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Genetic Analysis of Fluoroquinolone Resistant Genes in *Mycoplasma gallisepticum* Field Isolates

Sahar O. E[a]. and Sally H. Abou-Khadra[b,*]

[a]Mycoplasma Department, Animal Health Research Institute, Dokii, Giza.,[b]Microbiology Department, Animal Health Research Institute Zagazig branch.,Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt.

ABSTRACT

Mycoplasma gallisepticum (MG) causes economic losses to poultry industry all over the world. This study aimed to investigate the emergence of fluoroquinolone (FQ) resistance in MG field isolates using sequence analysis of genes encoding the subunits of the drug's target enzymes. The results showed that two MG isolates were resistant to fluoroquinolones. Amino acid mutations were recorded in *gyrA* at codons 59, 73, 157 and 174, *gyr*B at codons 415 in addition to Glu at 416 and *Par*C at codons 92 and 167. No mutations were observed in *gyr*E. Two-dimensional analyses illustrated that mutations in the resistant genes; *gyr*A, *gyr*B, and *Par*C were associated with alterations in the predicted protein structures of quinolone-resistance determining regions (QRDRs). These mutations were accompanied by elevation of minimum inhibitory concentration (MIC) profiles to FQ. It was concluded that the mutations in genes encoding DNA gyrase enzyme were the important causes for the appearance of FQ resistance MG isolates.

Keywords: *Mycoplasma gallisepticum*; MIC; Antimicrobial resistant genes; Mutation; Two-dimensional analyses.

Abbreviations*:*

MG: *Mycoplasma gallisepticum*
FQ: fluoroquinolone
QRDRs: quinolone-resistance determining regions
MIC: minimum inhibitory concentration
NCCLS: National Committee for Clinical Laboratory Standards

INTRODUCTION

Mycoplasmas are characterized by lack of cell wall, small size, and small genome bacteria (**Ferguson-Noel, 2013).** Within poultry industry, the power of *Mycoplasma gallisepticum* to infect both of the respiratory and reproductive tracts of poultry has made it a pathogen of highly economic concern (**Raviv and Ley, 2013**). Avian mycoplasmosis is diagnosed through using three main approaches: isolation and identification, determination of antibodies, and molecular detection of the organism's nucleic acid by PCR (**Kleven,1998**). The gold standard method for direct detection of the organism is culture it, but pathogenic avian mycoplasmas are slow growing, relatively fastidious

organisms, and might require up to 3 weeks for detectable growth **(Kelven, 2003)**. PCR represents a rapid and sensitive than culture methods. The 16SrRNA PCR method is commonly used for confirmation of mycoplasma infection in chickens

Antimicrobial agent plays a very important role in treating disease caused by *M. gallisepticum* **(Hannan *et al.* 1997; Ley and Yoder, 1997).** MG is known to be sensitive to antimicrobials that act on targets other than the bacterial cell wall, such as tetracyclines, macrolides, and quinolones **(Umar *et al.* 2017).**

In MG, the major target for quinolones is DNA gyrase (**Reinhardt *et al.* 2002** a). This enzyme belongs to type II topoisomerase family, which facilitates DNA unwinding at replication forks, while the main action of topoisomerase IV, is to remove the interlinking of daughter chromosomes at the completion of a round of DNA replication and allows their segregation into daughter cell **(Ruiz, 2003; Ambrozic *et al.* 2007)**.

Recently, the investigators have to return to the studies of mycoplasmas' proteome architecture as their proteins are orchestrated to provide cell survival. The cells of pathogenic mycoplasmas are characterized by the high adaptive potential to evade a host immune surveillance, acquire antimicrobial resistance, and to disseminate to new hosts **(Butenko *et al.* 2017)**. Protein structure comparison is fundamental in structural biology and bioinformatics. The overlap of two-dimensional (2D) maps of two structures can be easily calculated, providing a sensitive measure of protein structure similarity. Also, two-dimensional maps contain sufficient information to restore the 3D representation **(Pietal *et al.* 2007). Chernova *et al.* (2016)** mentioned that the 2D Bioinformatic identification of protein-coding DNA gyrase and topoisomerase IV targeted by fluoroquinolones antibiotics important for understanding the resistance of MG isolates for treatment.

So, our study aimed for identification of fluoroquinolone-resistant *Mycoplasma gallisepticum* isolated from broiler flocks and studying the sequential and proteomic mutation that occurred in *gyr*A, *gyr*B, *Par*C, and *Par*E genes and its predicted protein.

MATERIALS AND METHODS

Samples

One hundred samples were collected from broiler flocks, with a history of respiratory manifestations from different localities in (El-Sharkia Governorate) and not respond to treatment. Samples included 56 tissue samples (air sacs, tracheas, and lungs) and 44 choanal cleft swabs from diseased chicken.

Isolation and identification of *Mycoplasma* species

Mycoplasmas were isolated on PPLO broth and agar medium, as previously mentioned **(Sabry and Ahmed, 1975).** Mycoplasmas were differentiated from

Acholeplasma using digitonin test **(Freundt *et al.* 1973)**. Biochemical characterization of *Mycoplasma* isolates was performed using glucose fermentation and arginine deamination **(Erno and Stipkovits, 1973).**

***In vitro* antimicrobial susceptibility testing**

Antimicrobial susceptibility of ten *M. gallisepticum* field isolates were tested against fluoroquinolones such as enrofloxacin (ENFX), ciprofloxacin (CPFX), difloxacin (DIFX), gatifloxacin (GFLX), levofloxacin (LVFX) and ofloxacin (OFLX) (Oxoid, UK) using broth microdilution method **(Hannan, 2000)**. Antimicrobial concentrations ranged from 0.016 to 16 µg/ml. MIC results were interpreted according to National Committee for Clinical Laboratory Standards Institute **(** NCCLS **, 2002);** and Clinical & Laboratory, Standards, Institute **(CLSI, 2011)**. MIC50 and MIC90 were calculated using an orderly array method **(Hamilton-Miller, 1991)**.

Conventional polymerase chain reaction (PCR)

DNA extraction of *Mycoplasma* isolates

DNA extraction was performed for ten mycoplasmas recovered from broilers with respiratory manifestations according to **Ley and Yoder (1997)**.

