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Characterization of mycoplasmae isolated from chicken

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In the present investigation, the conventional methods, the serum plate agglutination test, a commercial enzyme-linked immunosorbent assay (ELISA) kit and polymerase chain reaction (PCR) test were used to detect *Mycoplasma* infections in broiler flocks in Egypt. The seroprevalence of *M. gallisepticum* antibodies in 12 broiler breeder flocks was 52.92% (634/1198) using ELISA. In hatched chicks from broiler breeder flocks, the serum plate agglutination test detected antibodies against *M. gallisepticum* in 52.86% (74/140) of the collected serum samples. Using cultural method, 14 *Mycoplasma* isolates were identified from tracheal swabs collected from the 12 broiler breeder flocks under the serological investigation. The isolates were characterized by PCR using primer for 16S/23S rRNA intergenic spacer region. Bands ranged from 464bp to 493bp were recorded among the field isolates, while *M. gallisepticum* F strain had a band at 935 bp. Sequence analysis of the PCR amplified fragment of 9 isolates showed that 2 isolates were *M. gallinaceum* while 7 isolates were *M. gallinarum.* Antigenic characterization of the 9 *Mycoplasma* isolates was investigated by Western blot test and the results were discussed.

Keywords: Mycoplasma, PCR, ELISA, Sequence, M. gallinarum, M. gallinaceum.

INTRODUCTION

Mycoplasmas are known to be broadly distributed cell wall-less prokaryotes with one of the smallest Known genomes among the species of the bacteria (Fadiel et al., 2007, El-Jakee et al., 2011). Mycoplamosis is one of the major problems among avian disease. Mycoplasmas cause decrease egg production and reduce feed conversion efficiency (Carpenter et al., 1981; Ley and Yoder, 1997). Production losses between 10 and 20% have been reported in layers (Bradbury, 2001). *M. gallisepticum* is a cause of respiratory disease and the most economically important of the avian *Mycoplasma*. The seroprevalence of *M. gallisepticum* infection in unvaccinated backyard chickens during the dry and wet seasons was 57.7 and 78.7% respectively in the year 2010 in Ethiopia (Chaka et al., 2012). As serology is not able to distinguish between strains so further studies are warranted to better understand the circulating strains, their interactions and their economic effect on backyard poultry production in Ethiopia (Chaka et al., 2012). In recent years PCR assays have become widely used as methods to confirm the presence of mycoplasmae in poultry flocks (Kajhn et al., 2009). *M. gallisepticum* and *M. synoviae* were detected in chickens using real-time TaqMan PCR protocols (Ferguson-Noel et al., 2012). Several fast growing *Mycoplasma* species, in particular *M. glycophilum, M. gallinaceum* and *M. pullorum*, were isolated frequently and were thought to be impeding the isolation of *M. gallisepticum* by outgrowing it (Bradbury et al., 2001). The study report the

isolation and characterization of mycoplasmae isolated from chickens using PCR, sequencing and Western blot techniques.

MATERIALS AND METHODS

Samples

Blood samples:

A total of 1198 serum samples collected from 12 broiler breeder flocks of different ages and 140 serum samples from hatched chicks were analyzed to detect *M. gallisepticum* antibodies using ELISA and rapid slide agglutination test respectively.

Tracheal swabs

A total of 2650 tracheal swabs from the 12 broiler breeder flocks and 350 tracheal swabs from hatcheries of the broiler breeder flocks, under serological investigation were examined for isolation of Mycoplasma species.

Detection of *M. gallisepticum* antibodies:

The serum samples of broiler breeder flocks were investigated for detection of *M. gallisepticum* antibodies using a commercial enzyme-linked immunosorbent assay (ELISA) kit produced by symbiotic, France. All the steps were applied as instructed by the manufacture. Also the serum samples of hatched chicks were investigated for *M. gallisepticum* antibodies using rapid slide agglutination test using stained *M. gallisepticum* antigen obtained from" Intervet International BV Boxneer, Holland".

Isolation and identification of *Mycoplasma* species

The tracheal swabs were collected using standard methods (Lierz et al., 2000). The swabs were inoculated in Modified Hay Flick's medium (Rosendal, 1994) and incubated at 37°C at 10% CO_2 for 3-5 days. 25 µl of broth was cultured on *Mycoplasma* agar medium and monitored every 24 hr for up to 7 days to detect the characteristic fried egg appearance of mycoplasmae. The suspected colonies were confirmed to be mycoplasmae using glucose fermentation, arginine deamination and digitonin sensitivity tests (Enro and Stipkovits, 1973). Also the growth inhibition test (Clyde, 1964) was applied.

