APPLICATION OF REAL TIME PCR AND DNA SEQUENCING FOR DIFFERENTIATION OF FIELD AND VACCINAL STRAINS OF *MYCOPLASMA GALLISEPTICUM*

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ABSTRACT

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Differentiation between field and vaccinal strains of *Mycoplasma gallisepticum*is important for the epidemiology of the disease. Real time PCR assay was applied to detect MG and further tested by mgc2 rt-PCR for F, 6/85 and TS-11 strains. Twenty four farms were positive for MG Out of the 50 farms, from which 12 were positive for F strain and 5 were positive for 6/85 strain, while 7 farms were MG positive but negative for live vaccine rt-PCR. DNA nucleotide sequencing for 20 positive cases for mgc2 gene confirmed the rt-PCR results, some point mutations were found in the isolated strains and grouped the sequenced samples into 4 groups (F, 6/85 and two field strain groups named field group A and B).

Key Words: *nucleotide, vaccines of Mycoplasma, rt-PCR, - Sequencing, point mutations*

INTRODUCTION

Mycoplasmas are the smallest known bacteria that are capable of replicating outside cells (Bradbury, 2008). MG infects a wide variety of gallinaceous birds including chickens, turkeys and pheasants (Christensen *et al.,* 1994). Live vaccines that have been used to control MG include F strain (Brown *et al.,* 1997), and 6/85 (Evans and Hafeez 1992) and TS-11 (Whithear *et al.,* 1990A). With the widespread use of live vaccines there is an increasing need to differentiate between vaccinal and field strains. Culture methods, are often labour- intensive and requirespecially formulated media, so the improvement of diagnostic tools for direct detection of mycoplasma was necessary (Mekkes and Feberwee 2005). Molecular techniques are cost effective and reliable means of differentiating vaccinal strains from field strains (Collet, 2005). Several techniques have been developed for MG strains differentiation. However, none of these methods have been as widely used as random amplified polymorphic DNA (Charlton *et al.,* 1999). But it has intrinsic problems that affect the reproducibility of the technique (Tyler *et al.,* 1997) thetest also needspure cultures of the target organism (Ferguson 2003). An alternative method should also allow MG strain discrimination at the level of clinical samples so avoiding the MG isolation step that is necessary for RAPD analysis. Nucleotide sequence analysis of a specified gene may allow the development of a PCR that is performed directly on clinical samples to detect MG. Some rt-PCR assays were innovated for MG strain differentiation as Taqmanprobes (Ravi*et al.,* 2008). Hybridization probes (Feberwee *et al.,* 2006) or High-resolution melting curve analysis (Ghorashi *et al.,* 2010). Recently, sequencinghave been used for studying the molecular epidemiology of MG (Ferguson *et al.,* 2005). In this study, we tested 2rt-PCR assays (MGA0319 and mgc2) in comparison with the mgc2 cPCR. The specificity and sensitivity of the PCR assays were determined. A field study for the surveillance of MG invaccinated and unvaccinated poultry farms was done, and the ability of rt-PCR to differentiate between vaccinal and field strains in the field was evaluated. Finally, sequence analysis of mgc2 gene of 20 selected positive samples for confirmation of rt-PCR results and for further epidemiological information.

MATERIALS and METHODS

Samples sources: The field study samples were divided according to vaccination and health status into 4 groups: chickens and turkey flocks vaccinated and unvaccinated diseased or not as described in table no 1 and 2.

We note that 10 tracheal swabs were collected from each flock and lungs just collected from some cases.

Table 1: Number, species and vaccination status of poultry flocks used in the study.

Species	Vaccinated (Type of vaccine)			Unvaccinated	Total
Chicken	19			19	38
	F	6/85	TS11		
	14	4	1		
Turkey	5 vaccinated with 6/85			7	12
Total	24			26	50

Table 2: Health status of the sampled flocks.

Spp.	Diseased		Apparently healthy		Total
	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated	
Chicken	4	10	15	9	38
Turkey	0	3	5	4	12
Total	4	13	20	13	
	17		33		50

The individual samples details types are described in Tables (3) to Table (6).

Table 3: Number and types of samples collected from different types of unvaccinated diseased birds from different sources.

