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Original Article

Mutations of domain V in 23S ribosomal RNA of macrolide-resistant *Mycoplasma gallisepticum* isolates in Egypt

Ahmed M Ammar[1], Norhan K Abd El-Aziz[1], Ahlam A Gharib[1], Hanaa K Ahmed[2], Amira E Lameay[3]

[1] *Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia, Egypt*
[2] *Genome Unit, Animal Health Research Institute, Dokki, Giza, Egypt*
[3] *Bacteriology Department, Animal Health Research Institute, Zagazig, Sharkia, Egypt*

Abstract

Introduction: Avian mycoplasmas impose a significant economic burden to the poultry industry. In recent years, macrolide-resistant *Mycoplasma gallisepticum* have occasionally been encountered in Egypt.

Methodology: This study was designed to document the involvement of macrolide-resistant *M. gallisepticum* in respiratory organs of chickens suffering respiratory problems. Concurrently, an exhaustive molecular characterization of the intrinsic resistance of recovered isolates to macrolides was done.

Results: Of 120 chickens showing respiratory problems, 14 (11.67%) *M. gallisepticum* were isolated and genetically identified; 8 of them were recovered from air sacs, 4 from lungs, and 2 from tracheas. Broth microdilution of all *M. gallisepticum* isolates showed various degrees of minimum inhibitory concentrations (MICs) against macrolides: erythromycin (0.25–32 µg/mL), tylosin (0.0625–4 µg/mL), and tiamulin (0.031–2 µg/mL). Nucleotide sequencing of domain V (peptidyl transferase region) of the 23S rRNA gene of macrolide-resistant *M. gallisepticum* isolates revealed transition mutations at positions 2068 and 2069 (corresponding to 2058 and 2059 in *Escherichia coli* numbering) in an isolate and at position 2067 (corresponding to 2057 in *E. coli* numbering) in three isolates as hot spots for macrolide resistance. Surprisingly, a transversion mutation at position 2621 (corresponding to 2611 in *E. coli* numbering) was reported in one of the recovered isolates as a first report.

Conclusion: Generation of new mutations is evidence for persistence of *M. gallisepticum* despite macrolide treatment. Periodic surveys to monitor for the possible appearance of resistant strains are recommended.

Key words: *M. gallisepticum*; broth microdilution; domain V mutations; sequence analysis

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Introduction

Mycoplasma gallisepticum, a major poultry pathogen, causes chronic respiratory disease in chickens and infectious sinusitis in turkeys, resulting in substantial economic losses in poultry farms throughout the world [1]. Moreover, *M. gallisepticum* infections are notifiable to the World Organization for Animal Health (OIE) [2]. Availability of other pathogens (such as *Escherichia coli*), increased environmental ammonia, high levels of dust, poor nutrition, immunosuppressive agents, and social stresses associated with intensive management play an important role in *M. gallisepticum* infections [3]. *M. gallisepticum* cytadhesin membrane surface protein (*mgc2*) is an important virulence factor, as it provides the pathogen for resisting host defenses, selective antibiotic therapy, and establishing chronic infection [4].

Currently, a variety of strategies are practiced to control *M. gallisepticum* infections either by chemotherapy, which is sometimes necessary in complement of strict biosecurity to preserve mycoplasma-free breeding flocks, or vaccination programs [5].

Notably, the macrolide class of antibiotics, including erythromycin, tylosin, and tilmicosin, is widely used in the veterinary field for prophylaxis and treatment of mycoplasmosis [6], but unfortunately, resistance has been readily developing in recent years [7]. The antibacterial activity of macrolides is due to inhibition of bacterial protein synthesis by binding to the 23S rRNA component of the bacterial 50S ribosomal subunit. Usually in bacteria with a small number of rRNA operons, such as mycoplasmas, acquired resistance to macrolides has been associated with mutations within domain II or V of the 23S rRNA

genes or in *rpl*D and *rpl*V, genes encoding ribosomal proteins L4 and L22 [8].