Molecular characterization of *Mycoplasma* isolates by PCR:

The DNA of the collected *Mycoplasma* isolates was extracted by Qiagen extraction kits (Sigma). Using universal primer of (Laureman et al., 1995), amplification was performed in a PCR machine (Perkin Elmer 9700), programmed for 40 cycles:94° C for 30 sec, 55 ° C for 120 sec,72 ° C for 120 sec, and the final incubation was at 72° C for 300 sec. Amplification reaction prepared in volume 50ul. Using species specific primer for *M. gallisepticum* (Nascimento et al., 1991), amplification was performed in a Thermal cycler (Perkin Elmer 9700), programmed for: 1 cycle at 94° C for 5 min, and 35 cycles at:94 ° C for 1 min, 55 ° C for 1min ,72 ° C for 2 min ,the final incubation was at 72° C for 5 min. Amplification reaction prepared in volume 50ul and the PCR products were analyzed using gel electrophoresis (Sambrook et al., 1989).

Sequence analysis:

Sequencing of the PCR products was done via the services provided by Lab technology company, Heliopolis - Egypt. The obtained nucleotide sequence comparison was done using the Bio edit sequence alignment software. Phylogenetic trees of the isolates were constructed using Molecular Evolutionary Genetics Analysis version 4 (Tamura et al., 2007).

Western blot assay

Antigenic characterization of *Mycoplasma* isolates was investigated by Western blot test using *M. gallisepticum* antibodies. The expressed proteins of the isolates were separated by SDS-PAGE and transferred electrophoretically from the SDS-gel to a nitrocellulose membrane according to procedures described and modified by Towbin and Gordan, (1984).

RESULTS

Detection of *M. gallisepticum* antibodies in broiler breeders and hatchery:

634 (52.92%) out of 1198 serum samples of broiler breeders were positive for the presence of *M. gallisepticum* antibodies using ELISA. The high positive percentages were observed in samples collected from the flocks aged 30 weeks or more (Table 1). Among the 140 serum samples of hatched chicks 74 samples (52.86%) were positive for *M. gallisepticum* using rapid slide agglutination as shown in Table 2.

Table 1: The percentage of positive results of *M. gallisepticum* antibodies in broiler breeder flocks using ELISA:

Flock	Age(week)	No. of serum samples examined	Positive samples	
			Number	%
F1	11	132	0	0
F2	76	100	100	100
F3	79	100	75	75
F4	75	100	80	80
F5	49	100	32	32
F6	38	180	168	93.33
F7	30	114	31	27.19
F8	25	52	8	15.38
F9	60	80	60	75
F10	87	80	31	38.75
F11	27	80	8	10
F12	30	80	41	51.25
Total		1198	634	52.92%

Table 2: The percentage of positive results of *M. gallisepticum* antibodies using rapid slide agglutination test (RSA) in hatched chicks:

Flock/hatch		Total no. of samples	RSA(no. of positive)	%
F1	First hatch	10	0	0
	Second hatch	10	0	0
F2		10	8	80
F5	First hatch	10	9	90
	Second hatch	10	1	10
F7	First hatch	10	7	70
	Second hatch	10	4	40
F8	First hatch	10	0	0
	Second hatch	10	4	40
F9	First hatch	10	9	90
	Second hatch	10	9	90
F11		10	9	90
F12	First hatch	10	4	40
	Second hatch	10	10	100
Total		140	74	52.86

Isolation and identification of *Mycoplasma* in hatched chicks from broiler breeder examined:

Culturing from the tracheal swabs from 14 birds showed the presence of typical *Mycoplasma* colonies after 3–4 days on solid media. Heavy growth was observed in broth after 2 days, and typical “fried egg” colonies were visible in agar media 3 days after the broth was inoculated. The positive samples were collected from three flocks out of 12 flocks at age 30, 49 and 75 weeks. There was no shedding of *Mycoplasma* among the 350 tracheal swabs collected from the two groups: group 1 represents hatched chicks of one of the flocks that were positive in serology and isolation, while group 2 represents the hatched chicks of the flocks that were negative for isolation of *Mycoplasma* and serologically positive.

