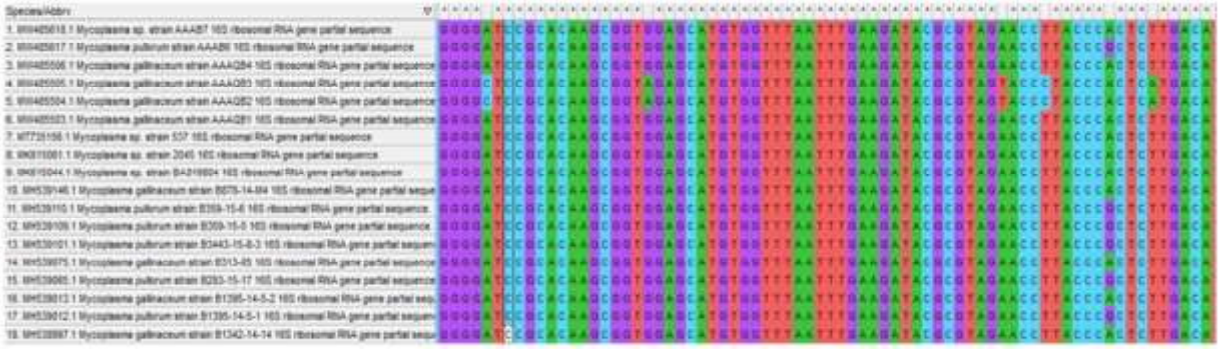

Fig.6: Analysis of Multiple sequence alignment of the incomplete16S rRNA gene, partial sequence of indigenous *Mycoplasma* spp. 16S rRNA gene, partial sequence isolates with for NCBI-Genbank*Mycoplasma* spp. based on analysis by ClustalW alignment by using (MEGA. X, multiple alignment assay tool). The multiple alignment analysis resemblance (*) and variances in 16S rRNA gene, partial sequence nucleotide sequences.

our several sequences sets every along in computationally manner in line with Goujon *et al*. (2010).

16S rRNA nucleotide sequence was submitted in NCBI and the local *Mycoplasma sp*. isolates AAAQB1, AAAQB2,AAAQB3 and AAAQB4 under accession number MW485503.1, MW485504,1, MW485505.1 and MW485506.1, respectively, were reveal strongly related to NCBI-Blast *Mycoplasma gallinaceum* (MH539075.1, MH539146.1, MH539013.1 and MH538997.1, South Africa strains), the local *Mycoplasma* sp. isolates (AAAB6) under accession number MW485617.1 were correlated closely to NCBI-Blast *Mycoplasma pullorum* (MH539065.1, MH539012.1, MH539101.1, MH539109.1 and MH539110.1, South Africa strain),the local *Mycoplasma* sp. isolate (AAAB7) under accession number MW485618.1 was exhibit close association to NCBI-Blast *Mycoplasma* sp. (MK615061.1, MK615044.1 and MT735156.1, Austria), of the current study as showed in Fig. 7.

PCR methods are simple and rapid methods to distinguish between *M*ycoplasma localized in tissues and *Mycoplasma* isolates in culture media and other bacterial species, on other hand 16S rRNA can recognized between *Mycoplasma* species depending on the blasted data sequence (Ferguson *et al*., 2005; Ghaniei, 2016; Ponnusamy *et al*., 2018).

After DNA sequencing, 900-1300 nucleotides of 16S rRNA sequence were obtained, BALSTN analysis, MSA along with phylogenetic tree. Fig. 6 and 7 confirmed that the *Mycoplasma* isolate for those isolates/strains aligned with each other, these results could suggest analogous ancestors as they may have developed and transmitted from certain areas of the globe; nevertheless, some isolates/strains have aligned in distinct branches of the tree may indicate a changeable process leading to emergence in somewhat variable strain which are not identical to another isolates/strains from the particular regions of world, may be due to exist of multiple incomplete copies or replicas along with varying dimensions of the gene zone that encode $COOH^{x}$ end of the protein, this variability made the ability to produce tens or even hundreds of thousands of variants (Browning *et al*., 2010).