PCR amplification and cycling protocol

PCR was done for detection *Mycoplasma* isolates using *16S r*RNA and mgc2 primers. In addition, *gyr*A, *gyr*B, *Par*C and *par*E amplified with genes specific primers designed on the basis of the genomic sequence of *M. gallisepticum* strain R (accession no. AE015450) (**Papazisi *et al.* 2003**)**.** The selected primers from the published papers are shown in (Table 1).

The amplified PCR product was electrophoresed on a 1.5% agarose gel in Tris-acetate-EDTA buffer **(Ewers *et al.* 2005)**. And 100-bp DNA ladder (Invitrogen, Carlsbad, CA) was used as a molecular weight marker.

Sequence analysis

DNA sequencing of *gyr*A, *gyr*B, *Par*C, and *par*E genes was conducted in both directions. The original sequence was trimmed to remove vague nucleotide sequences usually exist at the beginning of the sequencing reaction. Partial DNA sequences were submitted to the Gene Bank database and obtained accession numbers; KM076644 and KJ754683 for *gyr*A, KJ486460, and KJ754684 for *gyr*B, KJ778874 and KJ754685 for *Par*C and KM076645 and KM076646 for *parE*. Identification of homologies between nucleotide and amino acid sequences of the studied *Mycoplasma gallisepticum* DNA sequences and others published in GenBank were done using BLAST 2.2 search program (National Center for Biotechnology Information "NCBI" http://www.ncbi.nlm.nih.gov/). Comparisons of the obtained nucleotide sequences to *E. coli* sequences that published in GenBank were done using the BioEdit sequence alignment editor **(Hall, 1999)** and MegAlign, DNASTAR, Lasergene®, Version 7.1.0, USA. The phylogenetic trees were constructed using MegAlign for tree reconstruction of

sequences by Neighbor-joining method based on ClustalW **(Thompson *et al.*1994**). Sequence divergence and identity percentages were calculated by MegAlign. The structural character of GyrA, GyrB, ParC and ParE proteins sequences were identified by Protean (DNASTAR, Lasergene®, Version 7.1.0. USA).

Two-dimensional protein analysis of the QRDRs:

The deduced amino acid sequences of GyrA, GyrB and ParC proteins were analyzed using the PROTEIN subroutine in the DNASTAR software package (DNASTAR, Lasergene®, Version 7.1.0. USA). This subroutine uses the algorithm for predicting antigenicity **(Jameson and Wolf, 1988)**.

RESULTS AND DISCUSSION

***Mycoplasma* isolation rate .**

*Mycoplasma***,** belonging to the class Mollicutes**,** is a small free-living highly fastidious and slow growing micro-organism, **(Nicholas and Ayling, 2003).** One important feature of *M. gallisepticum* infection is that it can persist in the bird during all life, even in the presence of the humoral antibodies **(Stipkovits *et al.* 2011).**

In the present investigation, a recovery rate of MG isolation [(10/100)(10%)] obtained from respiratory organs of broilers suffered from respiratory manifestation in El-Sharkia Governorate**.** This result agreed with that recorded by several authors **(Ulgen and Kahraman, 1993; Dardeer, 1997; Mohamed, 1997; Saif-Edin, 1997; Sharaf, 2000; Mohammed, 2001)**. The present finding revealed that all examined mycoplasma isolates had *16S r*RNA, with a characteristic band at 185 bp (Fig.1a), as mentioned by **Lauerman (1998)**. Further confirmation was made using *mgc*2 primers for detection of the MG isolates (Fig.1b).

MICs of fluoroquinolone against *M. gallisepticum* isolates.

Among ten MG isolates, only two isolates were resistant to used fluoroquinolones, as mentioned before by **Reinhardt *et al*. (2002 b); Jian *et al*. (2012).** The MIC values for fluoroquinolones of tested MG isolates ranged from 0.063 to 8ug/ml as listed in **Table (2)**. These results were consistent with that reported by **Gerchman *et al*. (2008)**.

MIC50 and MIC90 values were lower for the human-use fluoroquinolones as compared to the veterinary-use agents. The MIC_{50} for both enrofloxacin and difloxacin was 0.25 µg/ml **(Table 3)**. These findings were lower than those recorded by **Gerchman *et al*. (2008)**. Also, the MIC_{90} for enrofloxacin was 0.5 µg/ml which was lower in comparison with previously mentioned **(Catania *et al.* 2019**). Gatifloxacin (GFLX) was the most effective fluoroquinolone against all MG isolates, that agreed with previously recorded by **Andriole, (2005).**

PCR amplification and DNA sequence analysis of quinolone-resistant determining region (QRDR) of MG isolates.

From all tested MG strains, 484-bp covering the entire QRDR of *gyr*A (Fig. 2a), a 580-bp fragment covering the region of *gyr*B (Fig.2b), 463-bp PCR products encompassing the regions of *Par*C and 440-bp fragment covering the region *par*E (Fig.2c and Fig.2d respectively) were obtained.

Genetic analysis of *gyr*A

Solid-phase sequencing of the amplified DNA revealed amino acid replacement at codon 59 (Histidine CAT→ Tyrosine TAT) and 157 (Isoleucine ATT→Valine GTT) in both analyzed isolates. Additionally, replacement at codon 73 (Valine GTT→ Alanine GCG) in SAAS.1 isolate and 174 (Isoleucine ATT→ Leucine TTG) in SAAS.12 isolate. Amino acids replacement at codons 157 and 174 were the first reported (Fig. 3a). **Reinhardt *et al.* (2002 a; b)** stated that His59→Tyr, Gly81→Ala, and Iso103→ Arg substitutions as favored mutational spots in *Gyr*A subunit**. (Jian *et al.* 2012)** found that a new mutation position at 136 involved in high-level fluoroquinolones resistance MG. **Lysnyansky *et al*. (2008)** observed that isolates with genotype changes at position 59 in *gyr*A remained sensitive to enrofloxacin but in this work fluoroquinolone resistant SAAS.12 isolate with MIC 4-8 ug/ml for fluoroquinolone antibiotics had amino acid substitutions at position 59 (His/Tyr) and this may attribute to the presence of other additional sites of mutation. A large number of mutations in *gyr*A and the presence of alterations in *Par*C play a vital role in developing resistance to ciprofloxacin in MG isolates **(Al-Agamy *et al.* 2012).** Interestingly, four silent mutations were recorded in both fluoroquinolone-resistant *M. gallisepticum* isolates at nucleotides; none of these resulted in amino acid substitutions.