Molecular characterization of the *Mycoplasma* isolates by PCR:

Bands ranged from 464 bp to 493 bp were recorded among the 14 *Mycoplasma* isolates using universal primer targeting 16s rRNA through the spacer region and beginning of 23srRNA gene for avian mycoplasmae, while the *M. gallisepticum* F strain had a band at 935 bp (Table 3 and Figure 1). Using specific Nascimento primers for *M. gallisepticum,* PCR revealed negative results among the isolates, while the F strain had specific

band at 732 bp (Figure 2).

Sequence analysis of the isolates:

The sequence analysis of the PCR product of 16srRNA among 9 isolates showed that 2 isolates were *M. gallinaceum* while 7 isolates were *M. gallinarum*. Nucleotide sequence and phylogenetic tree of the *Mycoplasma* isolates were constructed in comparison with some reference strains of mycoplasmae, as shown in Figures 3, 4, 5, 6 and 7. All sequences were analyzed and aligned; the percentage similarity of the DNA was calculated and a dendrogram was constructed.

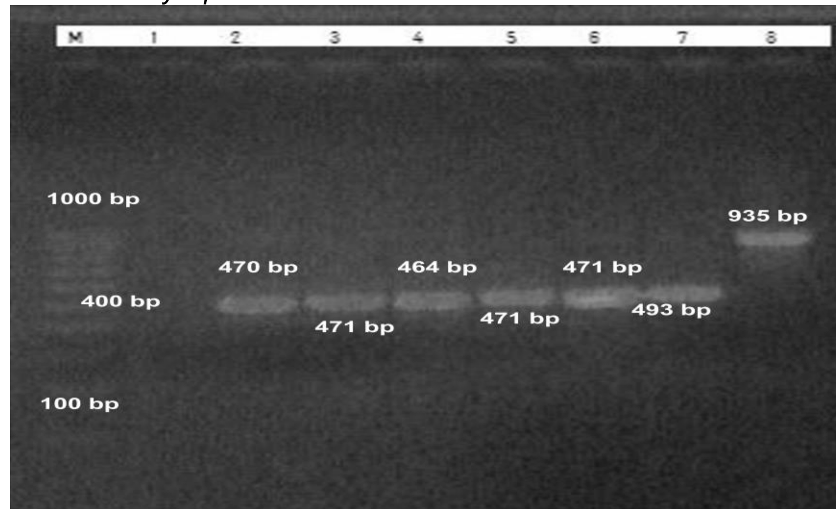

Figure 1: PCR amplified products among *Mycoplasma* isolates compared to *M. gallisepticum* F strain using universal primer. Lane M: 100 bp DNA marker (Qiagen), Lane 1: negative control, Lanes 2-6: *Mycoplasma* isolates had a band between 464-471 bp, Lane 7: *Mycoplasma* isolate had a band at 493 bp and Lane 8: *M. gallisepticum* F strain had a band at 935 bp.

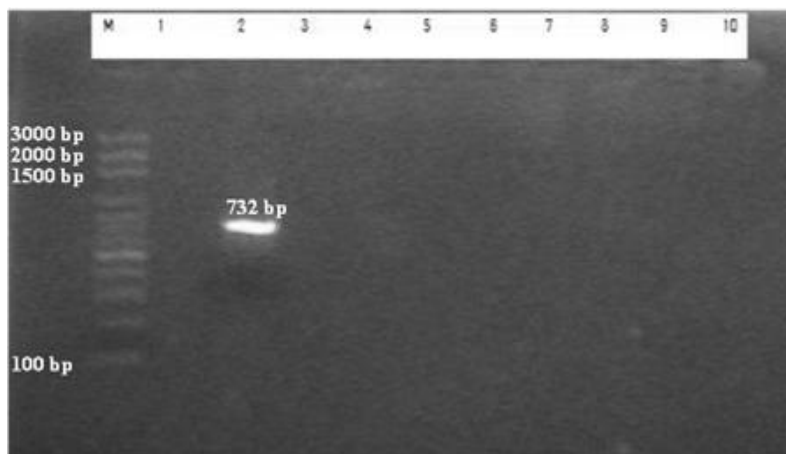

Figure 2: Results of PCR using Nascimento primers specific for *M. gallisepticum.* LaneM: 100bp marker (Qiagen), Lane 2: F strain of *M. gallisepticum* show specific band at 732 bp and Lanes 1 and 3-10: *Mycoplasma* isolates had no amplified band.