The ability to adapt 16S rRNA sequence to estimate similarities or dissimilarities between generation of phylogenetic associations is incontrovertible (Olsen and Woese, 1993), also in classification (Pettersson *et al*., 1994) mutate strains of organisms may play a critical role at certain junctures in evolution (Woese *et al*., 1980). *Mycoplasmas* have high mutation rates, suggesting that they are in a state of rapid evolution (Rogers *et al.,*1985), such as a new developing emergence *MG* strain that isolated from *Carpodacus mexicanus* (American house finches) in the nineties (Luttrell *et al*., 2001), losses are of hundreds of millions within few years followed the first outbreaks (Dhondt *et al*., 2007; Delaney *et al*., 2012), may be because certain branches of the *Mycoplasma* tree are distinguished by somewhat elevated mutation rates (Rogers *et al*.,1985) and as proved by Delaney *et al*. (2012) their study results propose that genome evolutionary development in pathogens can be enormously fast and may be go together with functional lake of CRISPRs.

The present results offer helpful information concerning the recent status of some nonpathogenic *Mycoplasma* isolates/strains in Al-Qadisiyah province, Iraq.

The results of numerous sequences arrangement of the partial DNA sequences of *Mycoplasma sp.* local isolates of the 16S rRNA gene (1500 bp) with further globe chosen reference of 16S rRNA gene which exemplify similar gene of current study exhibit high resemblance and identity in particular location in sequences arrangement, the obtainable data based on the BLAST to reveal the ratio of the identity which was 97-100% between the references strains and our Iraqi isolates.

This article was limited to non-pathogenic *Mycoplasma* species without reference to other identified bacterial species.

The nucleotide sequence data of current study based on 1500 bp 16srRNA proved the presence of *Mycoplasma gallinaceum, Mycoplasma pullorum* and *Mycoplasma* sp. in broiler chickens with seemingly not high prevalence rate. The current study results are supported by the results of other studies (Jordan *et al*., 1982; Koshimizu *et al*., 1982; Bradbury *et al*., 2001; Lobo *et al*., 2004; Beylefeld *et al*., 2018; Adeyemi *et al*., 2018), in fact these identified *Mycoplasmas* can not cause acute respiratory disease of chickens but the presence of clinical respiratory signs of the selected apparently diseased chickens may be attributed to other virulent *Mycoplasmas i.e*. *M. gallisepticum* or even *M. synoviae* or other bacterial or viral diseases, these nonpathogenic *Mycoplasmas* may have role as opportunistic agents could worsen the pathogenesis of the chief respiratory disease agent(s) as co-infection and mutual cooperation with other respiratory infections can lead more severe clinical respiratory findings (Levisohn and Kleven, 2000; Nascimento *et al*., 2005). So study of whole *M. gallinaceum* genome in upcoming studies is necessary to show the role of *M. gallinaceum* with other concurrent viral or bacterial infections (Abolink and Beylefeld, 2015). This point also must have applied on other nonpathogenic species such as *Mycoplasma pullorum* and *Mycoplasma gallinarum* in chickens especially no documented reference genome for *M. gallinarum* and *Mycoplasma pullorum* is presented yet.

Finally, culturing of these fastidious bacterial agents is challenging, time consuming and required special supplements with high cost and to be very patient; therefore, molecular identification technique by polymerase chain reaction is an satisfactory,conclusive and fast method compared with laboratory cultivation to diagnose infected birds, so this assay has been suggested by several reports for purposes of breeder flock monitoring, observation, investigation, surveillance, control and the decision of termination, because this technique provide high specification and excellent sensibility in the definitive diagnosis in addition to good ability of achievement of assessments on a large breeder flocks (Nascimento *et al.,* 1991; Kempf,1998; Buim *et al*., 2009) and part of solution to minimize economic damages in different types of poultry industry (Jalaladdini *et al*., 2014).

In this study we conclude and confirmed the utilization of the 16S rRNA gene analysis thought to be a typical standardization for bacterial classification and a means of worthwhile for distinguishing nucleotide sequence of several *Mycoplasma* spp.