Code	Species	Age/W	Breed	Town	Govern.	Sample
UNVD 1	Broiler chicken	5	Hubbard	Qotoor	Gharbia	10 T.S
UNVD 2	Broiler chicken	5	Hubbard	Sennouris	Faium	10 T.S
UNVD 3	Broiler chicken	5	Hubbard	Kafr El Zayat	Gharbia	10 T.S
UNVD 4	Broiler turkey	22	French	Ashmoun	Menoufia	10 T.S+4 lung
UNVD 5	Broiler turkey	22	Holland	Samalout	Menia	10 T.S+4 lung
UNVD 6	Broiler chicken	9	Sasu	Bepa	Benisuef	10 T.S
UNVD 7	Broiler chicken	6	Hubbard	Nasser	Benisuef	10 T.S
UNVD 8	Broiler chicken	6	Kobb	Tela	Menoufia	10 T.S
UNVD 9	Broiler chicken	4	Baladi	Zagazig	Sharkia	10 T.S
UNVD 10	Broiler chicken	4	Sasu	Beyala	Kafr El Sheikh	10 T.S
UNVD 11	Broiler chicken	5	Cobb	Ayat	Giza	10 T.S
UNVD 12	Broiler chicken	4	Hubbard	Hawamdya	Giza	10 T.S + 5 lung
UNVD 13	Broiler turkey	12	Holland	Malawy	Menia	10 T.S+ 5 lung

Table 4: Number and types of samples collected from different types of unvaccinated apparently healthy and birds from different sources.

Code	Breed/spp.	Age/w	Breed	town	Gov.	sample
UNVH 1	Broiler chicken	4	Kobb	El Bagour	Menoufia	10 T.S
UNVH 2	Broiler chicken	5	Hubbard	Dsook	Kafr El Sheikh	10 T.S +4 lung
UNVH 3	Broiler chicken	5	Cobb	Ayat	Giza	10 T.S
UNVH 4	Broiler chicken	4	Hubbard	Regwa	6th October	10 T.S
UNVH 5	Broiler chicken	8	Baladi	Zakazik	Sharkia	10 T.S
UNVH 6	Broiler chicken	6	Hubbard	Regwa	6th October	10 T.S
UNVH 7	Broiler chicken	8	Sasu	Maghagha	Menia	10 T.S
UNVH 8	Broiler chicken	5	Hubbard	Ashmoun	Menoufia	20 T.S
UNVH 9	Broiler turkey	8	Holland	Benimazar	Menia	10 T.S+ 4 lung
UNVH 10	Broiler turkey	15	French	Benimazar	Menia	10 T.S+ 4 lung
UNVH 11	Broiler turkey	17	Holland	Benimazar	Menia	10 T.S+ 4 lung
UNVH 12	Broiler chicken	4	Hubbard	Kafr El Zayat	Gharbia	10 T.S
UNVH 13	Broiler turkey	19	French	Elkata	6th October	10 T.S

Table 5: Number and types of samples collected from different types of vaccinated apparently healthy birds from different sources.

Code	Species	Age/w	Breed	Town	Govern.	Vacc.	Sample
VH 1	Layer breeder chicken	31	Luhman	Benha	Qalyubia	6/85	10 T.S
VH 2	Broiler breeder chicken	45	Buvens	Sadat	Menoufia	6/85	10 T.S
VH 3	Broiler breeder chicken	39	Luhman	Tanta	Gharbia	F	10 T.S
VH 4	Layer chicken	26	Luhman	Belbeis	Sharkia	F	10 T.S
VH5	Broiler turkey	22	French	Talkha	Dakahlia	6/85	10 T.S
VH 6	Broiler turkey	20	French	Met Ghamr	Dakhlia	6/85	10 T.S
VH 7	Layer chicken	32	Baladi	Kafr El Zayat	Gharbia	F	10 T.S
VH 8	Layer breeder chicken	38	Hubbard	Wadi El Natroun	Behera	F	10 T.S
VH 9	Layer breeder chicken	40	Hubbard	Wadi El Natroun	Behera	TS-11	10 T.S
VH 10	Broiler turkey	25	French	Hehia	Sharkia	6/85	10 T.S
VH 11	Broiler turkey	22	Holland	Mansoura	Dakahlia	6/85	10 T.S
VH 12	Layer turkey	25	Luhman	Tookh	Qalyubia	6/85	10 T.S
VH 13	Broiler breeder chicken	22	Hubbard	Benha	Qalyubia	F	10 T.S
VH 14	Layer breeder chicken	27	Baladi	Elmahala	Gharbia	F	10 T.S
VH 15	Layer breeder chicken	42	Baladi	Elshohada	Menoufia	F	10 T.S
VH 16	Layer chicken	22	Hubbard	Hawamdeya	Giza	F	10 T.S
VH 17	Layer chicken	29	Buvens	Elamrya	Alex	6/85	10 T.S
VH 18	Layer chickes	25	Baladi	Hehia	Sharkia	6/85	10 T.S
VH 19	Broiler chicken	19	French	Talkha	Dakahlia	F	10 T.S
VH 20	Layer breeder chicken	43	Hubbard	Talkha	Dakahlia	F	10 T.S
VH 21	Layer breeder chicken	33 w	Buvens	Met Ghamr	Dakhlia	F	10 T.S
VH 22	Broiler breeder chicken	24 w	Hubbard	Talkha	Dakhlia	F	10 T.S
VH 23	Layer chicken	65 w	Luhman	Talkha	Dakahlia	F	10 T.S
VH24	Layer chicken	26 w	Luhman	Belbeis	Sharkia	F	10 T.S