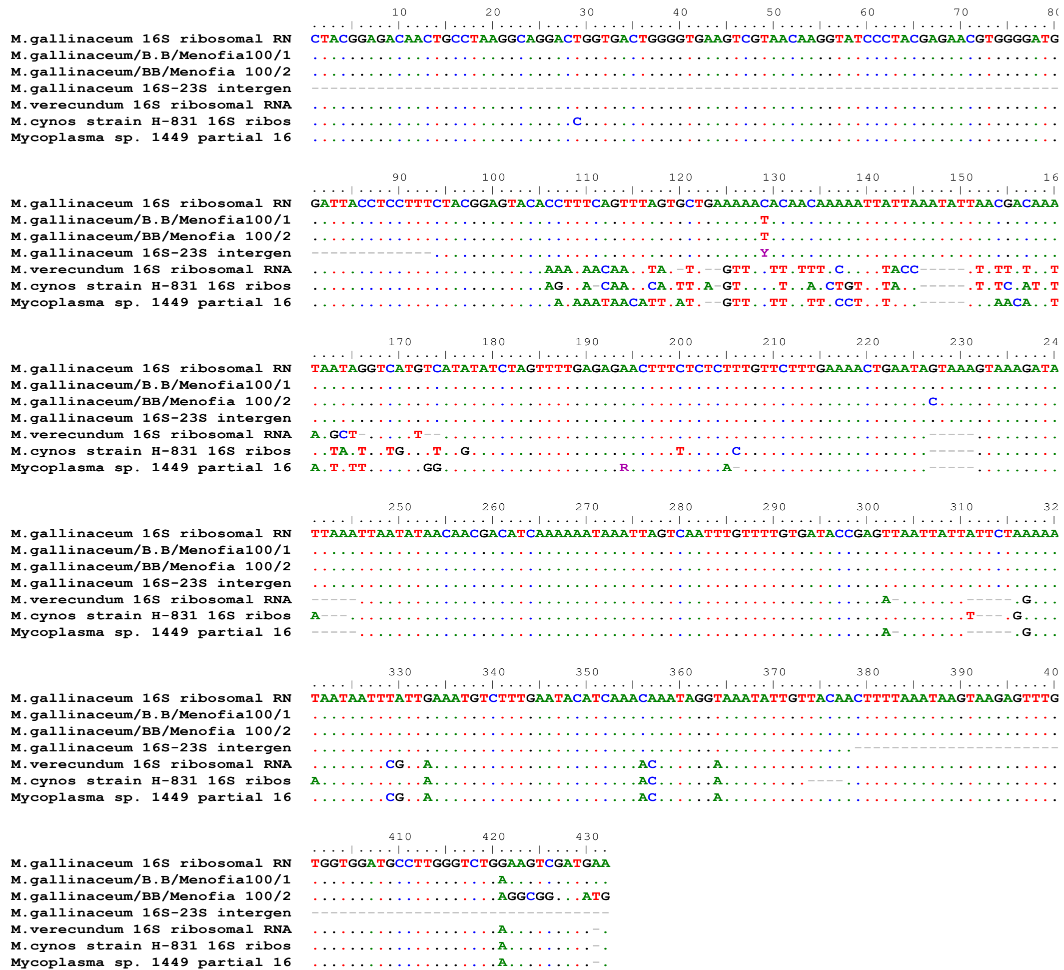

Figure 3: *M. gallinaceum* sequence graphic view showing minor basevariability

Table 3: PCR amplified bands among the 14 *Mycoplasma* isolates using 16srRNA of avian mycoplasmae

Isolate number	Age (weeks)	Band at bp
** Isolate 1	49	485 bp
** Isolate 2	49	493 bp
** Isolate 3	75	470 bp
** Isolate 4	30	471 bp
** Isolate 5	30	464 bp
** Isolate 6	38	471 bp
** Isolate 7	38	471 bp
Isolate 8	38	471 bp
** Isolate 9	38	464 bp
Isolate 10	38	464 bp
Isolate 11	38	470 bp
Isolate 12	30 weeks	470 bp
** Isolate 13	One day old	470 bp
Isolate 14	One day old	464 bp
*M. gallisepticum*F strain		935bp

