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Isolation and Molecular Characterization of *Mycoplasma gallisepticum* from Domestic Poultry in Sanliurfa, Turkey

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Abstract

THE aim of this study was to investigate the isolation of *Mycoplasma gallisepticum* from domestic poultry and the molecular characterization of the isolated strains. Tracheal swabs and tissue samples from 120 domestic poultry were used to isolate the pathogen. Seven *M. gallisepticum* were isolated from 120 samples (5.8%). Thirty of 62 (48.3%) chickens and 35 of 58 (60.3%) turkeys tested positive by PCR. PCR amplicons detected between 750-800 bp from the target gene regions of *mgc2*F and *mgc2*R and digested with the restriction enzymes *AluI*, *HaeII* and *HinfI* yielded two different PCR-RFLP types. Phylogenetic analysis of the *mgc2* gene region formed two groups. In addition, a deletion of 63 nucleotides in the base sequences of the *mgc2* gene was only detected in *M. gallisepticum* 6/85 and the isolates of group 1. Protein analysis of the isolates by SDS-PAGE clearly revealed bands with molecular weights of 120,100,70, 64, 67,56, 53,45,43 and 26 kDa in all isolates. In the WB analysis, antibodies against *M. gallisepticum*-specific proteins with molecular weights of 200, 120,100, 98,67, 64,40,35,26 kDa were detected. We concluded that further work on different genes of *M. gallisepticum* to distinguish more specifically between field isolates is needed based on the available data. In addition, naturally infected flocks can be distinguished from vaccinated flocks, which is a very effective method to control and monitor infection.

Keywords: CRD, *M. gallisepticum*, poultry mycoplasmosis, *mgc2* gene, western blot.

Introduction

The production of chicken meat and eggs is one of the most important ways in which livestock farming can meet the growing demand for animal-based food products caused by the growing world population. As a result, poultry farming has spread throughout the world. However, this expansion of the poultry industry has led to the emergence of several major diseases that cause billions of dollars in economic losses worldwide. Among these major bacterial diseases, mycoplasmosis stands out. *Mycoplasma gallisepticum* (*M. gallisepticum*), a virulent *Mycoplasma* species listed by the World Organisation for Animal Health (WOAH), is usually the causative agent of chronic respiratory disease (CRD), which causes severe economic losses in the poultry industry, especially in chickens, broilers and turkeys. The disease leads to reduced meat and egg production as well as chronic respiratory disease, including frothy eye discharge, nasal discharge, swelling of the infraorbital sinuses, swelling of the trachea and bronchi, thickening of the air sacs and accumulation of cheesy exudate [1-6]

Mycoplasmas spread very rapidly within a flock, and the entire population may become positive within one to two weeks after the appearance of clinical signs [7]. Adhesion of *M. gallisepticum* to host cells is a prerequisite for successful colonisation and pathogenesis. Typical genes unique to M. gallisepticum are genomic regions used for pathogen identification or strain differentiation, including genes such as *GapA*, *mgc2*, *CrmA* and *PvpA*, which are responsible for adhesion to the respiratory tract and host colonisation [3, 8, 9]. Many researchers have highlighted the usefulness of the *mgc2* gene in strain differentiation and emphasised its critical role during infection as an adhesion protein that can bind to host extracellular matrix (ECM) molecules [10-14]. Various methods are used to detect *M. gallisepticum*, and early diagnosis plays a crucial role in reducing and eliminating clinical symptoms. Although culture methods are recognised as the gold standard for the diagnosis of mycoplasma, serological methods and molecular techniques based on polymerase chain reaction (PCR) are often preferred for diagnosis [5, 15, 16].

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The aim of this study was to isolate local strains of *M. gallisepticum* from domestic poultry in the Şanlıurfa region of Turkey and to perform a molecular characterization of these isolates by comparison with the standard strain *M. gallisepticum* S6 and *M. gallisepticum* 6/85 vaccine strain using PCR, restriction fragment length polymorphism (RFLP-PCR), sequencing, phylogenetic analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB) methods.