Table 6: Number and types of samples collected from different types of vaccinated diseased birds from different sources.

Code	Species	Age/w	Breed	Town	Govern.	Vacc	Sample
VD 1	Layer chicken	36	Luhman	Kafrshukr	Qalyubia	F	10 T.S
VD 2	Layer chicken	16	Buvens	Kafrshukr	Qalyubia	F	10 T.S
VD 3	Layer chicken	24	Baladi	Abu Kebir	Sharkia	F	10 T.S
VD 4	Layer chicken	28	Baladi	Shebeen Elkanater	Qalyubia	F	10 T.S

Specificity of conventional PCR and rt-PCR: DNA of reference strains were used [MG (R, A5969, HF51, F, 6/85 and TS11), other mycoplasmas (MS, *M. meleagridis*, *M. bovis* and *M.bovigenitalium*) and reference bacterial strains (*S. aureus*, *E. coli*, *S.* Typhimurium, *C. jejuni*and *C. perferingens*)].

Detction limits of conventional PCR and rt-PCR: MG cultureswere serially diluted and colony counted according to Rodwell and Whitecomb (1983) Methods for direct and indirect measurement of Mycoplasma growth.] and their counts were 16 x 10^4, 8 x 10^5 and 16 x 10^5 CFU/ml for F, 6/85 and TS11, respectively.

MG culture: The samples were inoculated in Frey broth and agar medium and incubated at 37^{o}C. Mycoplasma colonies on agar plates were identified as MG according to OIE (2008).

Conventional PCR and rt-PCR:

DNA purification: DNA was extracted from 200µl of the pooled swabs and grinded lungs using the Qiamp DNA mini kit (Qiagen, Gmbh).

PCR amplification: It was done in a 25 µl reaction containing 12.5 µl of Quantitect probe rt-PCR buffer (Qiagen, Gmbh), 1 µl of each primer (20 pmol conc.), 4.5 µl of H_2O, and 6 µl of template. For rt-PCR, 0.125 µl of a 30 pmol probe was added. The cPCR reactions were performed in a Biometra T3 thermal cycler. The cPCR products were separated by electrophoresis on 2% agarose gel and photographed by a gel documentation system (Alpha Innotech, Biometra). While, rt-PCR was done in a Stratagen MX3005P that was used to amplify DNA and analyze the results using its own software.

Table 7: Oligonucleotide primers and probes sequences encoding for detection of mgc2 MGA0319 gene and gene mgc2 for Strain differentiation of Mycoplasma gallisepticum live vaccines.

Primer	Target gene	Primer sequence(5'-3')	Size of Amplicon (bp)
mgc2-F	**mgc2 Garcia *et al.*, 2004**	CGCAATTTGGTCCTAATCCCCAACA	**300 bp**
mgc2 -R		TAAACCCACCTCCAGCTTTATTTCC	
mglpU26	**MGA0319 Callison *et al.*, 2006**	CTA GAG GGT TGG ACA GTT ATG	**139 bp**
mglp164		GCT GCA CTA AAT GAT ACG TCA AA	
mglp probe		6-**FAM**-CAG TCA TTA ACA ACT TAC CAC CAG AAT CTG-tamra	
mgc2-rt-F	**mgc2 rt PCR Grodio*et al.*, 2008**	GGTCCTAATCCCCAACAAAGAAT	**127bp**
mgc2-rt-R		CTTGGTTGGTTCATATTAGGCATTT	
mgc2-rt-probe		6-**FAM** CCA CAG GGC TTT GGT GGC CCA-tamra	
F strain-for	**mgc2 gene Raviv *et al.*, 2008**	GTT CAA GAA CCA ACT CAA CCA	**112bp**
F strain-rev		GAT TAA GAC CGA ATT GTG GAT	
F strain-probe		6-**FAM** CAA CAA GGA TTT AAT CAA CCT CAG-tamra	
TS11-for		CTC AAG AAC CAA CTC AAC CA	**114bp**
TS11-rev		GGG GAT TAG GAA TAA ATT GCG GAT	
TS11-probe		**HEX**-CAG CCA GGA TTT AAT CAA CCT CAG-Tamra	
6/85-for		CTC AAG AAC CAA CTC AAC CA-Tamra	**112bp**
6/85-rev		GGA TGA GGA CCA AAT TGC GGA T	
6/85-probe		**CY5**-CAG CCA GGA TTT AAT CAA CCTCAG-Tamra	