** Isolates selected for sequence and antigenic characterization by western blot technique.

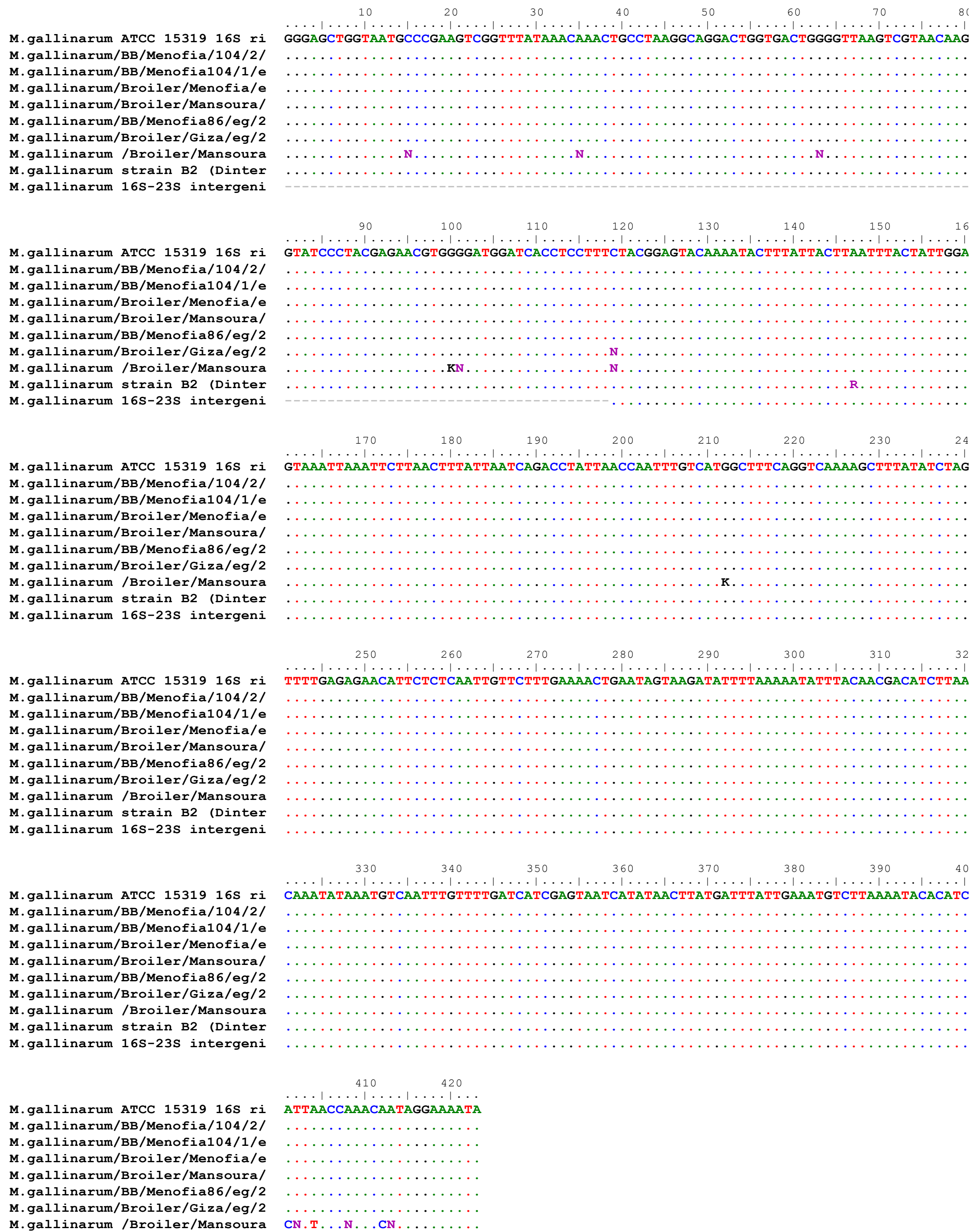

Figure 4: *M. gallinarum* sequence graphic view showing absence of base variability

M.cynos strain H-831 16S ribosomal RN...
Mycoplasma sp. 1449 partial 16S rRNA ...
M.verecundum 16S ribosomal RNA gene p...
M.gallinaceum BB Menofia 100 2 eg 2008
M.gallinaceum 16S ribosomal RNA gene ...
M.gallinaceum B.B Menofia100 1 eg 2008
M.gallinaceum 16S-23S intergenic spac...

43, 58, 72

2.0 1.5 1.0 0.5 0.0

Figure 5: The phylogenetic tree of *M. gallinaceum* in comparison with some reference strains of mycoplasmae. Neighbor joining phylogenetic tree constructed using Mega 4 software showing clustering of *Mycoplasma* isolates with a standard strain from the gene bank and indicate the high conservative nature of this part of *M. gallinaceum* genome.