Genetic analysis of *gyr*B

Sequencing of the amplified DNA for *gyr*B of both resistant isolates (SAAS.1 (*MG* EGY2014), SAAS.12) revealed amino acid replacement in the QRDR of *gyr*B at codon 415 (Glycine GGG → Methionine AUG) and codon 416 insertion Glutamic acid (GAA) (*E. coli* numbering). While isolate SAAS.1 (*MG* EGY2014) had another replacement at codon 325 (serine TCA→ leucine CTC) as the first report that may contribute to elevation of MIC profiles. And at 437 (aspartate GAC → asparagine AAT) (*E. coli* numbering) (Fig.4a), a similar result was detected previously by **Lysnyansky *et al*. (2008). Heddle and Maxwell (2002)** found that mutations in *gyr*B have been associated with quinolone resistance. **Yu *et al*. (2004); Hopkins *et al*. (2005); Jacoby (2005)** reported that the mutation frequency in *gyr*B is much lower compared to those for *gyr*A and *Par*C.

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Genetic analysis of *Par*C

A nucleotide sequence of the amplified DNA revealed amino acid replacement in the QRDR of *ParC* of MG SAAS.1 isolate at codon 92 (Serine AGT→ Threonine ACC) and outside QRDR of *Par*C at codon 167 (Glycine GGT→ Valine GTT) (according to *E. coli* numbering) as showed in Figure (5a) and both replacement positions were first reported. These findings were contrary to that reported by **Reinhardt *et al.* (2002 a; b); Lysnyansky *et al*. (2008); Jian *et al*. (2012); Lysnyansky *et al*. (2012)**.

Genetic analysis of *parE*

In our study, sequence analysis of the *par*E gene of both analyzed MG fluoroquinolones resistant isolates revealed no amino acid replacement. Similar results were obtained in other investigations **(Lysnyansky *et al.* 2008; Jian *et al.* 2012; Lysnyansky *et al.* 2012)**.

In this investigation, MG SAAS.12 isolate had no amino acid substitution in *Par*E or *Par*C and recorded a high level of resistance. These results disagreed with other observations **(Fitzgibbon *et al.* 1998)**. **Heisig (1996)** found that mutations in *Par*C or *par*E were observed only in mutants bearing at least one mutation in *gyr*A or *gyr*B and exhibiting an increase in the MIC of enrofloxacin. **(Jian *et al.* 2012)** stated that topoisomerase IV is a primary target of quinolones action in *M. hominis*, but DNA gyrase is a primary target of quinolone action in MG.

The obtained data explained why SAAS.12 had high MIC values of fluoroquinolones tested with only amino acid substitution in *gyr*A and *Par*C. **(Jian *et al.* 2012)** noted that two MG isolates exhibited no mutation in any target genes for fluoroquinolones or just one mutation in *Par*C but had high resistance to fluoroquinolones. **(Reinhardt *et al.* 2002 a; Reinhardt *et al.* 2002 b)** stated that mutations elsewhere in the topoisomerase genes or modifications in drug efflux systems may contribute to the resistant phenotype of these mutants.

(Bébéar *et al.* 1998; Bebear *et al.* 1999; Kenny *et al.* 1999; Bebear *et al.* 2000) mentioned that development of highly quinolones resistant mutant strains of *M. gallisepticum* required mutations in both topoisomerases, like for most bacteria.

Two-dimensional protein analysis of the QRDRs:

The prediction protein of GyrA showed amino acid substitution at positions 59, 73 and 157 (numbering according to *E. coli*) consequence by changes in antigenicity index of GyrA protein structures in SAAS 1 and at position 59 and 157 in SAAS 12 of examined MG isolates (Fig. 3b). In GyrB prediction protein amino acid substitution at position 415 and amino acid insertion at position 416 (numbering according to *E. coli*) lead to changes in antigenicity index of GyrB protein structures in both examined MG isolates (Fig.4b). In the case of Par*C* prediction protein, amino acid substitution at position 92 (numbering according

to *E. coli*) followed by a change in antigenicity index of ParC protein structure in SAAS.1 examined MG isolates (Fig.5b).

The two dimensional protein analysis of both fluoroquinolones drug target enzymes in two MG resistant isolates, illustrated some amino acids substitution in 2D prediction proteins of both GyrA, GyrB, and ParC in addition to insertion of one amino acid at codon 416 in ParC prediction protein. These findings were consistent with previously observed by **Chernova *et al.* (2016)**.

Conclusion

Finally, we can conclude that fluoroquinolones resistant *Mycoplasma gallisepticum* isolates arise mainly from an alteration in genes encoding DNA gyrase enzyme specially *gyr*A gene. The presences of mutation in *Par*C lead to elevation resistance to fluoroquinolones.

Declaration

Authors' contribution. All/both authors contributed equally to this work.

Acknowledgements. Preliminary results were presented as an Abstract at 10th Scientific Conference of the Egyptian Veterinary Poultry Association, Egypt, 4 -7 December 2017."

Competing interests. The authors declare that they have no competing interests.

