Global Veterinaria 18 (3): 215-220, 2017
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2017.215.220

Detection of Antimicrobial Resistant Genes in Mycoplasma Species Isolated from Layers

[1]Nasima M. Hider, [2]E.E. Abd elaziz, [2]Etab M. Abo Remila and [2]Salwa M. Helmy

[1]Department of Microbiology, Faculty of Veterinary Medicine,
Kafr El Sheikh University, Kafr El Sheikh, Egypt,
[2]Department of Mycoplasma, Animal Health Research Institute Dokki, Giza, Egypt

Abstract: Avian Mycoplasmosis is considered as one of the major economic problems facing poultry industry all over the world because of its significant losses which are mainly due to poor feed conversion and carcass condemnation at processing. This study comprises the examination of 210 tracheal swabs from commercial and local breed layer flocks in Kafr Elshiekh Governorate. Isolation percent of mycoplasma isolates varied from (5-80%). Sixteen *mycoplasma gallisepticum (MG*) isolates were identified from 210 tracheal swabs of 7 layer flocks with percentage 7.62% by PCR, while four *Mycoplasma synoviae* (*MS*) isolates were obtained with percentage 1.9% and six *Mycoplasma gallinarum(MGn)* was 2.86%. By PCR *MG* strains showed a characteristic band at 300 bp, *MS* showed a characteristic band at 1100 bp and *Mycoplasma gallinarum* targeting ISR gene giving a band about 540 bp.*MG* sensitive by Minimal Inhibitory Concentration (MIC) to tilmicosin and tylosin with mic (0.004 and 0.039 µg/ml) and it was resistant to erthythromycin with mic (20µg/ml), while it gives moderate resistance to ciprofloxacin, doxycycline and lincospectin respectively.*MGn* isolates were sensitive by disc inhibition to lincospectin, florfenicol and oxytetracycline while one strain was resistant to ciprofloxacin. PCR results of *MG* field isolates targeting resistance gene(Gyr.A) of quinolones at 500bp. It is advisable to use tilmicosin and tylosin for prophylactic and treatment purposes for *MG* control programs for chickens.

Key words: Mycoplasma · PCR · MIC · Resistant Gene · Layers

INTRODUCTION

Mycoplasma belonging to class Mollicutes. It is small free living highly fastidious and slow growing micro-organism [1]. mycoplasma typically cause respiratory diseases in their host and in chickens, the disease is characterized by coughing, nasal discharge and air sac lesions, but in some infections no clinical symptoms appear [2].*MG* infection start with colonization of the respiratory tract, tracheaitis and airsacculitis. Occasionally *MG* infection is also associated with conjunctivitis, salpingitis, arthritis [3]. *M.G* and *MS* can cause 20-30% reduction in egg production [4]. Furthermore egg with pimpled shells also associated with mycoplasma infections [5]. *MS* infection may cause drop in egg production of up to10 eggs fewer per hen [6, 7] study of eggshell apex abnormalities induced by *MS.* Infection with *MGn* cause Fatty liver hemorrhagic syndrome in commercial layers [8]. El-shater [9] isolated *M.G* (16.7%) and *MGn* (77.8%) from naturally infected chicken flocks.

Shaker [10] isolated *M. gallisepticum* (4.04%) and *M.synoviae* (8%). Eissa *et al*. [11] could isolate *M.S* 13.33%, while *M.G* was 6.66% from turkey pullet.Polymerase chain reaction (PCR) represent a rapid and sensitive alternative to traditional culture methods. Electrophoretic agarose gell of *Mycoplasma gallisepticum* using mgc2 primers [12]. PCR of *M.synoviae* using specific primers [13]. PCR of *M.gallinarum* targetting intergenic spacer region (ISR) [14]. Extensive use of quinolones such as ciprofloxacin, enrofloxacin and danofloxacin was the main cause of quinolones resistance, Jian *et al*. [15]. Quinolones inhibit DNA gyrase and topoisomerase IVactivities which are involved in DNA replication [16]. Mycoplasma acquired

Corresponding Author: Nasima Mohamed Hider, Department of Microbiology, Faculty of Veterinary Medicine, Kafr El Sheikh University, Kafr El Sheikh, Egypt. Tel: +1009845043.

resistance to macrolides has been associated with mutations domain II or V of the 23S rRNA genes or rPID and rPIV, genes encoding ribosomal proteins L4 and L22 [17]. PCR of *MG* field isolates targeting resistant gene(Gyr.A) of quinolones. so, the aim of this study is detection of antimicrobial resistant genes in *Mycoplasma gallisepticum* isolated from layers.