Material and Methods

Animal

This study was performed on samples from 120 domestic poultry of different age groups and sexes suspected of having mycoplasmosis based on clinical signs and necropsy findings. The samples were taken to the microbiology laboratory of the Faculty of Veterinary Medicine, Harran University, Şanlıurfa Region, Turkey. A total of 84 tracheal swabs were collected from chickens and turkeys, while 36 samples from different lesioned organs and tissues (lung, liver, spleen, heart, trachea, air sacs) and exudates from dissected animals were used for pathogen isolation.

Positive control strains

The *M. gallisepticum* S6 strain from the mycoplasma reference laboratory of the Pendik Veterinary Research Institute was used as a positive control. The vaccine strain *M. gallisepticum* 6/85 was purchased commercially (MSD Animal Health, USA).

Isolation of M. gallisepticum

M. gallisepticum was isolated according to the WOAH isolation method [5]. Dilutions prepared from swab samples and tissues were inoculated in tubes with 5 ml of Frey's broth and incubated under microaerobic conditions with 5-10% CO_2 at 37°C for 72 hours. After initial inoculation, samples showing colour changes and growth with significant contamination were filtered through 0.45 µm filters and then cultured on both solid and liquid media. After 3 days of incubation, the samples were examined under a stereomicroscope for colony formation. Suspicious colonies were confirmed by PCR.

DNA extraction

For DNA extraction, samples grown in liquid media were transferred to sterile Eppendorf tubes and centrifuged for 30 minutes at 14,000 g and 4°C. The supernatant was carefully discarded. The supernatant was carefully discarded and the pellet was resuspended in 25 µl ultrapure water. The tubes were boiled for 10 minutes and then cooled on ice. The samples were centrifuged again at 14,000 g for 5 minutes. The supernatant containing the DNA was transferred to separate tubes. The isolated DNA was stored at -20°C until PCR analysis [17]. The quality and quantity of the DNA was measured using a spectrophotometer (NanoDrop ND-1000).

PCR

The primers for *M. gallisepticum* used in this study are listed in Table 1. The PCR reaction mixture was prepared according to the method described by WOAH [5] and the samples were amplified using a thermocycler. The PCR products were electrophoresed on 1.5% agarose gels and the results were analysed under a UV transilluminator (UVCI-1100, MAJOR SCIENCE).

Statistical analysis

The chi-square test was used to determine statistical differences in the diagnosis of *M. gallisepticum*. The analyses were conducted with SPSS Statistics for Windows, version 20.0 (NY, USA).

Amplification and sequencing of the mgc2 gene region

The *mgc2* gene region (750-800 bp) was amplified for PCR-RFLP and sequencing according to the method described by Rajkumar et al. [18]. The PCR products were sequenced unidirectionally using the Big Dye Cycle Sequencing Kit v3.1 and an ABI PRISM 3130XL automated sequencer (Applied Biosystems) [19]. The sequences obtained were compared with similar sequences from GenBank using the BLAST programme (http://blast.ncbi.nlm.nih.gov) [20]. The sequences were analysed with ClustalX 2.1 for multiple alignment and MEGA-X for phylogenetic tree construction.

PCR-RFLP

The amplified PCR products were digested with Fast Digest *HaeII* (BfoI), *HinfI* and *AluI* restriction enzymes (Thermo Scientific, USA). The reaction mixtures were prepared according to the manufacturer's protocol. The PCR products were electrophoresed on a 2% agarose gel and analysed under a UV transilluminator (UVCI-1100, Major Science).