Consent to publish. Not applicable

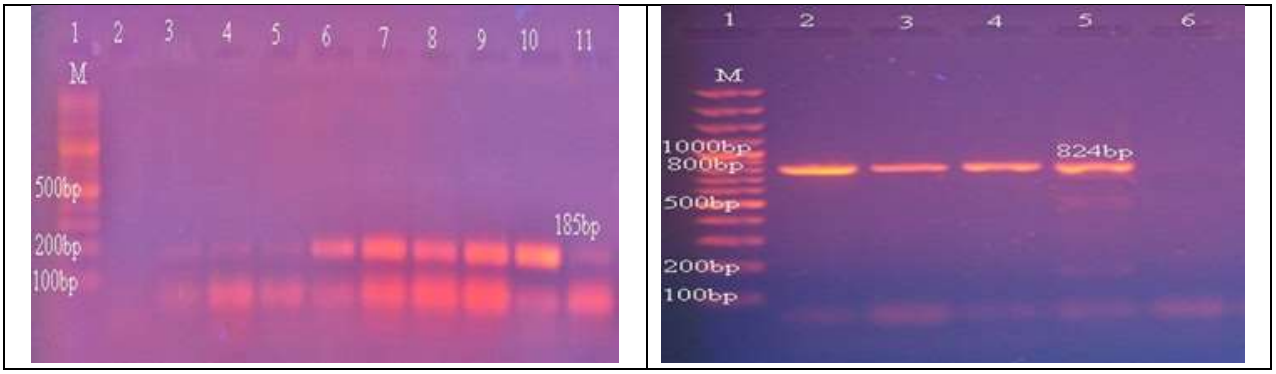

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Fig. 1a. Agarose gel electrophoresis of MG PCR product (16S*rRNA* gene). **Lane (1) M:** 100 bp DNA ladder"Marker", **Lane (2)** control negative, **Lane (11)** control positive MG reference strain (PG31). **Lanes (3:10):** positive MG isolates.	**Fig. 1b.** Agarose gel electrophoresis of MG PCR product (*mgc2* gene). **Lane (1) M:** 100 bp DNA ladder "Marker".**Lane(2):**control positive MG reference strain (PG31).**Lanes (3:5):** positive MG isolates. **Lane (6):** control negative

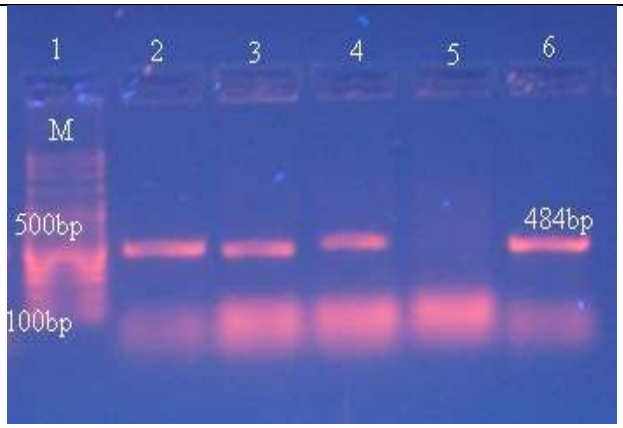
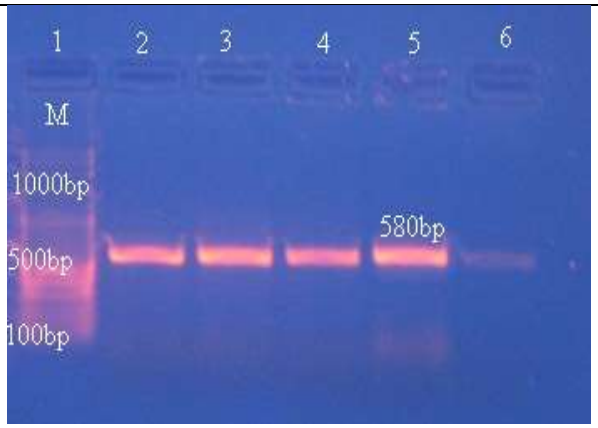

	

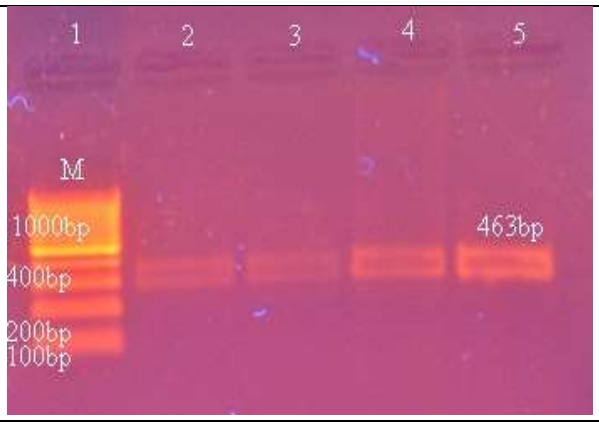
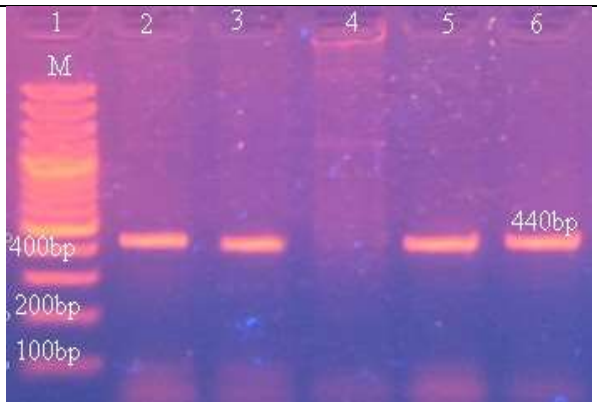
Fig. **2a.** Agarose gel electrophoresis of MG PCR product (*gyr*A gene). **Lane (1) M:** 100 bp DNA ladder "Marker". **Lanes (2,3,4)**: positive MG isolates. **Lane (5)**:control negative. **Lane(6)**: control positive MG reference strain (PG31).	**Fig. 2b.** Agarose gel electrophoresis of MG PCR product (*gyr*B gene).**Lane (1)M:** 100 bp DNA ladder "Marker". **Lanes (2,3,4)**: positive MG isolates. **Lane (5)**: control positive MG reference strain (PG31). **Lane (6)**:control negative
	

Fig. 2c. Agarose gel electrophoresis of MG PCR product (*Par*C gene). **Lane (1) M:** 100 bp DNA ladder "Marker". **Lanes (2,3,4)** positive MG isolates. **Lane (5)** control positive MG reference strain (PG31)**.**	**Fig. 2d.** Agarose gel electrophoresis of MG PCR product (*par*E gene). **Lane (1) M**: 100 bp DNA ladder "Marker". **Lanes (2,3,5)** positive MG isolates. **Lane (4)**:control negative. **Lane (6)** control positive MG reference strain (PG31).