MATERIALS AND METHODS

Samples: Two hundred and ten (210) tracheal swabs were collected from commercial and local layer breed from kafer-Elsheikh Governorate. All the samples were taken from flock with history of previous respiratory signs and drop in egg production.

Isolation and identification of Mycoplasma: Mycoplasma was isolated on specific medium PPLO described by Adler [18] and Erno and Stipkovits [19].

Biochemical Tests: Biochemical tests were performed as previously described by Erno and Stipkovits [19] and Freundt [20] these tests include digitonin sensitivity, glucose fermentation, arginine hydrolysis, film and spot production.

DNA Extraction and Purification: DNA extraction from the isolates was done as described by Fan *et al.* [21].

Primers Used for Detection of Mgc2 Gene [12]:

mgc2-2F (5′ CGC AAT TTG GTC CTA ATC CCC AAC A3′)
and mgc2-2R (5′TAA ACC CAC CTC CAG CTT TAT TTC C3′).

Amplification was 95°C for1min.followed by 40 cycles of 95°C for 20 sec, 60 °C for 40 sec. and 72 °C for 10 sec.

Primers and Amplification of the Target Sequence Intergenic Spacer Regions (ISRs) [14]: Using the following primer:

F (5′CGT TCT CGG GTC TTG TAC AC3′) and
R (5′CGC AGG TTT GCA CGT CCT TCA TCG 3′)

DNA amplification was accomplished with five cycles of denaturation at 94 °C for 15 sec, renaturation at 60 °C for 30 sec and elongation at 72 °C for 2 min, followed by 30 cycles with the same sequence, except for an extension of 2 sec per cycle in the elongation step.

Primers and amplification of the pMS122-11 [13]:

(5′GAA GGT ATT TAA TCA ACC TTG GCT G3′)
(5′TGT AAT GGC TCC AGC TCA AGC AGC T3′).

Amplification was at 95°C for2 min; this followed by 35 cycles of three different temperature and time(°C/min) segments of 94oc for 20 sec., 51 °C for 30 sec. and72 °C for 1 min. which corresponded to target DNA denaturation, primer annealing and primer extension.

Detection of Resistant Gene
Primers Used for Detection of Fluroro Quinolones Resistant Gene [22] of *Mycoplasma gallisepticum* gyrA with sequence

F: (5′GAG CTA GAA ACA TCA TTC ATG G3').
And R: (5' CCT ACA GCA ATA CCA CTT GAA 3').

PCR Amplifications Were Performed as Follows:

3 min at 95°C. 30 cycles of 95°C for 30 sec,56°C for 30 sec and 72°C for 45sec and 72°C for 10 min.

Minimal Inhibitory Concentration (MIC) of Some Antimicrobial Against Mycoplasma [23]

RESULTS

Isolation of Mycoplasma from Layer Flocks: Fifty six mycoplasma isolates were gained from 210 tracheal swabs of 7 layer flocks with different ages under examination with percent ranged from 5-80% from kafer El Elsheikh governorate. As in Table (2)

Biochemical Charachterization of Mycoplasma Isolated from Layer Flocks as in Table (3): Fifty mycoplasma isolates were glucose and arginine positive, 6 isolates were glucose negative while 6 isolates were arginine positive and 10 *mycoplasma* isolates were film and spot positive and 46 mycoplasma isolates were film and spot negative.

PCR Results of Isolates from Mycoplasma **Layers:** The 56 mycoplasma isolates from them by PCR results are 16 *MG*, 4 *MS, 6 Mycoplasma gallinarum* and 30 untyped as in Table (5).

16 *MG* isolates were identified from 210 tracheal swabs of 7 layer flocks with percentage 7.62% by PCR, while four *MS* isolates were obtained with percentage 1.9% and *Mycoplasma gallinarum* was 2.86%, *Mycoplasma* isolates appear as tiny smooth

Table 1: Antimicrobial used in sensitivity test:

Antibiotics test	Concentration	Company
Ciprofloxacin	20%	Arabco-med
Doxycycline	50%	Atco pharma
Erythromycin	20%	Mefeco
Licospectin	100/50	Pfizer
Streptomycin	100%	Mefco
Tilmicosin	30%	Arabco-med
Tylosin100%	100%	Arabco-med