References

Abolnik C and Beylefeld A (2015) Complete genome sequence of *Mycoplasma gallinaceum*. Genome Announc **3**(4): e00712-15. Dierks RE, JA Newman and BS Pomeroy 1967. Characterization of avian mycoplasma. *Ann. NY*

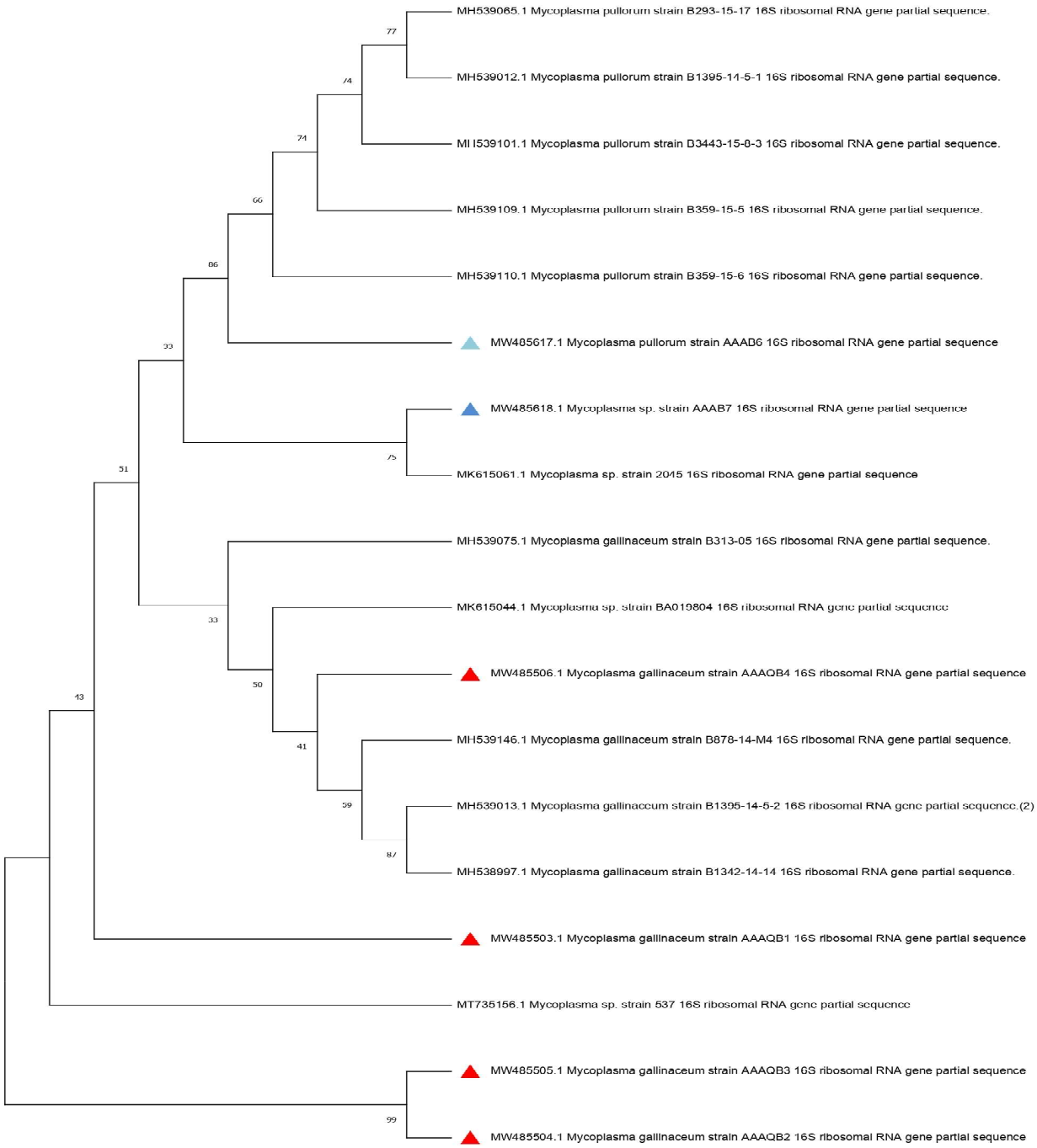

Fig.7: Analysis of Phygenetic tree according to the 16S ribosomal RNA gene partial sequence that utilized for *Mycoplasma* sp. detection of native *Mycoplasma* sp. isolates. applying test maximum parsimony tree in (MEGA X version).

Acad Sci. **143**:170-189.

Adeyemi M, Dauda AC, G Bwala AB and Abolnik C (2018) A comparative evaluation of the pathogenicity of *Mycoplasma gallinaceum* in chickens. *Avian Dis*. **62**:1-7.