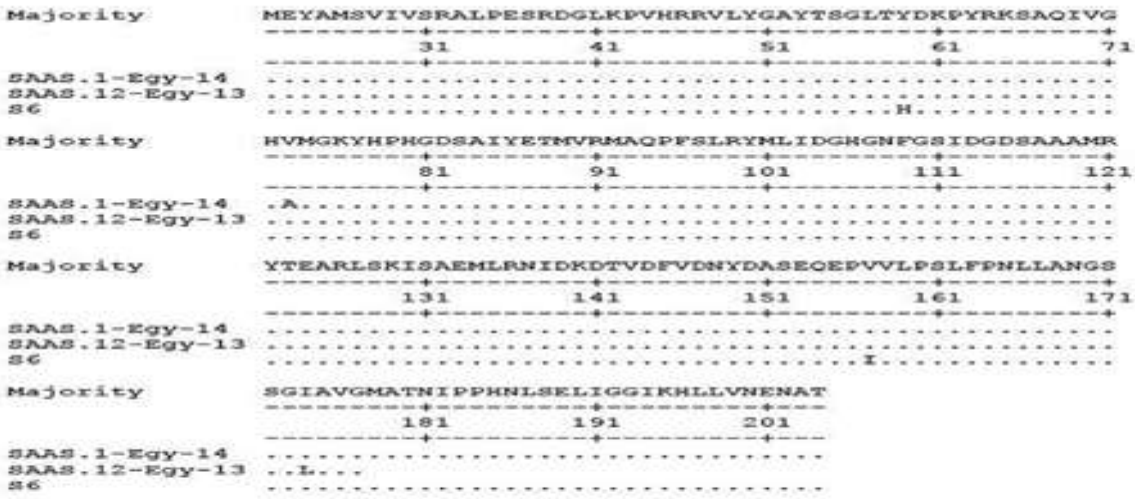


Fig. 3a. Amino acids sequence similarities for *M. gallisepticum gyr*A of the fluoroquinolones resistant MG isolates under study and the reference M. gallisepticum (S6) strains. Numbers refer to the amino acid positions in the *E. coli gyr*A sequence. The Tyr-59, Ala-73, Val-157, and Leu-174 in which mutations associated with fluoroquinolone resistance are founded.

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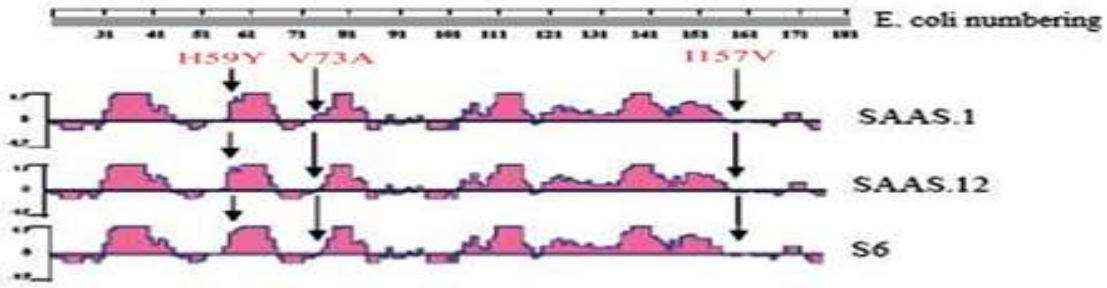

Fig. 3b. The secondary structures protein analysis (antigenicity index) for GyrA(amino acids numbering according to *E. coli* numbering) H: Histidine, Y: Tyrosine, V: Valine, A: Alanine, I: Isoleucine.

```
Majority        EGLTAIISIKMSEPQYQGQTKDRLGNTEVREFTNSVVSELLERFFLENPE
                ---------+---------+---------+---------+---------+
                       327       337       347       357       367
                ---------+---------+---------+---------+---------+
SAAS.1(EGY2014) -------.L.....................................................
SAAS.12-Egy-13  .........................................................
S6              .........................................................

Majority        EAAKITAKAVSAMFSRKRSEAALESARKSPFESASLPGKLADCITKDMEI
                ---------+---------+---------+---------+---------+
                       377       387       397       407       417
                ---------+---------+---------+---------+---------+
SAAS.1(EGY2014) .........................................................
SAAS.12-Egy-13  .........................................................
S6              .....................................................G-.

Majority        SELYIVEGDSAGGSAKSGRDRFYQAILPLRGKVLNVEKANHEKIFKNEEI
                ---------+---------+---------+---------+---------+
                       427       437       447       457       467
                ---------+---------+---------+---------+---------+
SAAS.1(EGY2014) ....................N....................................
SAAS.12-Egy-13  .........................................................
S6              .........................................................

Majority        RILIIAIGAGVNPEEFSLDKIRYNKIIIMTDADV
                ---------+---------+---------+----
                       477       487       497
                ---------+---------+---------+----
SAAS.1(EGY2014) ........................
SAAS.12-Egy-13  ....................................
S6              ....................................
```

Fig. 4a. Amino acids sequence similarities for *M. gallisepticum gyr*B of the fluoroquinolones resistant isolates under study and the reference *M. gallisepticum* (S6) strains. Numbers refer to the amino acid positions in the *E. coli gyr*B sequence. The Leu-325, Met- 415, Glu insertion- 416 and Asn-437 in which mutations associated with fluoroquinolone resistance are founded.