Table 2: Primary isolation of mycoplasma from layer flocks

Breed	Site of isolation	No. of samples	No.ofpositive samples	Digitonine	%of isolation
Farm1 layer	Trachea	50	40	+	80%
Farm 2 layer	Trachea	20	-	-	-
Farm 3 layer	Trachea	20	-	-	-
Farm 4 layer	Trachea	20	1	+	5%
Farm5 layer	Trachea	30	2	+	6.7%
Farm 6 layer	Trachea	20	5	+	25%
Farm 7 baladi layer	Trachea	40	6	+	15%
Farm 7 baladi layer	Trachea	10	2	+	20%
Total		*210*	*56*		*26.6%*

mycoplasma colonies appear under microscope as tinny smooth colonies with raised area, while the character of film and spot appear in case of *Mycoplasma synoviae* and *Mycoplasma gallinarum.*

Table 3: Biochemical charachterization of mycoplasma isolated from layer flocks

	Biochemical tests					
Number of isolates	Glucose		Arginine		Film and spot	
56	+	-	+	-	+	-
	50	6	6	50	10	46

Table 4: PCR results of mycoplasma isolates from layers

Number of Mycoplasma isolates	MG	MS	Mycoplasma gallinarum	Others
56	16	4	6	30 untyped

colonies on agar with a characterstic feature (Film and spot) in case of *Mycoplasma synoviae* and *Mycoplasma gallinarum***.**

Targeting *mgc2* gene of mycoplasma isolates, seven mycoplasma isolates giving specific band at 300 bp as in Fig: (1).

Three *Mycoplasma synoviae* isolates was identified giving specific band at 1100 bp as in Fig: (2).

As shown in Fig:(3), PCR product of field isolate isolated from chicken trachea that giving the possibility to be *MGn* Targeting ISR gene giving a band about 540 bp.

Minimal Inhibitory Concentrations (MICs) of Some Antibiotics Against *M. Gallisepticum* Isolates from Layer Flock: *MG* isolate was very sensitive to tilmicosin and tylosin with mic 0.004 and 0.039 µg/ml as in Table (5) and it was resistant to erthythromycin with mic 20 µg/ml,while it give moderate resistant to ciprofloxacin, doxycycline and lincospectin respectively.

Table (5), revealed that erythromycin, streptomycin were not effective on *MG*1while *MG*2 was resistant to ciprofloxacin, they had higher minimum inhibitory concentration (mic) (20,10,5 µg/ml) respectively than their C.max. Tilmicosin, tylosin were very effective *MG*1with mic 0.004 and 0.039 µg/ml receptively while tilmicosin was superior on the two *MG* isolate.

As in table (6), *Mycoplasma gallinarum isolates* was sensitive to lincospectin, florfenicol and oxytetracycline while strain 1 was resitant to ciprofloxacin.

PCR of *MG* Field Isolates Targeting Resistance Gene (Gyr.A) of Quinolones: As in Fig: (4), PCR results of *MG* field isolates targeting resistance gene (Gyr.A) of quinolones, where this gene was detected in these isolates in addition to control F strain with different degree and these result was similar to mic.

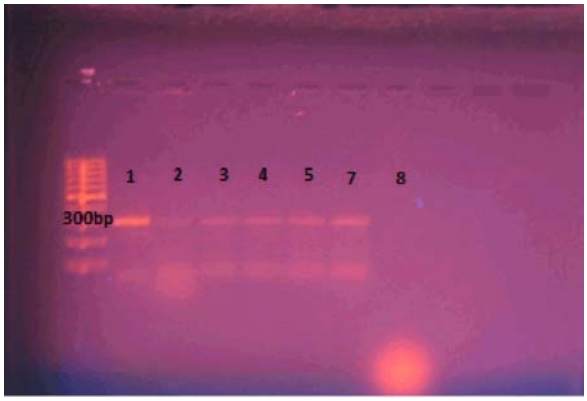

Fig 1: Electrophoretic agarose gell of *Mycoplasma gallisepticum* using mgc2 primers
1- 100bp DNA ladder
2- Control positive MG
3- 2-7 *MG* field isolates
4- 8- control negative