Al-Azawi IHS (2012) Cultural and molecular detection of Mycoplasmal urogenital infection in women. A Thesis submitted to the Council of the College of Medicine, University of Babylon as a Partial Fulfillment of the Requirement for the Degree of Doctorate Philosophy in Microbiology.

Ali EJ and Ali BH (2019) Isolation, identification and sequencing of *Mycoplasma galisepticum* by Culture and PCR in Baghdad city, Iraq. *Indian J. Public Hlth. Res. Develop*. **10**(8): 936-941.

Barber TL and J Fabricant (1971) A suggested reclassification of avian mycoplasma serotypes. *Avian Dis*. **15**:125-138.

Bencina D, Dorrer D and Tadina T (1987b) Mycoplasma species isolated from six avian species. *Avian Pathol*. **16**: 653-664.

Bencina D, Mrzei I, Tadina T and Dorrer D (1987a) Mycoplasma

species in chicken flocks with different management systems. *Avian Pathol*. **16**: 599-608.

Beylefeld A, Wambulawaye P, Bwala DG, Gouws JJ, Lukhele OM, Wandrag, DBR and Abolnik C (2018) Evidence for multidrug resistance in non-pathogenic Mycoplasma species isolated from South African poultry. *Appl. Environ. Microbiol*. doi:10.1128/AEM.01660-18.

Boonyayatra Sukolrat, Lawrence K Fox, John M Gay, Ashish Sawant and 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. *J. Vet. Diag. Investi*. **24**(1) 7-13.

Bradbury JM (2005) Gordon Memorial Lecture. Poultry mycoplasmas: sophisticated pathogens in simple guise. *Br. Poult. Sci.* **46**:125-136.doi.org/10.1080/00071660500066282.

Bradbury JM, Abdoul-Wahab OM, Yavari CA, Dupiellet JP and Bove JM (1993) *Mycoplasma imitans* sp. nov. is related to *Mycoplasma gallisepticum* and found in birds. *Int. J. Syst. Bacteriol.* **43**(4): 721-728.

Bradbury JM, Yavari CA and Dare CM (2001) Mycoplasmas and respiratory disease in pheasants and partridges. *Avian Pathol*. **30**:391-396.

Browning GFMS Marenda, PF Markham, AH Noormohammadi and KG 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.

Buim MR, E Mettifogo, J Timenetsky, S Kleven and AJP Ferreira (2009) Epidemiological survey on *Mycoplasma gallisepticum* and *M. synoviae* by multiplex PCR in commercial poultry. *Pesq. Vet. Bras*. **29**(7): 552-556.

Delaney NF, Balenger S, Bonneaud C, Marx CJ, Hill GE, Ferguson-Noel N, Tsai P, Rodrigo A, and Edwards SV (2012) Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. *PLoS Genet.* **8:** e1002511.

Dhondt AA, Dhondt KV, Hawley DM and Jennelle CS (2007) Experimental evidence for transmission of *Mycoplasma gallisepticum* in house finches by fomites. *Avian Pathol.* **36**: 205-208. display disease symptoms.

Dovc P, D Bencina and I Zajc (1991) Genotypic heterogeneity among strains of Mycoplasma gallinarum. *Avian Pathol*. **20**: 705-711.

Eden PA, Schmidt TM, Blakemore RP and Pace NR (1991) Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *Int. J. Syst. Bacteriol*. **41**: 324-325. doi:10.1099/00207713-41-2-324.

Ferguson NM, Hepp D, Sun S, Ikuta N, Levisohn S, Kleven SH and 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. *Microbiol*. **151**: 1883-1893.

Forrester CA, JM Bradbury, CM Dare, RJ Domangue, H Windsor, JB Tasker and AA Mockett (2011) *Mycoplasma gallisepticum* in pheasants and the efficacy of tylvalosin to treat the disease. *Avian Pathol*. **40**: 581-586.

Ghaniei A (2016) Molecular characterization of *Mycoplasma synoviae* isolated from broiler chickens of West Azarbaijan province by PCR of vlhA gene. *Vet. Res. Forum.* **7**(3) 197- 202.

Gharaibeh S and Hailat A (2011) *Mycoplasma gallisepticum* experimental infection and tissue distribution in chickens, sparrows and pigeons. *Avian Pathol*. **40**: 349-354.

Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J and Lopez R (2010) A new bioinformatics analysis tools framework at EMBL–EBI. *Nucleic Acids Res.* **38** (Web Server issue): 695-699.