In Egypt, *M. gallisepticum* infection represents a serious problem in chickens, especially in the absence of hygiene conditions and vaccination programs [9]. In light of the above and as a consequence of scarce data on macrolide resistance of *M. gallisepticum* in Egypt, the current study was conducted to assess the prevalence of *M. gallisepticum* in chickens in Sharkia province, Egypt, and to further characterize the respective mutations of domain V in the 23S rRNA gene conferring macrolide resistance in recovered isolates.

Methodology

Clinical specimens

One hundred and twenty chickens showing respiratory manifestations were collected randomly from different areas in Sharkia province, Egypt, over a two-year period, from March 2012 to May 2014, to be examined. Lung, trachea, and air sac tissue specimens of each diseased chicken were placed into separate sterile containers and then transported to the laboratory in an icebox within 24 hours for mycoplasma isolation.

Isolation and identification of M. gallisepticum

Half a gram of each tissue specimen was cut into small pieces and ground with sterile sand to be cultivated on pleuropneumonia-like organism (PPLO) broth and agar base media (CM0403/CM0401, Oxoid, Hampshire, England, UK) with mycoplasma-selective supplement G (SR0059, Oxoid, Hampshire, England, UK), as described previously [10]. Conventional identification of mycoplasma by digitonin test [11], glucose fermentation, and arginine deamination [12] was then performed. Furthermore, genomic DNA used for polymerase chain reaction (PCR) was purified using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions. All mycoplasma isolates were confirmed as *M. gallisepticum* by PCR using species-specific primers for 16S rRNA and *mgc*2 genes, following previously published methods [13,14].

Determination of minimum inhibitory concentrations (MICs)

In vitro susceptibilities for erythromycin, tylosin, and tiamulin (Sigma-Aldrich, St. Louis, USA) against *M. gallisepticum* isolates were determined by a broth microdilution method as previously described [15], following the guidelines recommended by Hannan [16]. Serial twofold dilutions from 0.0625–32 μg/mL for erythromycin and 0.031–16 μg/mL for tylosin and tiamulin, prepared in PPLO broth containing 10^4 to 10^5 CFU/mL of *M. gallisepticum*, were placed in custom-designed 96-well microtiter plates (Corning, New York, USA). The positive and negative controls comprising only *M. gallisepticum* organisms and PPLO broth, respectively, were also included in each plate; the microplates were then sealed with adhesive sheets, incubated aerobically at 37°C and examined daily for five to seven days. MIC is defined as the lowest concentration of antibiotic that prevents a color change in the medium at the time when the antibiotic-free growth control showed a color change. Interpretation of MIC values for the concerned antimicrobials in this study was performed using previously published criteria [16].

Touch-up gradient PCR amplification and sequence analysis of domain V in the 23S rRNA gene

The gene encoding domain V of the 23S rRNA gene of macrolide-resistant *M. gallisepticum* isolates was amplified using two internal primers, MG-23S-1F (CACAGCTCTATGCTAAATCGC) and MG-23S-1R (GGTCCTCTCGTACTAAG) in the touch-up gradient PCR technique based on a previously published protocol with some modifications [17]. PCR was carried out in a total reaction volume of 25 μL. The reaction mixtures consisted of 12.5 μL of DreamTaq Green Master Mix (2X) (Fermentas, Waltham, USA), 0.1 μL of 100 pmol of each primer (Sigma-Aldrich, St. Louis, USA), 7 μL of template DNA, and nuclease-free water up to 25 μL. The touch-up gradient PCR amplification program was applied in a PTC-100 programmable thermal cycler (Peltier-Effect Cycling, MJ Research Inc., UK) as follows: 10 cycles of 95°C for 30 seconds, 45°C for 30 seconds, and 72°C for 30 seconds, followed by 30 cycles of 95°C for 1 minute, 46°C for 1 minute, and 72°C for 1 minute.