M.gallinarum Broiler Mansoura1 eg 2009
M.gallinarum strain B2 (Dinter) 16S r...
M.gallinarum BB Menofia86 eg 2008
M.gallinarum ATCC 15319 16S ribosomal...
M.gallinarum BB Menofia104 1 eg 2008
M.gallinarum BB Menofia 104 2 eg2008
M.gallinarum Broiler Mansoura eg 2009
M.gallinarum Broiler Menofia eg 2009
M.gallinarum Broiler Giza eg 2010
M.gallinarum 16S-23S intergenic space...

3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

Figure 6: The phylogenetic tree of *M. gallinarum* in comparison with some reference strains of mycoplasmae. Neighbor joining tree constructed using mega 4 software showing clustering of all samples with the standard ATCC strain and indicate the high conservative nature of this part of *M. gallinarum* genome.

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                                         10        20        30        40        50        60        70        80
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M.gallinaceum 16S ribosomal RN   XTETTA*GRTGDWGEVVTRYPYENVGMDYLLSTEYTFQFSAEKHNKNY*ILTTNNRSCHISSFERTFSLCSLKTE**SKD
M.gallinaceum/B.B/Menofia100/1   ......*....................................Y....*..........................**...
M.gallinaceum/BB/Menofia 100/2   ......*....................................Y....*..........................Y*...
M.gallinaceum 16S-23S intergen   .------------------------------X...........X....*..........................**...
M.verecundum 16S ribosomal RNA   ......*............................KITIYXXLLIY.LX-XIIISX.XX................X-X..
M.cynos strain H-831 16S ribos   ......*............................KLXIHFX*.KTVLX-XINIIML.YV...............X-X..
Mycoplasma sp. 1449 partial 16   ......*.............................*ITFIXLL.YLFX-XKQIIL.WD.....X...X......X-X..