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. Cli. Microbiol*. **43**(7): 3431-3434.

Islam A, A Aslam, ZI Chaudhry, MUD Ahmed, HU Rehman, K Saeed and I Ahmad (2011) Pathology of *Mycoplasma gallisepticum* in naturally infected broilers and its diagnosis through PCR. *Int. J. Agric. Biol*. **13**: 835-837.

Jalaladdini, Sayed Mohammadhosein, Sayed Ali pourbakhsh and Babak kheirkhah (2014) Isolation and identification of Mycoplasma gallisepticum in chickensbn from industrial farms in Kerman province. *Int. J. Adv. Biol. Biomed. Res.* **2**(1): 100-104.

Jordan FTW and MM Amin (1980) A survey of mycoplasma infections in domestic poultry. *Res. Vet. Sci*. **28**:96-100.

Jordan FTW, H Erno, GS Cottew KH Hinz and L Stipkovits (1982) Characterization and taxonomic description of five mycoplasma serovars (serotypes) of avian origin and their elevation to species rank and further evaluation of the taxonomic status of *Mycoplasma synoviae*. *Int. J. Syst. Bacteriol*. **32**: 108-115.

Jordan FTW (1983) Recovery and identification of avian mycoplasmas. In: Methods in Mycoplasmology, Vol. 2. Eds. by Tully JG and Razin S New York: Academic Press. pp. 69-79.

Kahya S, Yýlmaz O, Eyigor A, Temelli S and Carlý KT (2015) Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by Real-Time PCRs and *Mycoplasma gallisepticum*-antibody detection by an ELISA inchicken breeder ûocks. *Kafkas Universitesi Veteriner Fakültesi Dergisi*. **21**(3): 361-366.

Kempf I (1998) DNA amplification methods for diagnosis and epidemiological investigations of avian Mycoplasmosis, *Avian Pathol.* **27**:1, 7-14, doi: 10.1080/03079459808419268.

Khalifa KA, ES Abdelrahim, M Badwi and AM Mohamed (2013) Isolation and molecular characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in chickens in Sudan. *J. Vet. Med.* Article ID 208026, 4 pages.

Kizil O and Ozdemir H (2006) Clinical, haematological and biochemical studies in goats naturally infected with Mycoplasma agalactiae. *Bull. Vet. Inst. Pulawy*. **50**: 325-328.

Kleven SH (2008) Mycoplasmosis. In: A laboratory manual for the isolation, identification and characterization of avian pathogens, 5th ed. L Dufour-Zavala, DE Swayne, JR Glisson, JE Pearson, WM Reed, MW Jackwood and PR Woolcock, eds. American Association of Avian Pathologists, Athens, GA. Jacksonville, Florida. pp. 59-64.

Kobayashi H, Oethinger M, Tuohy MJ, *et al*. (2009) Limiting false-positive polymerase chain reaction results: detection of DNA and mRNA to differentiate viable from dead bacteria. *Diagn Microbiol. Infect. Dis*. **64**: 445-447.

Koshimizu K, H Kotani, T Magaribuchi, T Yagihashi, K Shibata and M Ogata (1982) Isolation of urea plasmas from poultry and experimental infection in chickens. *Vet. Rec*. **110**:426-429.

Leigh SA and JD Evans (2009) Detection of *Mycoplasma gallinarum* by Real-Time PCR. *Int. J. Poult. Sci.* **8**(2): 108-111.

Levisohn S and Kleven SH (2000) Avian mycoplasmosis (*Mycoplasma gallisepticum*). *Rev. Sci. Tech*. **19**: 425-442.

Lobo E, MC Garcia, H Moscoso, S Martinez and SH Kleven (2004) Strain heterogeneity in Mycoplasma pullorum isolates identified by random amplified polymorphic DNA techniques. *Span J. Agri. Res*. **2**:500-503.

Luttrell MP, Stallknecht DE, Kleven SH, Kavanaugh DM, Corn JL and Fischer JR (2001) *Mycoplasma gallisepticum* in house finches (*Carpodacus mexicanus*) and other wild birds associated with poultry production facilities. *Avian Dis.* **45**: 321-329.

Moalic PY, I Kempf, F Gesbert and F Laigret (1997) Identification of two

pathogenic mycoplasmas as strains of *Mycoplasma pullorum*. *Int. J. Syst. Bacteriol*. **47**:171-174.