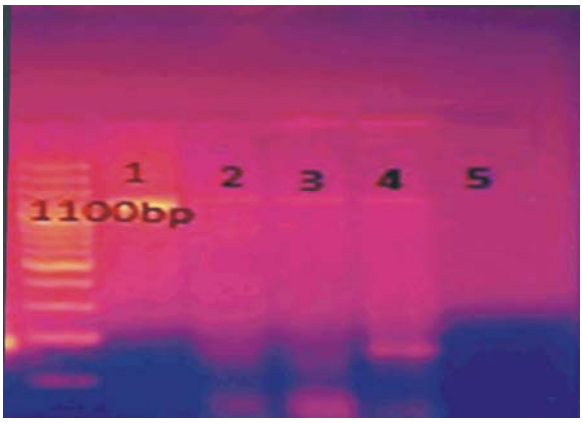

Fig. 2: Elecrophoretic gell of *Mycoplasma synoviae* using specific primers
1- 100 bp DNA ladder
2- Control positive MS
3- 2- 4 field MS samples
4- 5- control negative

Table 5: Results of *MG* strain sensitivity to some antimicrobials with first well concentration 25 µg/ml

	Sensitivity test/ µg/ml		
Antimicrobial	MG 1	MG 2	C. max
Ciprofloxacin	1.25	5	2.44
Doxycycline	1.25	1.25	4.47
Erythromycin	20	0.625	2.44
Lincospectin	2.5	5	8.13
Streptomycin	10	2.5	21
Tilmicosin	0.004	0.625	2.09
Tylosin	0.039	1.25	2.09
Control	culture +	Culture+	

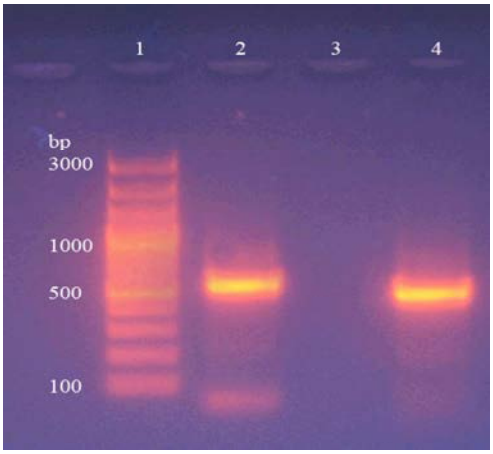

Fig. 3: Agarose gell electrophores of *Mycoplasma gallinarum* targeting intergenic spacer region (ISR).
1- 100bp DNA ladder
2- Control positive mycoplasma
3- control negative
4- Sample positive

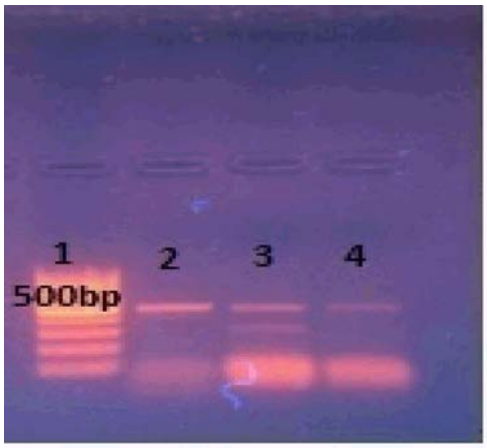

Fig. 4: Elctrophoretic gell of PCR results of resistance gene of quinolones
1- 100bpDNA ladder
2- Positive control F strain
3- Field isolate MG1
4- Field isolate MG2

Table 6: Sensitivity test by disc inhibition for *Mycoplasma gallinarum* isolates

Antibiotic	Conc/disc	Strain 1 mgr/mm	Strain 2 mgr/mm
Ciprofloxacin	CIP5	Resistant	1.5
Erythromycin	E15	1.5	1.2
Florofenicol	FFC30	2	1.8
Iincospectin	LIN100/50	2.4	2
Oxytetracyclin	OT30	1.8	1.5
Spiramycin	SP100	1.5	1.3