PCR products (879 bp) were visualized on ethidium bromide-stained agarose gels, and then purified from the gel using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The sequencing reaction was conducted with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, PerkinElmer, Foster City, USA) in an ABI 3130 automated DNA Sequencer (Applied BioSystems, Carlsbad, USA). Sequence editing, consensus, and alignment construction were performed using BioEdit software package version 7.0.4.1 [18]. Numbering of the nucleotide substitutions in domain V of the 23S rRNA sequenced amplicon is based on the sequence of the

respective gene in *E. coli* (Figure 1). Consistent use of the *E. coli* system facilitates comparison between the different organisms and avoids the discrepancies in some of the other notation systems [19].

Nucleotide sequence accession numbers

The nucleotide sequences of domain V in the 23S rRNA gene of macrolide-resistant *M. gallisepticum* isolates under study were assigned GenBank accession numbers KT020843, KT020844, KT020845, KT020846 and KT153253.

Results

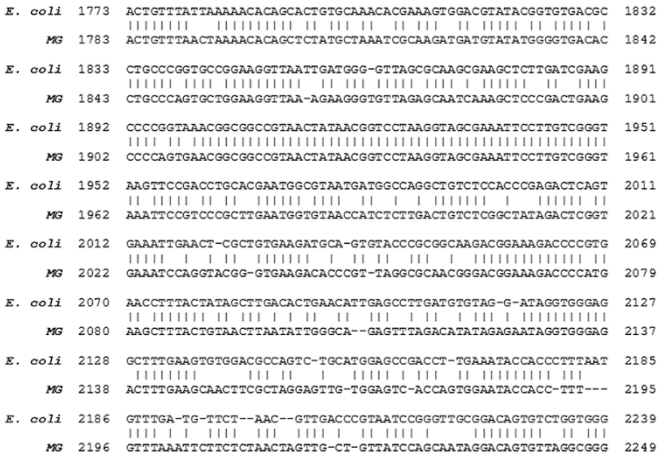
Prevalence of M. gallisepticum infection

On the basis of cultural and biochemical properties, 14 out of 120 (11.67%) examined chickens with various respiratory problems in Sharkia province, Egypt, had *M. gallisepticum* infection. All isolates were genetically confirmed by PCR to have the 16S rRNA gene of *M. gallisepticum*; six of them only possessed the *mgc*2 gene. *M. gallisepticum* isolation from respiratory organs of diseased chickens showed that air sacs were the main site of multiplication of the microorganism (57.1%); lungs were the second site of isolation (28.6%), followed by tracheas (14.3%).

In vitro activities of macrolides against M. galliseptium isolates

The broth microdilution method on 14 *M. gallisepticum* isolates recovered from chickens experiencing respiratory disorders showed various degrees of MICs against macrolides: erythromycin (0.25–32 µg/mL), tylosin (0.0625–4 µg/mL), and tiamulin (0.031–2 µg/mL) (Table 1). By comparing these results with MIC breakpoints, all isolates were found to be sensitive to tylosin and tiamulin, which are still drugs of choice for *M. gallisepticum* infections, while five isolates (35.7%) were resistant to erythromycin, indicating a frequent use of such antibiotic in chicken farms in order to prevent, control, or treat respiratory diseases.

Figure 1. Partial sequences alignment of domain V of 23S rRNA gene of *E. coli,* accession no. V00331.1 and *M. gallisepticum* (*MG*) strain R (low), accession no. NR_076192.1 from GenBank database showing numbering divergence in 10 nucleotide bases.



Sequence analysis of the domain V in the 23S rRNA gene of macrolide-resistant M. gallisepticum