SDS-PAGE

SDS-PAGE analysis was performed according to the method described by Laemmli [21]. The protein concentration of the mycoplasma suspensions was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Protein samples were mixed with Laemmli 2X buffer (Sigma Lot #SLBT2607) and boiled for 5 minutes. A stacking gel (4%) and a resolving gel (12%) were prepared and the samples were subjected to gel electrophoresis. After electrophoresis, the gel was removed from the electrophoresis tank and stained with Coomassie

Blue staining solution (Thermo) on a horizontal shaker (MR-12, BIOSAN) for 3-4 hours.

Western blotting (wet electrophoretic transfer)

The proteins separated by SDS-PAGE were transferred to a 0.2 µm nitrocellulose membrane (NM) using the blotting method (Sigma). The transfer was performed in a Trans-Blot Vertical Electrophoresis System (Hoefer PS300-B, USA) at constant 40 V for 1–16 hours [22]. After transfer, the NM was incubated overnight at +4ºC in blocking solution (Tris-buffered saline + 3% bovine serum albumin). Twenty millilitres of TBST (Tris-Base Immunoblot Wash Buffer-TBST) was prepared and 1/100 diluted CRD-positive primary antibody solution was added. The mixture was incubated for at least 2 hours at room temperature on a horizontal shaker. Then 20 ml of TBST containing 1/5000 diluted goat anti-chicken IgY (AP) (Thermo, PA1-30369) labelled with alkaline phosphatase secondary antibody was added and incubated for 1 hour at room temperature. BCIP (Sigma), NBT (Roche) and alkaline phosphatase buffer (0.1 M Tris-HCl; 0.1 M NaCl; 5 mM $MgCl_2$, pH 9.5) were used for substrate preparation. NBT solution (66 µl) was mixed with alkaline phosphatase solution (10 ml) and 33 µl BCIP solution was added. The mixture was applied to the NM. After the formation of antibody bands against the proteins, the reaction was stopped by washing with distilled water.

Results

Culture and PCR

As a result of the culture method, *M. gallisepticum* was isolated from 7 out of 120 samples of domestic poultry in Şanlıurfa region, with 3 samples from chickens and 4 from turkeys (5.8%) (Figs. 1). By PCR, 65 out of 120 samples (54.2%) were found to be positive for *M. gallisepticum*. Specific DNA was detected in 30 of 62 chicken samples (48.3%) and 35 of 58 turkey samples (60.3%). The M. *gallisepticum* suspect samples (4, 9, 66, 68, 79, 95 and 120) isolated by culture were also confirmed positive for *M. gallisepticum* by PCR at 185 bp (Table 2, Figs. 2). Statistically, there was a significant difference in detection rates between PCR and culture methods for *M. gallisepticum* in tracheal and post-necropsy samples ($p<0.001$).

Accession numbers of the strains isolated in this study and registered in Genbank (NCBI): OR392423.1, OR392425.1, OR392424.1, OR392422.1, OR392421.1, OR392420.1, OR392419.1.

PCR-RFLP, Sequencing, and Phylogenetic Analysis

The amplified *mgc2* gene region from 7 *M. gallisepticum* field isolates, *M. gallisepticum* 6/85 vaccine strain, and *M. gallisepticum* S6 strain was digested with *AluI, HaeII,* and *HinfI* restriction enzymes. The resulting fragment sizes are shown in Table 3. The images of the electrophoresis gels are shown in Figs. 3, 4. and 5.

The sequencing results showed the following base lengths for the samples: MG4; 714 bp, MG79; 782 bp, MG9; 721 bp, MG68; 716 bp, MG95; 716 bp, MG120; 712 bp, MG66; 782 bp, *M. gallisepticum* 6/85; 718 bp, and M. gallisepticum S6; 787 bp. These were analysed for the digestion regions and base lengths of the sequence. Two different PCR-RFLP profiles were observed due to differences in the number and size of fragments obtained. As with the sequencing results, most isolates (MG4, 9, 68, 95, 120) showed the same PCR-RFLP profile, while isolates MG66 and MG79 showed different PCR-RFLP profiles. The vaccine strain *M. gallisepticum* 6/85 showed a very similar RFLP profile to isolates MG9, MG68, MG95, MG4 and MG120.