DNA sequencing: mgc2 sequencing for selected 20 positive cases (five 6/85 cases, seven F cases and 8 field cases) were performed. PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). The sequence reaction was done using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) and the sequence reaction was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan). A phylogenetic tree was created by the Meg Align module of Lasergene DNA Star.

RESULTS

Specificity test: MGA0319 and mgc2rt-PCR assays amplified DNA from MG strains only. Also mgc2 cPCR assay yielded specific bands for MG strains only. Also, all of the F, 6/85 and TS-11 rt-PCR assays were specific for its related strains.

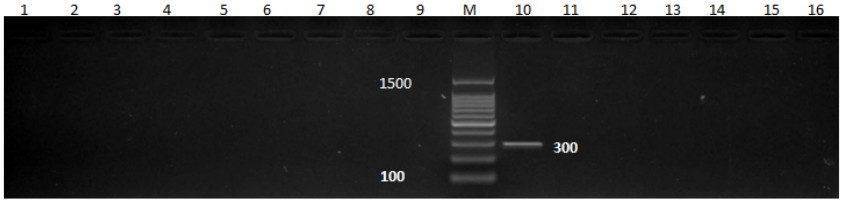

Photo.(1a) Gel electrophoresis result of mgc2 gene cPCR specificity test (1st part).

Electrophoresis result of mgc2 coiventional PCR specificity test. No amplification was detected except for the positive control . (1) M. bovigenitalium , (2) S. Typhimurium, (3) C. jejuni, (4) S. aureus, (5) E.coli, (6) C. perferingens, (7) ILT, (M) marker 100 plus , (8) IB , (9) IBD , (10) positive control, (11) AIV, (12) NDV, (13) REO , (14) POX , (15) Marek's, (16) Negative control.

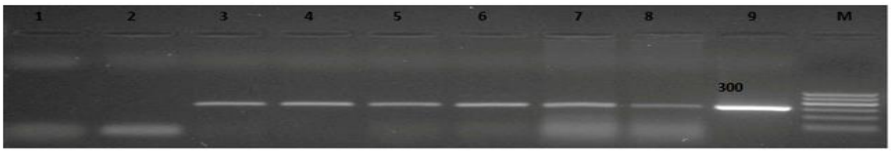

Photo.(1b) Gel electrophoresis result of mgc2 gene cPCR specificity test (2nd part).

Electrophoresis result of mgc2 cPCR specificity test. Positive amplification of 300 bp fragment was detected in MG strains. (L) Ladder 100-600 bp (1) *M. bovis.*, (2) A5969 strain, (3) HF51 strain, (4) TS11, (5) MG inac, (6) F VAX, (7) 6/85, (8) MS, (9) R strain.