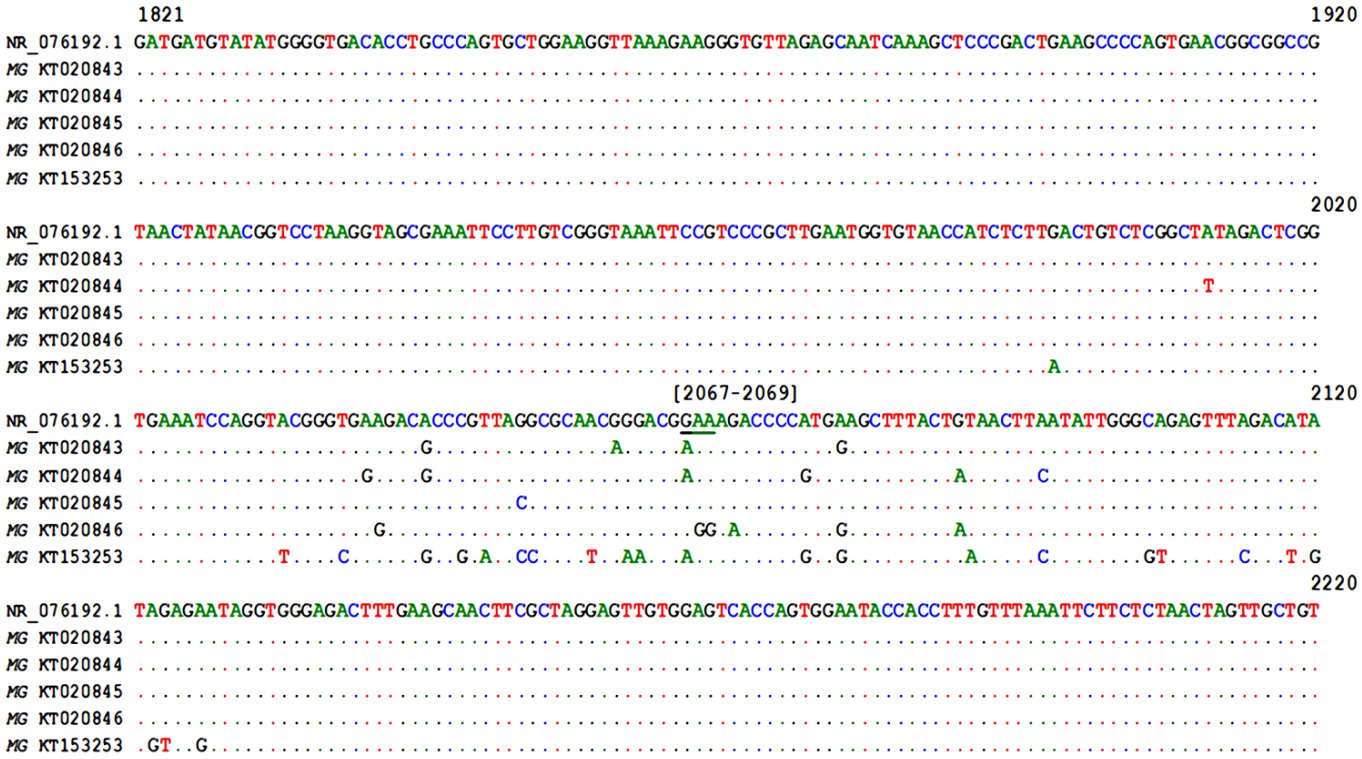
To investigate the possibility that point mutations in the central loop of domain V in the 23S rRNA gene are responsible for intrinsic resistance to macrolides, the regions from nucleotide 1758 to 2684 of five erythromycin-resistant *M. gallisepticum* isolates were amplified, sequenced, and characterized. Sequence

Table 1. MICs of macrolide antibiotics for *M. gallisepticum* isolates from chickens.

***M. gallisepticum* isolates**	**Source**	**MIC value (µg/mL)**		
		Erythromycin	**Tylosin**	**Tiamulin**
*MG*1	Lung	2	1	0.125
*MG*2	Air sac	4	0.25	0.25
*MG*3	Air sac	1	0.125	0.062
*MG*4	Lung	32 R	4	0.5
*MG*5	Trachea	4	0.125	0.125
*MG*6	Air sac	32 R	2	0.5
*MG*7	Air sac	4	0.125	0.062
*MG*8	Lung	0.25	0.125	0.125
*MG*9	Air sac	0.5	0.0625	0.031
*MG*10	Lung	16 R	0.5	2
*MG*11	Trachea	16 R	1	0.25
*MG*12	Air sac	2	0.125	0.125
*MG*13	Air sac	2	0.25	0.5
*MG*14	Air sac	32 R	4	0.5