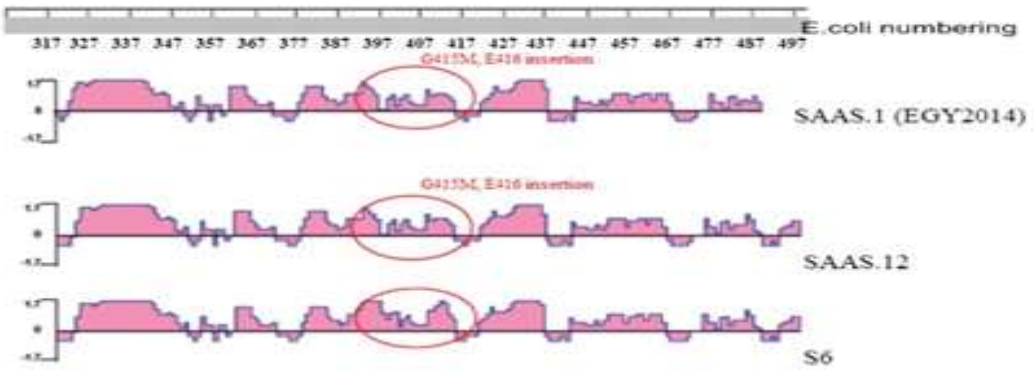

Fig. 4b. The secondary structures protein analysis (antigenicity index) for GyrB (amino acids numbering according to *E.coli* numbering) G: Glycine, M: Methionine, E: Glutamic acid.

```
Majority          ERALPDIRDGLKPVQRRVLYGMYNLGLYYNKSYRKSAATVGEVIGKFHPH
                  ---------+---------+---------+---------+---------+
                           37        47        57        67        77
                  ---------+---------+---------+---------+---------+
SAAS.1-Egy-13     ..................................................
SAAS.12-Egy-13    ..................................................
S6                ..................................................

Majority          GDSSIYEALVRMTQSWKNNIPLIDMQGNNGSIDGDNAAAMRYTEARLSHY
                  ---------+---------+---------+---------+---------+
                           87        97        107       117       127
                  ---------+---------+---------+---------+---------+
SAAS.1-Egy-13     ..............T...................................
SAAS.12-Egy-13    ..................................................
S6                ..................................................

Majority          GNLMLENINKETVNFVNNFDDSEVEPTILPSLLPNLLVNGSTG
                  ---------+---------+---------+---------+---
                           137       147       157       167
                  ---------+---------+---------+---------+---
SAAS.1-Egy-13     ..........................................V...
SAAS.12-Egy-13    ..........................................
S6                ..........................................
```

Fig. 5a. Amino acids sequence similarities for *M. gallisepticum ParC* of the fluoroquinolones resistant MG isolates under study and the reference M. gallisepticum (S6) strains. Numbers refer to the amino acid positions in the *E.coli ParC* sequence. The Thr-92 and val- 167 in MG SAAS.1 which mutations associated with fluoroquinolone resistance are founded and no mutation in MG SAAS.12.

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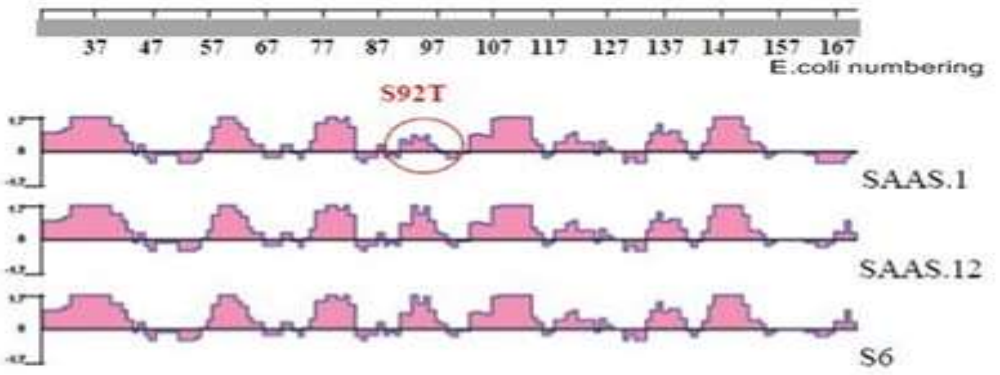

Fig. 5b. The secondary structures protein analysis (antigenicity index) for ParC (amino acids numbering according to *E.coli* numbering) S: Serine, T: Threonine.

Table (1): Oligonucleotide primers used for detection 16S rRNA, *mgc*2, and quinolone-resistance genes.

Primer Designation	Sequence (5'-3')	Amplified Product Size	Reference
16s RNA F	GAGCTAATCTGTAAAGTTGGTC	185 bp	**(Lauerman, 1998)**
16S RNA R	GCTTCCTTGCGGTTAGCAAC		
*mgc*2-F	GCT TTG TGT TCT CGG GTG CTA	824 bp	**(Ferguson *et al.* 2005)**
*mgc*2-R	CGG TGG AAA ACC AGC TCT TG		
*gyr*A-F	GAGCTAGAAACATCATTCATGG	484 bp	**(Lysnyansky *et al.* 2008)**
*gyr*A-R	CCTACAGCAATACCACTTGAA		
gyrB-F	CTGACGGTAAGATTAGCAAAG	580-bp	**(Lysnyansky *et al.* 2008)**
gyrB-R	GACATCAGCATCGGTCATGA		
*par*E-F	CTGAAGGTGGAAGTCATGA	440 bp	**(Lysnyansky *et al.* 2012)**
*par*E-R	GTTGCCAATCCCACAACCT		
ParC-F	GATCTTGATGATATCATGTCAC	463 bp	**(Lysnyansky *et al.* 2008)**
ParC-R	CCAGTTGAACCATTAACGAGT		

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Table (2): MIC range, MIC_{50}, and MIC_{90} of fluoroquinolones against *M. gallisepticum* isolates.

Antimicrobial agent	MIC(µg /ml)		
	Range [a]	MIC_{50} [b]*	MIC_{90} [c]*
Enrofloxacin	0.063- 4	0.25	0.5
Difloxacin	0.125- 8	0. 25	1
Ciprofloxacin	0.063- 4	0.125	0.125
Gatifloxacin	0.063 – 8	0.063	0.25
Ofloxacin	0.063 – 8	0.125	0.25
Levofloxacin	0.063 – 8	0.25	0.25

a Range of the MIC values.

b The MIC at which 50% of the isolates are inhibited

c The MIC at which 90% of the isolates are inhibited.

*Both b and c were calculated by an orderly array method.