A phylogenetic tree was constructed using the sequences of the *mgc2* gene region of the 7 *M. gallisepticum* field isolates and the 15 *M. gallisepticum* strains/isolates from GenBank (Figs. 6). Based on the phylogenetic analysis of the *mgc2* gene region, five highly similar field isolates were grouped as group 1 (MG4, 9, 68, 95, 120) and two field isolates formed another group as group 2 (MG66, 79). Each group showed high similarity with some *M. gallisepticum* strains and isolates from different countries (Table 4). The isolates in groups 1 and 2 showed 96-99% similarity with isolates from South Africa and Brazil and 96-100% similarity with isolates from India, the USA and Australia. The isolates in both groups showed high similarity with the *M. gallisepticum* S6, 6/85 and TS-11 vaccine strains (Table 5). The percentage similarity of isolates within group 1, group 2 and between groups and their similarity to vaccine and reference strains are shown in Table 6.

It was also noted that the field isolates in Group 1, such as the *M. gallisepticum* 6/85 vaccine strain, had a 63-nucleotide deletion in the *mgc2* gene, which was not observed in the isolates in Group 2 or the *M. gallisepticum* S6 strain (Figs. 7).

SDS-PAGE and Western blot

The protein profiles of the 7 *M. gallisepticum* field isolates, the vaccine strain *M. gallisepticum* 6/85 and the standard strain *M. gallisepticum* S6 obtained after electrophoretic separation are shown in Figs. 8. Protein bands at 120, 100, 70, 64, 67, 56, 53, 45, 43 and 26 kDa were clearly visible in all isolates from group 1 and group 2. The isolates MG68 and MG95 from group 1 and MG79 and MG66 from group 2 formed an additional band at 28 kDa. The isolates from Group 2 also formed a band at 15 kDa. All strains, including the vaccine strain *M. gallisepticum* 6/85 and the standard strain *M.*

gallisepticum S6, showed the described protein bands.

The antibody profiles against *M. gallisepticum* specific proteins in the WB analysis are shown in Figs. 9. As in the other analyses, the field isolates from Group 1 and Group 2 showed a high degree of similarity and produced similar antibody profiles against most of the proteins. Prominent antibody bands against p200, p120, p100, p98, p67, p64, p40, p35 and p26 were observed in all isolates from group 1 and group 2. In addition, group 1 isolates also produced antibodies against p56. No banding was observed in the WB analysis performed with CRD negative control chicken serum.

Discussion

The pathogenic mycoplasma species responsible for CRD, *M. gallisepticum*, causes infections mainly in chickens and turkeys, but also in other avian species such as partridges, peacocks, quails and pheasants [23]. Various methods have been used for the detection of mycoplasma infections. The culture method is considered by the WOAH to be the 'gold standard' for the isolation and identification of mycoplasmas [5, 23]. Despite the disadvantages of the culture method, such as time consuming and loss of viability of the pathogen in the sample, it is considered necessary for diagnosis [5]. Muhammad et al [24] found that the successful isolation of pathogens using the culture technique is highly dependent on proper sample collection and storage. Since *Mycoplasma* organisms are very sensitive, they are quickly inactivated if they remain in the environment for a long time. Therefore, careful sampling and immediate processing in culture media are essential to maintain the viability of the microorganism. In addition, Kleven [25] emphasised that *Mycoplasma* pathogens are very sensitive during incubation in culture media and that changes in pH can lead to pathogen inactivation. In this study, contamination-induced colour changes were observed in liquid cultures, and cultures with contamination were filtered through 0.45 µm filters and recultured. It was observed that samples that could not be isolated despite second or third passages, but were positive by PCR, could not be isolated due to pH changes. It was also found that the risk of contamination of the medium is minimised when samples are collected under sterile conditions and handled carefully, increasing the isolation rate of the pathogen.