MG: Mycoplasma gallisepticum; MIC: minimum inhibitory concentration; R: resistant

Figure 2. Alignment of 23S rRNA gene of *M. gallisepticum* strain R (low) from GenBank database, accession no. NR_076192.1 and erythromycin-resistant *M. gallisepticum* isolates under study. Partial sequence of domain V from position 1821–2220 are presented. The nucleotides are numbered on the basis of *M. gallisepticum* and identical nucleotides are indicated by dots. Multiple nucleotide changes were recorded in all isolates and hot spot mutations were underlined.



analysis of domain V of the 23S rRNA gene revealed several mutations in all examined isolates. The results are summarized in Table 2, along with MICs, and in Figure 2. Interestingly, many transition mutations were recorded within a highly conserved region of the central loop of domain V that is believed to be part of the binding site for macrolide antibiotics. According to *E. coli* numbering, the G2057A detected in two mutants (numbers 1 and 2) was expected to cause a drastic fall in drug binding, as this mutation lead to a disruption of the rRNA structure with an opening of the stem preceding the single-stranded portion of the peptidyl transferase loop. An additional A2058G mutation (mutant number 4), the most widespread mutation

Table 2. Characteristics of erythromycin-resistant *M. gallisepticum* mutants from respiratory organs of chickens.

MG mutants	Source	MIC value (µg/mL)			Nucleotide change in 23S rRNA[a]	Accession number
		ERY	TYL	TIA		
*MG*4	Lung	32	4	0.5	G2051A[b], G2057A[b] A2035G[c], A2070G[c], A2509G[c], T2605C[c]	KT020843
*MG*6	Air sac	32	2	0.5	G2057A[b] A2001T[c], A2030G[c], A2035G[c], G2067A[c], G2080A[c], A2087C[c], G2464T[c], C2610T[c]	KT020844
*MG*10	Lung	16	0.5	2	T2606C[b] G2043C[c], A2292C[c]	KT020845
*MG*11	Trachea	16	1	0.25	A2031G[c], A2058G[c], A2059G[c], G2061A[c], A2070G[c], G2080A[c], C2621G[c]	KT020846
*MG*14	Air sac	32	4	0.5	G1988A[b], G2057A[b], C2096G[b], A2108T [b] T1867G[c], G1933T[c], C1963A[c], G1975A[c], A1978T[c], A2023T[c], T2028C[c], A2035G[c], C2038G[c], T2040A[c], G2043C[c], G2044C[c], A2049T[c], G2052A[c], G2053A[c], A2067G[c], A2070G[c], T2081A[c], A2087C[c], A2097T[c], A2104C[c], A2110G[c], A2112G[c], G2113T[c], A2116G[c], T2181C[c], T2201C[c]	KT153253

MG: *M. gallisepticum*; MIC: minimum inhibitory concentration; ERY: erythromycin; TYL: tylosin; TIA: tiamulin; [a] *E. coli* numbering; [b] Silent mutation; [c] Sense mutation.

occurring under clinical therapy in resistant pathogens, is highly correlated with decreased susceptibility of *M. gallisepticum* to macrolides. Adenosine 2058 is the key nucleotide involved in macrolide interaction on the ribosome, and any alteration in this site or corresponding site would prevent the attachment of the macrolide to its binding site. Moreover, A2059G mutation on the rrnB operon of mutant number 4 could lead to a macrolide resistance phenotype with a return to high-level resistance to erythromycin. The C2611G mutation site in mutant number 1 was expected to produce a moderate effect.