DISCUSSION

In the present investigation recovery rate of *MG* was (7.62%) from Kafr elshiekh Governorate, *MS*(1.9%)and *MGn* recovery rate is (2.86%).These results agree with that recorded by several authers [10,11,24]. For *MS* isolation ratio (1.9) by PCR but Shaker [10] could isolate *M.synoviae* by 8% while [11] isolate *M.synoviae* by 13.33%.*M.gallinarum* was isolated from layers with percentage2.86% this is dis-agreement with El-shater [9] who isolate *M.gallinarum* by (77.8%) and Mansour [24] by (17%). In the present study, PCR technique was used for identification of mycoplasma in infected layer flocks. The examined *M. gallisepticum* strains showed a characteristic band at 300 bp, this result is agreement with those obtained by Lysnyansky *et al.* [12], El-Shater *et al.* [25] and Loolmani *et al*. [26]. Elecrophoretic gell of *mycoplasma synoviae* showed a characteristic band at1100 bp, this result is agreement with Zhao and Yamamoto [13] who identified *MS* by PCR with characteristic band at1.1 kbp. *Mycoplasma gallinarum* was identified biochemically and by PCR, where PCR results gave band at 540bp, which agreement with Ramírez *et al.* [14] and Abd El-Aziz and Abd-Alla [27] who isolates MG from turkey. PCR results of *MG* field isolates targeting resistance gene (gyr.A) of quinolones, where this gene was detected in these isolates at 500bp, this result agree with Lysnyansky *et al.* [22]

CONCLUSION

Mycoplasma gallisepticum (MG) is a persistent, highly transmissible chicken pathogen.It predisposes the birds to other infection yielding significant losses in performance and associated economics to all sectors of the poultry industry. depended in their culture on PPLO media which appears as tinny smooth with raised area from center (Fried egg) appearance under stereromicroscope, differentiation from achloplasma using digitonin test where mycoplasma is sensitive to digitonin showing marked inhibition zone surround the disc. Biochemical identification proved that *M.gallisepticum and Mycoplasma synoviae are* glucose positive and Argnine negative while *M.gallinarum was* arginine,Film and Spot positive. Extensive use of quinolones such as ciprofloxacin, enrofloxacin and danofloxacin was the main cause of quinolones resistance. This study was investigated for molecular detection of mutation inquinolone resistance determining regions(QRDRs) of quinolone resistant *MG* field isolates and resistance to macrolides. Finally quinolones antibiotic has been used as the routine of treatment and prophylaxis in layer flocks lead to appearance of quinolones resistant mycoplasma isolates as found in this work and this finding indicates alarmingly that the quinolones will become useless with in the next few years. This study cocluded that it is advisable to use tilmicosin and tylosin for prophylactic and treatment purposes for *MG* control programs for chickens.

ACKNOWLEDGMENT

The auther would like to thanks Department of Mycoplasma, Animal Health Research Institute Dokki, Giza, for financial support and necessary facilities to carry out this work.

REFERENCES

1. Nicolas, R. and R.D. Ayling, 2003. Mycoplasma bovis :diseas,diagnosis and control. Research in Vet. Sci., 74(2): 105-112.
2. Feberwee, A., D.R. Mekkes, D. Klinkenberg, J.C. Vernooij, A.L. Gielkens and J.A. Stegeman, 2005a. An experimental model to quantify horizontal transmission of Mycoplasma gallisepticum. Avian Pathol., 34: 355-361.
3. Winner, F., R. Rosengarten and C. Citti, 2000. In vitro cell invasion of Mycoplasma gallisepticum. Infection and Immunity, 68(7): 4238-4244.
4. North, M.O., 1984. Breeder Mangment In Commercial Chicken Production manual. The Avi. Publishing Company . Inc . Westport, Connectict, pp: 240-243,298-321.
5. Branton, S.L., B.D. Lott, W.R. Maslin and E.J. Day, 1995. Fatty liver hemorrhagic syndrome observed in commercial layers fed diets containing chelated minerals. Avian Dis., 39: 631-635.
6. Khan, M.I., K.M. Lam and R. Yamamoto, 1987. Mycoplasma gallisepticum strain variations detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Avian Diseases., pp: 315-320.
7. Feberwee, A., J.J. De Wit and W.J. Landman, 2009. Induction of eggshell apex abnormalities by Mycoplasma synoviae: field and experimental studies. Avian Pathology, 38(1): 77-85.
8. Burnham, M.R., E.D. Peebles, S.L. Branton, M.S. Jones and P.D. Gerard, 2003. Effects of F-strain Mycoplasma gallisepticum inoculation at twelve weeks of age on the blood characteristics of commercial egg laying hens. Poultry science., 82(9): 1397-1402.