In addition to the culture method, serological methods also have disadvantages, such as cross-reactions that lead to false positive or false negative results. Due to these limitations, molecular methods, especially PCR diagnostic techniques, are more practical and preferable [5, 15]. Several researchers have reported that PCR is a reliable, accurate and successful method for the detection of mycoplasma pathogens [26-28]. In a study by Bağcıgil [26], *M. gallisepticum* was detected in 3 of 96 tracheal samples by culture and in 47 samples by PCR. Giram et al. [29] reported that between 2017 and 2021, 233 (6.44%) of 3620 suspected mycoplasma samples from tracheal and cloacal swabs in India were positive for *M. gallisepticum* by PCR. In another study by Mahmmoud et al. [30], 79 (85.9%) of 92 lung tissue samples from chicken farms were found to be positive for *M. gallisepticum* by PCR. Marouf et al. [31] detected *M. gallisepticum* in 206 (62%) of 332 samples from suspect poultry on farms, of which 175 (85%) were confirmed by PCR. Branton et al. [32] found that contamination of tracheal swabs collected from poultry fed or in contact with feed affected isolation rates. In this study, the lower isolation rates compared to the PCR results for tracheal swab samples were likely due to contamination from the feed material.

In this study, 120 tracheal swabs and necropsy tissue homogenates were collected from domestic poultry of different ages and sexes. *M. gallisepticum* was isolated by culture from 7 (5.8%) of the samples and specific *M. gallisepticum* DNA was detected by PCR in 65 (54.2%) of these samples. The results indicate that the lower isolation rate compared to PCR results may be related to factors such as previous antibiotic treatment in most of the domestic poultry species submitted for diagnosis, contamination of materials collected from feed, sampling during the chronic phase of infection, or insufficient pathogen detectable in tissues. Statistically, there was a significant difference between the positivity rates of PCR and culture for *M. gallisepticum.* PCR had a higher positivity rate than culture for the same samples, which makes PCR a reliable test for the diagnosis of *M. gallisepticum* due to its practicality, speed, low cost and high specificity and sensitivity.

One of the aims of molecular typing is to distinguish pathogenic strains from live vaccines. Due to the widespread use of *M. gallisepticum* vaccines, it has been emphasised that some diagnostic methods should be used to distinguish live vaccine strains from *M. gallisepticum* field strains, and these methods are necessary to advance epidemiological studies [33]. To differentiate *M. gallisepticum* strains and isolates antigenically and epidemiologically, many researchers have favoured the PCR-RFLP method using different restriction endonuclease enzymes [18, 34, 35]. In this study, a PCR-RFLP method using the restriction enzymes *HinfI*, *HaeII* and *AluI* was used to differentiate *M. gallisepticum* vaccine and field isolates.

In PCR-RFLP, the amplified *mgc2* gene is one of the genes encoding major surface proteins related to cytoadhesin used for genotyping. Rajkumar et al. [18] stated that *mgc2* PCR-RFLP can be a useful method to obtain important information for

epidemiological studies on the diversity and distribution of *M. gallisepticum* in India and for the prevention and control of CRD. Lysnyansky et al. [36] developed a rapid test in Israel to distinguish *M. gallisepticum* field isolates from TS-11 and 6/85 vaccine strains using *mgc2*F and *mgc2*R primers directly from tracheal swab samples without *M. gallisepticum* isolation and using the restriction enzymes *HaeII* and *SfaN1* for PCR-RFLP. Mathengtheng [34] analysed 10 reference strains of *M. gallisepticum* using the enzymes *PstI, BsmI, CfrI, ClaI,* and *HinfI* with the *mgc2* PCR-RFLP method and reported that differences between isolates were detected based on the RFLP profiles.