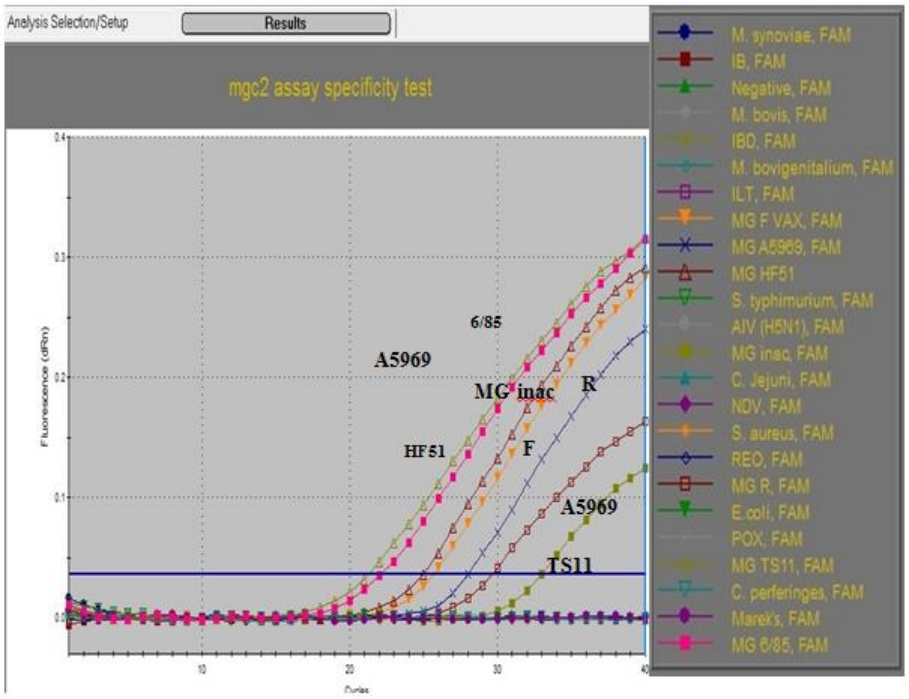

FIG. 1A

Figure (1A & 1B) Amplification curves of specificity test of the MG (1A mgc2 gene and 1B (MGA0319 gene) rt-PCR assay.

Amplification curves shows positive results for MG strains and negative results for other strains.

Amplification plot generated by StratageneMX3005P software. The fluorescence emission intensity is plotted on the Y axis versus the cycle number on the X axis.

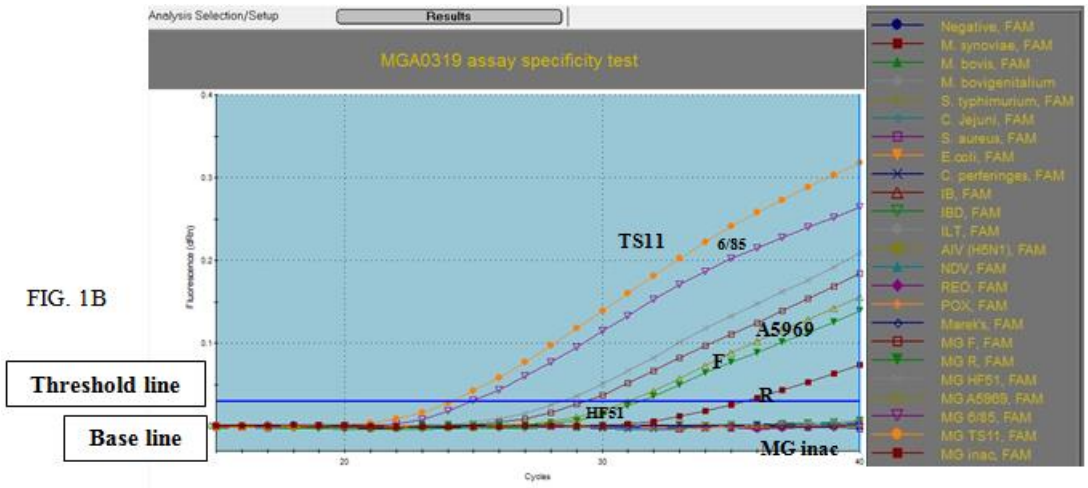

FIG. 1B

Sensitivity test:
TheMGcPCR and rt-PCR showed variable detection limits, as shown in Table (4).

Table 8: Results of sensitivity test of MG.

Assay	MG rt-PCR assay		Strain differentiating rt-PCR			mgc2 cPCR
	MGA0319 Gene	mgc2 gene	F	6/85	TS-11	
Detection limit (CFU / mL)	**50**	**14**	**18**	**12**	**20**	**70**

Field study results:
Results of different tests for different field groups are shown in table (9).

Table 9: Collective results of all the farms of the field study with different tests.

Group	Isolation	mgc2 cPCR	MGA0319 rt-PCR	mgc2 rt-PCR	Live vaccine rt-PCR		
					F	6/85	TS-11
VH	5/20	6/20	6/20	10/20	5/10/20	5/10/20	0/10/20
UNVH	0/13	2/13	4/13	4/13	4/4/13	0/4/13	0/4/13
VD	2/4	3/4	3/4	2/4	2/2/4	0/2/4	0/2/4
UNVD	3/13	4/13	7/13	8/13	1/8/13	0/8/13	0/7/13
Total positive	10/50 (20%)	15/50 (30%)	20/50 (40 %)	24/50 (48%)	12/50 (24%)	5/50 (10%)	0/50 (0)
% in relation to MG positive cases					12/24 (50%)	5/24 (16.67%)	0/24 (0)

VH: vaccinated healthy group UNVH: unvaccinated healthy group VD: vaccinated diseased
UNVD: unvaccinated diseased group
Also, the results of different tests in relation to the birds' health statuses are shown in **table (10).**

Table 10: Results of the field study with different PCR tests in relation to health status of the birds.