                                         90        100       110       120       130       140
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....
M.gallinaceum 16S ribosomal RN   IKLI*QRHQKIN*SICFVIPS*LLF*K**FIEMSLNTSNK*VNIVTTFK*VRVCGGCLGSGSR*
M.gallinaceum/B.B/Menofia100/1   ....*.......*........*...*.**...........*........*..........E..*
M.gallinaceum/BB/Menofia 100/2   ....*.......*........*...*.**...........*........*..........EAGY
M.gallinaceum 16S-23S intergen   ....*.......*........*...*.**...........*.....X-----------------
M.verecundum 16S ribosomal RNA   X-..*.......*.......RX.X-X.**.VK......KQ*E.......*..........E..X
M.cynos strain H-831 16S ribos   XX..*.......*........*.F-*.K*..K......KQ*E..X-...*..........E..X
Mycoplasma sp. 1449 partial 16   X-..*.......*.......RX.X-X.**.VK......KQ*E.......*..........E..X
```

Figure 7: Amino acid graphic view of *M. gallinaceum*.

Table 4: Western blot analysis of the 9 sequenced isolates

Isolate number	*Mycoplasma* species	No. of bands of western blot	Band at
1	*M. gallinaceum*	2	50,153 kDa
2	*M. gallinaceum*	2	48,177 kDa
9	*M. gallinarum*	4	49,71,116,148 kDa
6	*M. gallinarum*	4	48,77,118,150 kDa
7	*M. gallinarum*	5	47,71,119,158,384 kDa
3	*M. gallinarum*	4	47,70,117,150 kDa
4	*M. gallinarum*	5	132,179,316,397,483 kDa
5	*M. gallinarum*	5	129,179,310,377,509 kDa
13	*M. gallinarum*	5	126,176,294,419,528 kDa

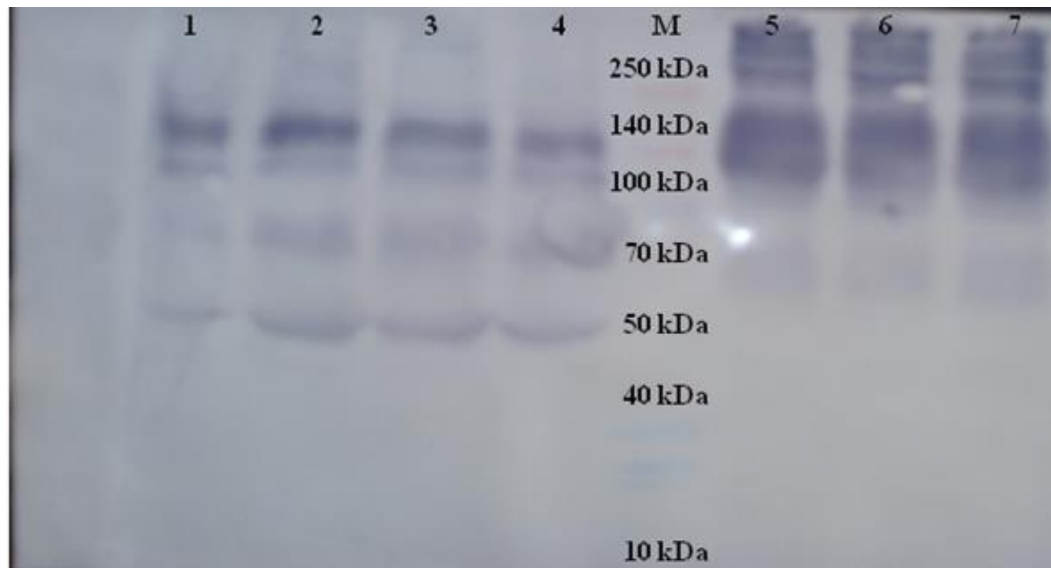

Figure 8: Western blot analysis of the sequenced isolates of mycoplasmae.Lane M: marker, Lanes 1 & 2: *M. gallinaceum* and Lanes 3-7: *M. gallinarum.*

Antigenic Characterization of *Mycoplasma* isolates by Western blot using anti *M.* gallisepticum antibodies:

Antigenic characterization of 9 *Mycoplasma* isolates was investigated by Western blot test using anti *M. gallisepticum* antibodies as shown in Table 4 and Figure 8. Analyses were applied using lab image program for band analysis (lab image version 2.7.0).

Discussion:

Avian mycoplasmosis causes considerable economical losses to the poultry industry, especially in chickens all over the world. *M. gallisepticum* is responsible for chronic respiratory disease in chickens. In broiler, it causes reduction in weight gain, decrease in feed conversion efficiency, increase in mortality rate and increased condemnations in slaughter houses (Gharaibeh and Al Roussan, 2008, Jalaladdini, 2014).

Historically, chicken flocks are usually monitored for *M. gallisepticum* infection by serology using rapid plate agglutination then confirmed by ELISA, or isolation (Kleven, 1998).

In the present study 52.92% (634/1198) of serum samples collected from broiler breeder flocks were positive to *M. gallisepticum* antibodies using ELISA. The high positive percentages were observed in samples collected from the flocks aged approximately 30 weeks or more as shown in Table 1. Serological tests are useful for monitoring *M. gallisepticum* infection in a flock and for taking prophylactic measures to control *M. gallisepticum* infection early in *M. gallisepticum* free poultry (Osman et al., 2009). Thirty six out of the 49 chicken flocks (73.5%) tested by

Gharaibehand Al Roussan (2008) using ELISA had positive antibody titer for *M. gallisepticum*. The presence of *M. gallisepticum* and *M. synoviae* antibodies was examined in 77 and 290 flocks, respectively by Kapetanov*et al*. (2010), significant number of flocks were marked positive *M. gallisepticum* during rear (19.05%) and in commercial broilers (8%). Seasonal variation of prevalence of *M. gallisepticum* infection was observed by(Sarkar et al., 2005), the prevalence was higher (62.44%) in winter season and lower (53.10%) in summer season. The seroprevalence of *M. gallisepticum* infection among the serum samples collected from the hatched chicks of the investigated farms was 52.86% (74/140) using rapid slide agglutination test (Table 2). The result supported the earlier investigations of Pradhan (2002) and Dulali (2003) who reported 57.15 and 52% seroprevalence of *M. gallisepticum* infection in chickens respectively. The hatched bird tested were less than 5 weeks of age so the titers may be due to maternal antibodies from parent flocks.

Using culture method a total of 2650 tracheal samples were examined among the serologically investigated farms for detection of mycoplasmae. Also 350 tracheal swabs collected from two groups: group 1 represents hatched chicks of one of the flocks that were positive in serology and isolation while group 2 represents the hatched chicks of the flocks that were negative for isolation of *Mycoplasma* and serologically positive were investigated for detection of mycoplasmae. 14 isolates could be isolated (Abbas et al.,2018).