In this study, PCR products of the *mgc2* target gene region of 7 isolated *M. gallisepticum* field strains, the *M. gallisepticum* 6/85 vaccine strain and the *M. gallisepticum* S6 strain were amplified using *mgc2*F and *mgc2*R primers and then digested with *AluI, HaeII* and *HinfI* restriction enzymes. Fragment sizes generated by the *mgc2* PCR-RFLP method were analysed and the differences and similarities between the isolates and the vaccine strain were evaluated. Different fragment sizes were obtained after digestion with *AluI, HaeII* and *HinfI*. Based on the differences in the number and size of these fragments, two different PCR-RFLP types were identified. Most of the isolated strains MG9, MG4, MG68, MG95 and MG120 showed the same PCR-RFLP profile, while isolates MG66 and MG79 showed a different PCR-RFLP profile. The *M. gallisepticum* 6/85 vaccine strain showed very similar RFLP profiles to the field isolates MG4, MG9, MG68, MG95 and MG120.

In the study by Bíró et al. [37], the *AluI* enzyme was applied to the *amplified M. gallisepticum crmC* gene and the fragments (350 and 297 bp) formed between the field and reference strains were analysed. In contrast to this study, the *AluI* enzyme was applied to the *mgc2* gene of *M. gallisepticum* in the present study. While a similar profile was observed in most field isolates, only field isolate MG120 formed a different fragment compared to the other field isolates and the reference strains used. Zakeri et al. [35] reported that in the diagnostic *M. gallisepticum* assay using *mgc2* PCR-RFLP *HaeII* cuts at 270 bp for TS-11 but not for field isolates. Rajkumar et al. [18] applied PCR-RFLP using the enzyme *HaeII* to the *mgc2* target gene of 9 *M. gallisepticum* field isolates and *M. gallisepticum* 6/85 and TS-11 vaccine strains and found that this enzyme cut the 6/85 vaccine strain and 3 *M. gallisepticum* field isolates at 270 bp. As in the mentioned studies, the *mgc2* gene region was amplified and PCR-RFLP with restriction enzymes was performed. The same gene regions and *HaeII* enzyme were used, but in this study, after PCR amplification of the *mgc2* gene region between 700-800 bp with the *HaeII* restriction enzyme, the RFLP results showed that field isolates MG4 at 637 and 77 bp, MG9 at 644 and 77 bp, MG68 at 639 and 77 bp, MG95 at 640 and 76 bp, MG120 at 638 and 74 bp, MG79 at 777 and 7 bp, and the *M. gallisepticum* 6/85 vaccine strain at 638 and 80 bp. However, no restriction was observed with the MG66 isolate and the standard *M. gallisepticum* S6 strain. The *mgc2* PCR-RFLP results show differences between the isolates. This proves the diversity of *M. gallisepticum* in the region. It is also known that there is no licenced avian mycoplasmosis vaccine used in breeding flocks in the country. It was concluded that PCR-RFLP can be used as a reliable, useful and rapid method to differentiate between vaccine strains and field isolates in epidemiological studies and for diagnostic and economic purposes.

Isolates and strains differ in their pathogenicity due to phenotypic and genotypic variations [11]. Sequence analysis of specific genes allows for more precise differentiation and better resolution [38]. In this study, the *mgc2* base sequences of *M. gallisepticum* field isolates and reference strains were determined for a more accurate epidemiologic analysis. The obtained sequence data were analysed with ClustalX Multiple Sequen and MEGA-X, and a phylogenetic tree was constructed.

In sequence analysis studies, Rajkumar et al. [18] reported that four distinct groups were formed phylogenetically as a result of *mgc2* gene sequence analysis of 13 *M. gallisepticum* field isolates. They reported that the isolates of group 4 showed 100% similarity to the vaccine strain MG 6/85. In this study, based on the phylogenetic analysis of the *mgc2* gene region, five highly similar field isolates were grouped into group 1 (MG4, 9, 68, 95, 120) and two field isolates were grouped into group 2 (MG66, MG79). The isolates in group 1 and group 2 showed high similarity to the *M. gallisepticum* 6/85 vaccine strain, with similarity percentages of 99.5% and 97.3%, respectively.