Nascimento E, V Pereira, M Nascimento and M Barreto (2005) Avian mycoplasmosis update. *Rev. Bras. Cienc. Avic*. **7:**1-9.

Nascimento ER, R Yamamoto, KR Herrick and RC Tait (1991) Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. *Avian Dis*. **35**: 62-69.

Nicholas RAJ, Ayling RD and McAuliffe L (2008) Isolation and Growth of Mycoplasma of Ruminants. In: NicholasRAJ, Ayling RD, McAuliffe L., Editors. Mycoplasma Diseases of Ruminants. Cab International, 3-14.

OIE (2008) Manual of Diagnostic tests and vaccines for terrestrial animals. Chapter 2.3.5: Avian Mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*), pp. 482-496.

Olsen GJ and Woese CR (1993) Ribosomal RNA: a key to phylogeny. *Faseb J*. **7**: 113-123.

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

Ponnusamy P, T Lurthu Reetha, BSM 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.

Poveda JB, Carranza J, Miranda Am Garrido, A Hermoso M, Fernandez A and Domenech J (1990) An epizootiological study of avian mycoplasmas in Southern Spain. *Avian Pathol*. **19**:4: 627-633.doi: 10.1080/03079459008418718

Quinn PJ, Carter ME, Markey B and Carter GR (2002) The Mycoplasmas. In: Clinical Veterinary Microbiology, Mosby, Virginia Tech, and Blacksburg, USA. pp. 320-326.

Raji AI, Moller C, Litthauer D, van Heerden E and Piater LA (2008) Bacterial diversity of biofilm samples from deep mines in South Africa. *Biokemistri*. **20**(2): 53-62. http://www.bioline.org.br/bk.

Rauf M, Chaudhary ZI, Younas M, Anjum AA, Ali MA, Ahmad AN and Khan MUR (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.

Raviv Z and DH Ley (2013) *Mycoplasma gallisepticum* infection. In: Diseases of Poultry. DE Swayne, JR Glisson, LR McDougald, LK Nolan, DL Suarez and VL Nair eds. Wiley-Blackwell, Ames, Iowa. pp. 877-893.

Rawadi, G.A. (1998) Characterization of mycoplasmas by RAPD fingerprinting. *Methods in Mol. Biol*. **104**: 179-187.

Razin S and Vincent PC (1983) Biochemical and enzymatic test in Mycoplasma identification. Sugar fermentation. In: Method in mycoplasmology. Ed. By Razin and G Tully. Academic Press. 337-339.

Razin S, D Yogev and Y Naot (1998) Molecular biology and pathogenicity of mycoplasmas. Microl. *Mol. Biol. Rev*. **62**:1094-1156.

Reece RL, L Ireland and PC Scott (1986) Mycoplasmosis in racing pigeons. *Aust Vet J*. **63**:166-167.

Rogers MJ, J Simmons, RT Walker, WG Weisburg, CR Woese, RS Tanner, IM Robinson, DA Stahl, G Olsen, RH Leach and J Maniloffi (1985) Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. *Proc. Natl. Acad. Sci. USA*. **82**: 1160-1164.

Shah-Majid M (1987) A case-control study of *Mycoplasma gallinarum* in the male and female reproductive tract of indigenous fowl. *Isr. J. Med. Sci*. **23**:530.

Shimizu T, Numano K and Uchida K (1979) Isolation and identification of Mycoplasmas from various birds: an ecological study. *Jap. J. Vet. Sci*. **41**: 272-282.

Stipkovits L and I Kempf (1996) Mycoplasmoses in poultry. *Rev. Sci. Tech. OIE*. **15**:1495-1525.

Tomar P, Singh Y, Mahajan NK, Jindal N and Singh M (2017) Molecular detection of Avian mycoplasmas in poultry affected with respiratory infections in Haryana (India). *Int. J. Curr. Microbiol. App. Sci*. **6**(6): 2155-2162.

Woese CR, Maniloff J and Zablen LB (1980) Phylogenetic analysis of the mycoplasmas *Proc. Natl. Acad. Sci. USA*. **77**: 494-498.

Young G, Turner S, Davies JK, *et al*. (2007) Bacterial DNA persists for extended periods after cell death. *J. Endod*. **33**: 1417-1420.