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Table 3

Mutations in topoisomerase genes of two analyzed *M. gallisepticum* strains and their antimicrobial susceptibilities to fluoroquinolones

MG isolates	MIC (µg/ml)						Mutations positions in QRDRs*		
	ENFX	DIFX	CPFX	GFLX	OFLX	LVFX	*gyr*A	*gyr*B	*ParC*
SAAS.1	4	8	4	8	8	8	His 59-Tyr Val 73-Ala Ile 157-Val	Ser 325-Leu Asp 437-Asn Gly 415-Met insertion -416 Glu	Ser 92-Thr Gly 167-Val
SAAS.12	4	8	4	8	8	4	His 59-Tyr Ile 157-Val Ile 174-Leu	Gly 415-Met insertion -416 Glu	--

MIC: minimum inhibitory concentration; ENFX: enrofloxacin; DIFX: difloxacin; CPFX: ciprofloxacin; GFLX: gatifloxacin ;OFLX: ofloxacin; LVFX: levofloxacin.

*DNA sequences were analyzed and translated to amino acid sequences and numbering according to *E. coli* amino acid sequences numbering.

His: Histidine, Thr: Threonine, Gly: Glycine, Ala: Alanine, Asn: Asparagine, Tyr: Tyrosine, Val: Valine, Leu: Leucine, Ser: Serine, Ile: Isoleucine, Met: Methionine, Glu: Glutamic acid.

REFERENCES

Al-Agamy, MHM, AM Shibl and HH Radwan (2012). Detection of mutations in quinolone-resistant determining regions in clinical isolates of *Escherichia coli* from Saudi Arabia. African Journal of Biotechnology 11, 1054-1058.

Ambrozic, JA, R Keber, K Zerjavic, T Orazemand and M Grabnar (2007). Emergence of the quinolone resistance-mediating gene aac (6')-Ib-cr in extended-spectrum-beta-lactamase-producing *Klebsiella* isolates collected in Slovenia between 2000 and 2005. Antimicrobial agents and chemotherapy 51, 4171-4173.

Andriole, VT (2005). The quinolones: past, present, and future. Clinical Infectious Diseases 41, S113-S119.

Bebear, C, H Renaudin, A Charron, D Gruson, M Lefrancois and C Bebear (2000). In Vitro Activity of Trovafloxacin Compared to Those of Five Antimicrobials against *Mycoplasmas* Including *Mycoplasma hominis* and *Ureaplasma urealyticum* Fluoroquinolone-Resistant Isolates That Have Been Genetically Characterized. Antimicrobial agents and chemotherapy 44, 2557-2560.

Bébéar, CM, H Renaudin, A Charron, JM Bové, C Bébéar and J Renaudin (1998). Alterations in topoisomerase IV and DNA gyrase in quinolone-resistant mutants of *Mycoplasma hominis* obtained in vitro. Antimicrobial agents and chemotherapy 42, 2304-2311.

Bebear, CM, J Renaudin, A Charron, H Renaudin, B De Barbeyrac, T Schaeverbeke and C Bebear (1999). Mutations in the g*yrA, ParC,* and *parE* genes associated with fluoroquinolone resistance in clinical isolates of *Mycoplasma hominis.* Antimicrobial agents and chemotherapy 43, 954-956.

Butenko, I, A Vanyushkina, O Pobeguts, D Matyushkina, S Kovalchuk, A Gorbachev, N Anikanov, G Fisunov and V Govorun (2017). Response induced in *Mycoplasma gallisepticum* under heat shock might be relevant to infection process. Scientific reports 7, 11330.

Catania, S, M Bottin, A Fincato, M Gastaldelli, A Barberio, F Gobbo and e Al. (2019). Evaluation of minimum inhibitory concentrations for 154 *Mycoplasma synoviae* isolates from Italy collected during 2012-2017. PLoS ONE 14, e0224903.

Chernova, O, E Medvedeva, A Mouzykantov, N Baranova and V Chernov (2016). Mycoplasmas and their antibiotic resistance: The problems and prospects in controlling infections. Acta Naturae (англоязычная версия) 8.

Clinical, And, Laboratory, Standards, Instituteand (ClSI)(2011). Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement, in: M100-S21, C.d. (Ed.), Wayne, Pennsylvania, USA, pp. 42-46.

Dardeer, M (1997). Efficacy of different serological techniques in serodiagnosis of avian Mycoplasmosis., PHD. Poultry Dept.,Faculty of Vet.Med.,Cairo University,

Erno, H and L Stipkovits (1973). Bovine mycoplasmas: cultural and biochemical studies. I. Acta Veterinaria Scandinavica 14, 436.

Ewers, C, T Janßen, S Kießling, H-C Philipp and LH Wieler (2005). Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian diseases 49, 269-273.

Ferguson-Noel, N (2013). Mycoplasmosis. Diseases of poultry, 13th ed., DE Swayne, JR Glisson, LR McDougald, LK Nolan, DL Suarez, and VL Nair, eds. John Wiley & Sons, Ames, Iowa.

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

Fitzgibbon, JE, JF John, JL Deluciaand DT Dubin (1998). Topoisomerase Mutations in Trovafloxacin-Resistant *Staphylococcus aureus*. Antimicrobial agents and chemotherapy 42, 2122-2124.

Freundt, E, B Andrews, H Ernø, M Kunze and F Black (1973). The sensitivity of Mycoplas-matales to sodium-polyanethol-sulfonate and digitonin. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene 225, 104-112.

Gerchman, I, I Lysnyansky, S Perk and S Levisohn (2008). In vitro susceptibilities to fluoroquinolones in current and archived *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates from meat-type turkeys. Veterinary microbiology 131, 266-276.

Hall, TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucleic acids symposium series. [London]: Information Retrieval Ltd., c1979-c2000., pp. 95-98.

Hamilton-Miller, J (1991). Calculating MIC50. Journal of Antimicrobial Chemotherapy 27, 863-864.

Hannan, P, G Windsor, A De Jong, N Schmeer and M Stegemann (1997). Comparative susceptibilities of various animal-pathogenic mycoplasmas to fluoroquinolones. Antimicrobial agents and chemotherapy 41, 2037-2040.

Hannan, PC (2000). Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary *Mycoplasma* species. Veterinary research 31, 373-395.