Health status	Isolation	mgc2 cPCR	MGA0319 rt-PCR	mgc2 rt-PCR	Live vaccine rt-PCR		
					F	6/85	TS-11
Healthy	5/33 (15.15%)	8/33 (24.50%)	10/33 (30.3%)	14/33 (42.50%)	9/14/33 (64.28%)[a] (27.27%)[b]	5/14/33 (35.7%)[a] (15.15%)[b]	0/14/33 0 0
Diseased	5/17 (29.4%)	7/17 (41.20%)	10/17 (58.80%)	10/17 (58.80%)	3/10/17 (40%)[a] (23.52%)[b]	0/10/17 0 0	0/10/17 0 0

a: Percent in relation to MG positive cases only b: Percent in relation to all farms

DNA sequencing results:
4 out of the 5 positive 6/85 samples: Healthy cases VH1, VH5, VH17 and VH18 (Accession no.JX981926, JX981928, JX981929 and JX981930, respectively) showed 100% identity and query coverage to 6/85 strain (AY556231.1). However, one sample Healthy VH2 (JX981927) showed 100% identity and 99% query coverage as it had a mutation from thymine to adenine (Position 243) which changed the N (Asparagine) amino acid to K (Lysine) causing a missense non conservative mutation.

Six positive F strain samples VH4, VD3, VD4, UNVD2, UNVD9 and UNVH4 (Acc. No.JX981931, JX981934, JX981935, JX981936, JX981937 and JX981933, respectively) showed100% identity to F strain (CP001873.1), while one sample VH20 (JX981932) had 99% identity as it had a silent mutation due to a nucleotide change from thymine to cytosine.

The DNA sequence grouped the 8 field strains samples into 2 groups. The 1st one called Field group A included 5 samples UNVD6, UNVD7, UNVD12, UNVD13 and UNVH12 (Acc. No. JX981939, JX981940, JX981941, JX981942 and JX981938, respectively) that showed great relation to some Egyptian strains as Eis6-T-10 (HQ591357.1) and some Israelian strains as UHP1 (AY556297.1) and YBS2 (AY556298.1).

The 2nd group was called Field group B, which included 2 identical samples UNVD3 (JX981943) and UNVD4 (JX981944) and another variant sample UNVD8 (JX981945). All of the group B strains showed great relation to the Pakistanian strain EgPK10AP08 (FJ395202.1), the Egyptian strain EGY/67240/CK08 (HQ143372.1) and S6 (AY556229.1). However, there was a mutation from the Pakistanian strain in 1 position in all samples (Position 36) that has lead to a mutation from I (Isoleucine) to M (Methionine) and another 3 silent mutations in the variant sample (JX981945).

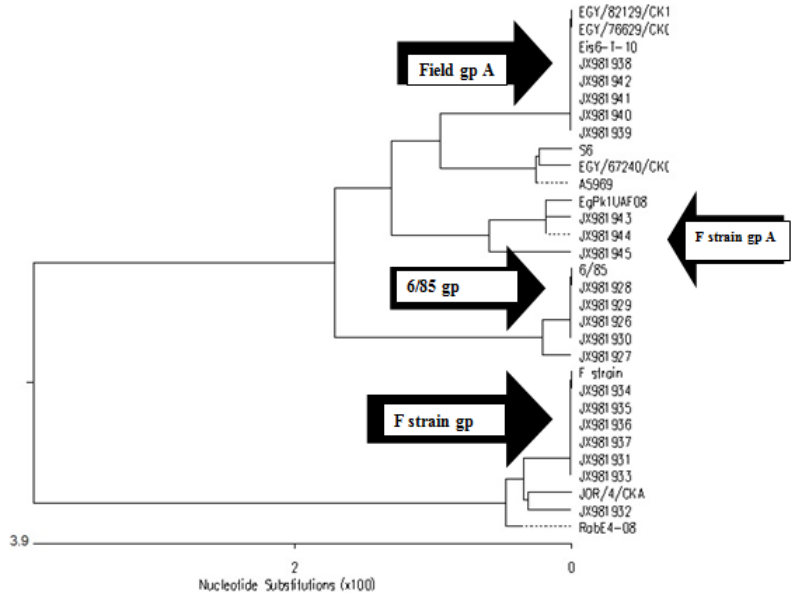

Fig. 1: phylogenetic tree of all field study samples.