9. El-shater, S.A.A., 1986. Some studies on chronic respiratory disease in fowls Ph.D.Thesis, Poultry department, faculty of Vet. Med., Ass. Univ.
10. Shaker, M.M., 1995. Microbiological Studies on mycoplasma infection in poultry. Ph.D. Thesis, Fac. Vet. Med., Cairo Universiyty.
11. Eissa, S. I. and M. A. Dardeernd Abo-Norag, 2000. Application of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for identification of mycoplasma infection in turkeys with special reference to treatment. Veterinary Medical Journal Giza, 48(2): 197-206.
12. Lysnyansky, I., M. García and S. Levisohn, 2005. Use of mgc2-polymerase chain reaction–restriction fragment length polymorphism for rapid differentiation between field isolates and vaccine strains of Mycoplasma gallisepticum in Israel. Avian Diseases., 49(2): 238-245.
13. Zhao, S. and R. Yamamoto, 1993. Detection of Mycoplasma synoviae by polymerase chain reaction. Avian Pathology, 22(3): 533-542.
14. Ramírez, A.S., C.J. Naylor, C.A. Yavari, C.M. Dare and J.M. Bradbury, 2011. Analysis of the 16S to 23S rRNA intergenic spacer region of Mycoplasma synoviae field strains. Avian Pathology., 40(1): 79-86.
15. Li Jian, Lu Dian Hong, Liu ZhiJie, Zhang XiaoHua, Wei FeiLong, Liu YaHong and Jiang HongXia, 2012. Role of mutations in DNA gyrase and topoisomerase IV in fluoroquinolones-resistance of Mycoplasma gallisepticum obtained in vitro and in vivo. Journal of Animal and Veterinary Advances., 11(13): 2327-2332.
16. Wang, M., Q. Guo, X. Xu, X. Wang, X. Ye, S. Wu and M. Wang, 2009. New plasmid-mediated quinolone resistance gene, qnrC, found in a clinical isolate of Proteus mirabilis. Antimicrobial Agents and Chemotherapy, 53(5): 1892-1897.
17. Vester, B. and S. Douthwaite, 2001. Macrolide resistance coferred by base substitution in 23rRNA. Antimicrob.Agents Chemother., 45(1): 1-12.
18. Adler, H.E., J. Fabricant, R. Yamamoto and J. Berg, 1958. Symptoms on chronic respiratory disease of poultry. Isolation and Identification of pleuropneumonia- like organism of Avian origin. Am. J. Vet. Res., 19: 440-447.
19. Erno, H. and L. Stipkovits, 1973.Bovine Mycoplasmas, cultural and biochemical studies. Acta-Vet. Scand., 14: 450-463.
20. Freundt, E.A., 1973. Culture media for classic mycpoplasma. In:Methods in mycoplasmology. Razin, S. and Tully. J. G.eds. 1: 127-135.
21. Fan, H. H., S. H. Kleven and M. W. Jackwood, 1995. Application of polymerase chain reaction with arbitrary primers to strain identification of Mycoplasma gallisepticum. Avian Diseases., 729-735.þ
22. Lysnyansky, I., I. Gerchman, S. Perk and S. Levisohn, 2008. Molecular characterization and typing of enrofloxacin-resistant clinical isolates of Mycoplasma gallisepticum. Avian Diseases., 52(4): 685-689.
23. Senterfit, L.B., D. Taylor-Robinson, J.A. Robertson, C. Bebear and G. Laber, 1983. Antibiotic sensitivity testing of mycoplasmas. Methods in Mycoplasmology., 2: 397-401.
24. Mansour, A.F.A., 1995. Studies on the Prevalence of Avian Mycoplasma in Egypt and their economical importance. M.V.Sc.Thesis, Poultry Department, Fac.of. Vet. Med., Cairo.Univ.
25. El-Shater, S.A.A., S.I. Eissa and A.M. Hassan, 2000. Detection of pathogenic mycoplasmas (M. gallisepticum and M. synoviae) in samples from chickens and turkeys by polymerase chain reaction (PCR) and identification by arbitrarily primed PCR (AP-PCR). Egyptian Journal of Agricultural Research, 78(1, Special Issue), 57-68.
26. Loolmani, F.S., Seyyed Ali pourbakhsh, Mansour Banani and Said Charkhkar, 2014. Phylogenetic analysis of mgc2 gene of Mycoplasma isolates from broiler breeder flocks in Tehran province,Iran. European journal of Zoological Research, 3(2): 37-42.
27. Abd El-Aziz, E.E. and S.H. Abd-Alla, 2014. Isolation and molecular characterization of Mycoplasma from turkey sinusitis with special reference to treatment in dakahlia governorate. Animal Health Research Journal, 2(4): 282-290.