Discussion

Mycoplasma gallisepticum has long been recognized as a common respiratory pathogen, especially in chickens, causing considerable economic hardship on the poultry industry [20]. It possesses a specialized tip structure by which it attaches to the respiratory epithelium (cytadhesion), which is considered necessary for successful colonization and, consequently, the possibility to invade cells. The chronic nature of mycoplasma infections demonstrates a failure of the host immune system to deal effectively with these organisms [21]. The macrolide class of antibiotics is widely used in the veterinary field for prophylaxis and treatment of mycoplasmosis; however, different phenotypes of intrinsic resistance of mycoplasmas have readily developed [17].

In the current study, 14 (11.67%). *M. gallisepticum* isolates were isolated and identified from respiratory organs of 120 chickens showing various respiratory problems using standard bacterial culture and PCR methods, which are known as the gold standard for *M. gallisepticum* diagnosis [20]. The recovery rates of *M. gallisepticum* vary greatly among countries. Lower results were recorded in a previous study in Egypt (10%) [22]; however, higher isolation rates were reported in other studies in Pakistan (27.6%) [23] and Algeria (21.67%) [24]. The difference in isolation rates may be attributed to the fact that *M. gallisepticum* may be endemic in certain areas due to defect in control programs and biosecurity measures [25]. Interestingly, our results revealed higher occurrence of *M. gallisepticum* infection in air sacs of diseased chickens (57.1%) as the main multiplication site for the microorganism, which is consistent with a previous study [26].

The liquid method for MICs against *M. gallisepticum* was used in this study because of its simplicity and convenience compared to the agar or solid method [16]. Furthermore, the inhibitory zone of the agar method against *M. gallisepticum* cannot be determined for some antibiotics [27]. The microbiological criterion was used for interpretation of MIC results, since no Clinical and Laboratory Standards Institute breakpoints for erythromycin, tylosin, and tiamulin are available for the avian pathogen *M. gallisepticum* [16]. Herein, all *M. gallisepticum* isolates showed excellent susceptibilities to tylosin (0.0625–4 μg/mL) and tiamulin (0.031–2 μg/mL); meanwhile, 35.7% of recovered isolates exhibited erythromycin resistance. Generally, a previous study reported that tilmicosin and tylosin are effective against *M. gallisepticum* infection, and resistance of the microorganism to tylosin has been reported to develop slower than resistance to erythromycin [28].

The interactions of macrolides have mainly been mapped by chemical footprinting experiments [29] and X-ray crystallography [30] to the peptidyl transferase region within domain V of the 23S rRNA gene. In the current study, sequence analysis of domain V of the 23S rRNA gene revealed several mutations in all examined isolates. For instance, an erythromycin-resistant *M. gallisepticum* isolate (*MG*11) harbored an adenine to guanine substitution at position 2059 and 2058; those mutations appeared to be essential for macrolide binding. In nucleotide position 2057, a guanine to adenine transition was reported in three erythromycin-resistant *M. gallisepticum* isolates. Actually, those mutations were the most frequently reported in the erythromycin-resistant isolates of *M. gallisepticum* genetically characterized [17,31] and have been previously reported as hot spots for macrolide resistance in other mycoplasmas [32,33]. Based on our results, transition can be considered the predominant type of mutation in *M. gallisepticum.* This may be due to the structural difference between purine and pyrimidine. Additionally, a transversion substitution of cytosine by guanine at position 2611 was found in an isolate (*MG*11), which was expected to have a more moderated effect, as reported previously in other mycoplasmas [34].

Conclusions

This is the first description of macrolide-resistant isolates of *M. gallisepticum* in diseased chickens in Egypt. The intrinsic erythromycin resistance was certainly linked to the G2057A, A2058G, and A2059G transitions. Moreover, this is the first characterization of *M. gallisepticum* that harbors a transversion mutation at position 2611 in the 23S rRNA gene.

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Corresponding author

Norhan K Abd El-Aziz
Department of Microbiology, Faculty of Veterinary Medicine
Zagazig University
Elemam Elshafey street 77, Mubarak district
44519, Zagazig
Sharkia, Egypt
Phone: +20 055 2262477; +20 122 6369943
Fax: +20 055 2283683
Email: nourhan_vet@yahoo.com; nourhan_vet@zu.edu.eg

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