The isolates as well as *M. gallisepticum* F strain were characterized using PCR. There are many different PCR methods applied for mycoplasmae detection. The PCR methods used for *M. gallisepticum* detection are based on the amplification of different gene fragments, mostly the 16SrRNA gene, while other PCR assays amplify other *M. gallisepticum* genes (Raviv*et al*., 2007).The 16S rRNA gene is a highly conserved region with low levels of genetic variation, which reduces the likelihood of detection lack of some *M. gallisepticum* strains (Weisburgetal., 1989).

Figure 1and Table 3 show an amplified product between 464 – 493 bp among the examined isolates and 935 bp among the *M. gallisepticum* F strain using 16S rRNA primers. Lauremane*t al*. (1995) recorded that the amplicon of *M. gallisepticum* was the largest (899bp), and its size could be used as a distinct identifying characteristic. They added that the amplicon of *M. iowae*was the smallest (425bp), while the amplicon sizes of the other *Mycoplasma* species could be put into three size groups, such as *M. iners* and *M. gallopavonis*(554 and 543bp, respectively), *M. gallinarum* and *M. meleagridis* (465 and 475 bp, respectively), and *M. gallinaceum, M. synoviae*, and *M. pullorum* (490, 490 and 516 bp, respectively).

Using (Nascimento et al., 1991) primers specific for *M. gallisepticum,* PCR revealed negative results among the isolates, while the F strain had specific band at 732 bp as shown in Figure 2.The PCR products generated from the avian *Mycoplasma* species were between 400-600 bp with the exception of *M. gallisepticum* (1000bp) and *M. imitans*(3000bp) as recorded by (Ramirez et al., 2007).

The sequence analysis of the PCR product of 16S/23SrRNA showed that 2 isolates were *M. gallinaceum* while 7 isolates were *M. gallinarum* as in Table 3. Several fast-growing *Mycoplasma* species, in particular *M.glycophilum*, *M.gallinaceum* and *M.pullorum*, were isolated by (Bradbury et al., 2001) and were thought to be impeding the isolation of *M. gallisepticum* by outgrowing it.

M. gallinaceum sequence graphic view (Figure 3) shows minor base variability, Neighbor joining phylogenetic tree constructed using Mega 4 software showing clustering of *Mycoplasma* isolates with a standard strain from the gene bank and indicate the high conservative nature of this part of *M. gallinaceum* genome (Figure 5).*M. gallinarum* graphic view (Figure 4) showing absence of base variability, Neighbor joining phylogenetic tree constructed using Mega 4 software clustering of all samples with the standard ATCC strain and indicate the high conservative nature of this part of *M. gallinarum* genome (Figure 6). ISR sequences of the 21 avian *Mycoplasma* type strains analyzed by (Ramirez et al., 2007) ranged from 178 to 341bp with 16 of them being between 250 and 350 bp. The Genbank/EMBL accession numbers for the ISR sequences of the 21 avian *Mycoplasma* species determined were AJ780982 to AJ781002.

The locus of the ISRs examined by (Ramirez et al.,2007) were not assigned, however ,some polymorphisms were detected in *M. columbinasale, M .columbinum, M .columborale, M. corogypsi, M. gallinaceum, M. pullorum*and *M. synoviae*and insertion/deletions in *M. columborale, M. pullorum*and *M. synoviae.*

A PCR procedure using universal *Mycoplasma* primers for amplification of the 16S/23S ribosomal RNA (rRNA) intergenic spacer region in *Mollicutes* has provided a sensitive

assay for detection of mycoplasmas (Harasawa et al., 1993). In the scope of epizootiology, only the prevalence of *M. gallisepticum* in poultry tends to decrease while infection with other *Mycoplasma* occurs even more frequently (Kapetanov et al., 2010).

In the present study the isolates were investigated by Western blot for detection of immunogenic antigens using polyclonal antibodies as shown in Table 4 and Figure 8. It is clear that 6 out of 14 isolates had a band at 47-50 kDa. Immunoblotting techniques contributed to the identification of immunogenic species-specific proteins, which contributed to improve the serodiagnostic tests.

CONCLUSION

Several fast growing *Mycoplasma* species as *M. glycophilum, M. gallinaceum* and *M. pullorum*, were impeded the isolation of *M. gallisepticum.* Characterization of mycoplasmae isolated from chickens using PCR techniques reveals negative *M. gallisepticum* results among the isolates. The sequence analysis of the PCR product of 16srRNA among 9 isolates showed that 2 isolates were *M. gallinaceum* while 7 isolates were *M. gallinarum*.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

EJ and SM designed experiments and reviewed the manuscript. RH performed the phenotypic bacteriology work. EJ, SM and BHA applied the molecular and Western blot techniques. All authors read, revised and approved the final version. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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