Heddle, J and A Maxwell (2002). Quinolone-binding pocket of DNA gyrase: role of GyrB. Antimicrobial agents and chemotherapy 46, 1805-1815.

Heisig, P (1996). Genetic evidence for a role of *ParC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. Antimicrobial agents and chemotherapy 40, 879-885.

Hopkins, KL, RH Davies and EJ Threlfall (2005). Mechanisms of quinolone resistance in *Escherichia coli* and Salmonella: recent developments. International journal of antimicrobial agents 25, 358-373.

Jacoby, GA (2005). Mechanisms of resistance to quinolones. Clinical Infectious Diseases 41, S120-S126.

Jameson, B and H Wolf (1988). The antigenic index: a novel algorithm for predicting antigenic determinants. Bioinformatics 4, 181-186.

Jian, L, L Dian-Hong, L Zhi-Jie, Z Xiao-Hua, W Fei-Long, L Ya-Hong and J Hong-Xia (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.

Kelven, K (2003). Avoiding spread of airborn infections. Health Estate. 57, 37-38.

Kenny, GE, PA Young, FD Cartwright, KE Sjöström and WM Huang (1999). Sparfloxacin selects gyrase mutations in first-step *Mycoplasma hominis* mutants, whereas ofloxacin selects topoisonmerase IV mutations. Antimicrobial agents and chemotherapy 43, 2493-2496.

Kleven, S (1998). Mycoplasmas in the etiology of multifactorial respiratory disease. Poultry Science 77, 1146-1149.

Lauerman, L (1998). Mycoplasma PCR assays. Nucleic acid amplification assays for diagnosis of animal diseases, 41-42.

Ley, D and H Yoder (1997). *Mycoplasma gallisepticum* infection, In B. W. Calnek (ed.), Diseases of poultry, 10th ed ed. Press, Ames, Iowa, Iowa State University . , pp. 194–195.

Lysnyansky, I, I Gerchman, S Levisohn, I Mikula, A Feberwee, N Ferguson, A Noormohammadi, J Spergser and H Windsor (2012). Discrepancy between minimal inhibitory concentration to enrofloxacin and mutations present in the quinolone-resistance determining regions of *Mycoplasma gallisepticum* field strains. Veterinary microbiology 160, 222-226.

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, 685-689.

Mohamed, R(1997). Some studies on *Mycoplasma gallisepticum* in broiler chicken in Egypt, Poultry and fish Dept., Faculty of Vet. Med. Zagazig University. , Faculty of Vet. Med, Zagazig University.

Mohammed, E(2001). Diagnostic studies on avian mycoplasmosis with special reference to *Mycoplasma synoviae*. , Poultry Dept., Fac. Of Vet. Med.,. Cairo University, Vet. Med., Cairo University.

National, Commitee, For, Clinical, Laboratory, Standardsand (Nccls) (2002)). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, in: NCCLS document M31-A2. National Committee for Clinical Laboratory Standards (Ed.), approved standard-second edition ed, Wayne, PA.

Nicholas, R and R Ayling (2003). *Mycoplasma bovis*: disease, diagnosis, and control. Research in veterinary science 74, 105-112.

Papazisi, L, TS Gorton, G Kutish, PF Markham, GF Browning, S Swartzell, A Madan, G Mahairas and SJ Geary (2003). The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain Rlow. Microbiology 149, 2307-2316.

Pietal, MJ, I Tuszynska and JM Bujnicki (2007). PROTMAP2D: visualization, comparison and analysis of 2D maps of protein structure. Bioinformatics 23, 1429-1430.

Raviv, Z and D Ley (2013). *Mycoplasma gallisepticum* infection, p 877–893. Diseases of poultry. Wiley-Blackwell, Ames, IA.

Reinhardt, A, C Bébéar, M Kobisch, I Kempf and A Gautier-Bouchardon (2002 b). Characterization of mutations in DNA gyrase and topoisomerase IV involved in quinolone resistance of *Mycoplasma gallisepticum* mutants obtained in vitro. Antimicrobial agents and chemotherapy 46, 590-593.

Reinhardt, A, I Kempf, M Kobisch and A Gautier-Bouchardon (2002 a). Fluoroquinolone resistance in *Mycoplasma gallisepticum*: DNA gyrase as primary target of enrofloxacin and impact of mutations in topoisomerases on resistance level. Journal of antimicrobial chemotherapy 50, 589-592.

Ruiz, J (2003). Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. Journal of Antimicrobial Chemotherapy 51, 1109-1117.

Sabry, M and A Ahmed (1975). Evaluation of media and cultural procedure for the primary isolation of *Mycoplasma* from female genitalia of farm animals. J. Egypt. Vet. Med. Assoc 35, 18-36.

Saif-Edin, M (1997). Situation of mycoplasma infections among chickens in Upper Egypt with evaluation of different diagnostic techniques. ASSIUT Veterinary Medical Journal 37, 54-67.

Sharaf, MAA(2000). Studies on some *Mycoplasma* species in poultry at Menoufiea Governorate, Microbiology Dept., Fac. of Vet. Med.,. Zagazig University., Fac. of Vet. Med., Zagazig University.

Stipkovits, L, J Biro, N Erdei and S Szathmáry(2011). Past, present and future of *M. gallisepticum* infection, in: Veterinary Medical Research Institute of Hungarian Academy of Sciences , B., Hungary.www.wpas-aeca es.D 1-10. (Ed.).

Thompson, JD, DG Higgins and TJ Gibson (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research 22, 4673-4680.

Ulgen, M and M Kahraman (1993). Comparative bacteriological and serological studies on avian Chronic Respiratory Disease (CRD). . Veterinarium 4, 33-35.

Umar, S, M Munir, Z Ur-Rehman, S Subhan, T Azam and M Shah (2017). Mycoplasmosis in poultry: update on diagnosis and preventive measures. World's Poultry Science Journal 73, 17-28.

Yu, X, M Susa, C Knabbe, RD Schmid and TT Bachmann (2004). Development and validation of a diagnostic DNA microarray to detect quinolone-resistant *Escherichia coli* among clinical isolates. Journal of clinical microbiology 42, 4083-4091.