DISCUSSION

The present study results showed that cPCR was more sensitive than isolation. This result agreed with Kempf *et al.* (1993); Saif-Edin, (1997); El Shater and Oraby, (2001) as their results showed that cPCR was more sensitive than isolation in different samples. The present study showed that rt-PCR yielded 20 and 24 positive samples for mgc2 and MGA0319 assays, while cPCR showed only 15 positive samples. The two tests agreed with each otherin 94.04% of samples. And both of the rt-PCR assays were sensitive than the cPCR assays. These results confirmed the results obtained in the sensitivity test of the validation step, this could be due to the determination of the CT value within the logarithmic phase of the amplification reaction, instead of the end point determination used by conventional systems, also detection of result by a computerized system in rt-PCR is much better than visual detection of bands in cPCR. The present study results differed from that of Callison *et al*. (2006) as they found that MGLP and cPCR assays had similar results for detection of MG. However, it agreed with Mekkes and Febrewee

(2005), who found that the rt-PCR detection limit was 10 to 1000 times lower than that of cPCR.

The specificity test showed that themgc2 rt-PCR, MGA0319 rt-PCR assays and the mgc2 cPCR were specific for MG strains. This was consistent with that obtained by Garcia *et al.* (2004); Callison *et al.* (2006); Grodio *et al.* (2008) who have tested these assays respectively and had similar results. Also our results agreed with those of Raviv *et al.* (2008) who found similar results for the live vaccinal strain assays. Also the result of the current study concerning the relation between the results of mgc2 cPCR and MGA0319 rt-PCR in the field study was close to that found by Callison *et al.* (2006) who found the agreement between the two tests was 97.74%, while it was 96.42% in our study. The mgc2 rt-PCR assay showed a detection limit of 14 CFU/ml, this result was close to that of Grodio *et al.* (2008), who found it less than 10 copies per reaction when tested with MG DNA. The MGA0319 rt-PCR 50 CFU/ml detection limit was also close to that of Callison *et al.* (2006) who reached the 25 copies detection limit. The mgc2 cPCR sensitivity test resulted in the study done by Garcia *et al.* (2004) was 40 CCU/reaction which can't be compared with our's because of the different unit. The detection limits for 6/85, F, TS-11 assays were 12, 18 and 20 CFU/ml, respectively. These limits were also so close to that reached by Raviv *et al.* (2008) who found the detection limits about 10 copies/reaction. From our antimortum findings, some cases in our study had severe respiratory signs, but it showed weak positive result in mgc2 and MGA0319 rt-PCR. This was described by Carli and Eyigor (2003), who found that the swabs taken by scraping trachea of necropsied birds are much better than live chicken swabs. This explains the negative results of the vaccinated birds like cases VD1, The strong positive rt-PCR results for some cases in which we could collect lung samples like UNVD2 confirmed this idea. Also, the negative results shown by the vaccinated flocks like VD1 may be due to vaccination failure. Case UNVD 13 was also positive for MG and negative by isolation. This result may agree with the idea of Ley and Yoder (1997) who reported that MG infections in turkeys resulting in mild clinical disease are unusual, While this result disagreed with Kempf *et al.* (1997) who stated that mild or subclinical cases of MG can be observed naturally and experimentally in chickens and turkeys.

Although some cases as UNVD 1 and UNVD 3 were collected from diseased flocks, they were MG negative which may be due to infection with other respiratory microorganisms. Furthermore, cases like UNVH4 and UNVD9 were unvaccinated but they were F strain positive. This was explained by Kleven (2002), who stated the possible vertical and lateral transmission of F strain within or between farms.

The mgc2 gene was the target for DNA sequencing as itwas characterized by the presence of different nucleotide insertions/deletions, which may be helpful for strains differentiation. While, the MGA_0319 gene encodes a predicted conserved surface lipoprotein (Ferguson *et al.,* 2005). The 6/85 VH2 DNA sequence was closely related to the MG TLS-2 strain (JN113387.1), this strain was isolated from Israelian broiler breeder farm and was sensitive to tylosin, tilmicosin and enrofloxacin as reported by Gerchman *et al.* (2011) this may confirm that this mutation didn't increase the virulence. This was explained by Steinage *et al.* (2003) who reported that 6/85-like isolates was recovered from vaccinated and unvaccinated contact chickens long after vaccination. And also agreed with Zaki *et al.* (2004) who studied the safety of MG 6/85 vaccine after backpassage in turkeys and found that both the original and the back passaged strains were apathogenic in turkeys.

Silentmutation in the F strain sample VH20 can be an explanation for the apparently healthy state found in this farm. But this mutation lead to sub grouping of VH20 in another subgroup that contained the Jordanian strain JOR/4/CKA (HQ143378.1), which was reported as F strain by Gharaibeh *et al.* (2011) who found that it was indistinguishable from F strain by RAPD and IGSR sequencing. The 5 field group A samples showed 100% identity with the Egyptian field strains Eis6-T-10, which was isolated from turkey farm and had an identity around 94-99% to Israelian strains as reported by Eissa *et al.* (2011). The phylogenetic tree showed that the field group A samples were also related to some geographically related field strains as the Israelian chicken strain (UHP1) and turkey strains YBS2. The field group A strains had different pathogenic profile as it didn't cause symptoms in case UNVH12. However, this may be related to the dose and time of infection in relation to time of sampling. The field group B samples were related to many field strains, but the most related one was the Pakistanian strain EgPK10AP08, which was isolated from a 22w layer farm, which showed rales, sinusitis and was related to S6, A5969. The field group B samples were also related to some Egyptian field strains as EGY/67240/CK08 (HQ143372.1). Collectively, the mgc2 DNA sequence was able to divide the 20 sequenced samples into the 4 mentioned groups. It was helpful to confirm the results of the live vaccine rt-PCR assays, and also to detect mutations within these vaccinal strains, also to give an image about some of the field strains that are circulating in the Egyptian chicken and turkey farms. The results showed that rt-PCR was valuable to differentiate live vaccinal strains. However, there were some point mutations in the vaccinal strains 6/85 and F strain which didn't lead to the rise of higher virulent strains, and also the rt-PCR assays were still able to detect these vaccinal strains. While, the detection of these

mutations will not be done by rt-PCR unless repeated in many strains and lead to the development of rt-PCR assay to detect these mutations. The same idea can be applied for the field strains after collection of data from different studies that can help to design new field strain rt-PCR assays. Continuing the studies related to this idea will help to make a database about the Egyptian MG state, which may help to assess the immune protection efficiency of the current, used MGvaccines, and may also lead to the creation of new homologous or heterologous vaccines for the Egyptian field.

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استخدام تفاعل انزيم البلمرة المتسلسل فى الوقت الحقيقى وإختبار الكشف عن التتابع النيكلوتيدى للتمييز بين العترات الحقلية واللقاحية للميكوبلازما جاللسبتكم

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التمييز بين العترات الحقلية واللقاحية للميكوبلازما جاليسبتكم هام لمعرفة وبائية المرض. فى هذه الدراسة تم استخدام إختبار تفاعل انزيم البلمره المتسلسل الكمى فى الوقت الحقيقى الخاص بالجينات mgc2 و MGA0319 للكشف عن الميكوبلازما جالليسيبتكم وذلك لإختبارها فيما بعد بإختبار تفاعل انزيم البلمره المتسلسل الكمى فى الوقت الحقيقى لجين mgc2 للكشف عن العترات F و 6/85 و TS-11. ٢٤ مزرعة كانت ايجابية للميكوبلازما جالليسيبتم من ٥٠ مزرعة دواجن محصنة وغير محصنة للميكوبلازما جالليسيبتكم كانت منهم ١٢ مزرعة إيجابية للعترة F و ٥ مزرعة إيجابية للعترة 6/85. لكن كانت هناك ٧ مزارع إيجابية للميكوبلازما جالليسيبتكم لكن سلبية لإختبار تفاعل انزيم البلمره المتسلسل الكمى فى الوقت الحقيقى الخاص بالعترات اللقاحية الحية. إختبار الكشف عن التتابع النيوكليتيدى للحامض النووى ل ٢٠ حالة إيجابية لجين mgc2 أكد نتائج إختبار تفاعل انزيم البلمره المتسلسل الكمى فى الوقت الحقيقى وجمع العينات المختبرة للتتابع النيوكليتيدى للحامض النووى في ٤ مجموعات (F و 6/85 و مجموعتين للعترات الحقلية تم تسميتهم مجموعة حقلية أ و ب).

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