Abdelwhab [39] reported that the *M. gallisepticum* isolate showed 97%, 97% and 87% similarity to the vaccine strains (6/85, TS-11, F strain) based on sequence analysis and concluded that this strain was a field isolate and not a vaccine strain. Loolmani et al. [40] reported that after sequence and phylogenetic analysis of 15 *M. gallisepticum* isolates, two distinct groups were formed, with each group showing high similarity (up to 99.3%) to certain *M. gallisepticum* strains and isolates from other countries, including some isolates from South Africa and America. They also reported 99.6% similarity to the *M. gallisepticum* S6 reference strain, 97.3%-99.3% similarity to the TS-11 vaccine strain, and 97.3%-98.3% similarity to an *M. gallisepticum* isolate from Iran. Al-Mahmoudi et al. [41] performed sequence analysis and phylogenetic reconstruction on four samples that tested positive for *M. gallisepticum* by PCR and found that these

samples were 99% similar to strains from South Africa. Giram et al. [29] sequenced the *mgc2* gene regions of two *M. gallisepticum* samples (MGH01, MGM01) and found that both isolates were similar to previously documented Indian strains, but showed no similarity to vaccine strains (TS-11, 6/85 and S6).

In this study, the field isolates in groups 1 and 2 showed 96-99% similarity to isolates from South Africa and Brazil and 96-100% similarity to isolates from India, USA and Australia. In addition, the field isolates showed high similarity to *M. gallisepticum* vaccine strains (6/85, TS-11). Isolates in group 1 and group 2 showed 99.5% and 97.3% similarity to the *M. gallisepticum* 6/85 vaccine strain and 89%-90% and 100% similarity to the TS-11 vaccine strain, respectively. Isolates from group 1 showed high similarity to the *M. gallisepticum* 6/85 vaccine strain, whereas isolates from group 2 showed complete (100%) similarity to the TS-11 vaccine strain. The percentages of similarity to the *M. gallisepticum* S6 strain were 96%-96.5% for group 1 and 97.2% for group 2. As seen in this study and in previous research, the majority of the isolated *M. gallisepticum* field strains showed high similarity to strains from South Africa and the USA. The phylogenetic analysis suggests that one of the reasons for the similarity between the isolated strains and those from different countries around the world is the significant role of migratory wild birds, which are key agents in spreading infectious pathogens from one region to another. The location of the Şanlıurfa region along migratory bird routes further emphasises its importance in the spread of infection [42].

Compared to other bacteria, the ribosomal RNAs of *Mycoplasma* species possess several conserved oligonucleotide sequences, indicating their evolutionary origin [43]. Therefore, nucleotide polymorphisms and deletions between field isolates are not unexpected. Rajkumar et al. [18] reported that 63 nucleotides were deleted in Group II isolates following sequencing and sequence analysis. Similar to this study, field isolates from group 1 also showed the deletion of 63 nucleotides of unknown origin. Similar polymorphisms were also observed in the *M. gallisepticum* 6/85 vaccine strain. Gene size polymorphisms due to insertions and/or deletions are common in *M. gallisepticum* strains [10]. Vaccine strains from India, Israel and South Africa (6/85 and F strains) and isolates have been reported to have deletions at the carboxy (C) terminal of the *mgc2* gene [10, 44]. In the current study, the majority of *M. gallisepticum* field isolates with deleted *mgc2* gene regions showed high similarity to strains from India, South Africa and the *M. gallisepticum* 6/85 vaccine strain.

SDS-PAGE, a molecular technique, is used to identify proteins associated with virulence and phenotypic diversity of different *M. gallisepticum* strains [45]. Khan et al. [46] stated that high sensitivity SDS-PAGE and/or RFLP methods can distinguish *M. gallisepticum* strains by direct comparison of DNA from samples with protein band profiles. The major polypeptide bands resulting from SDS-PAGE treatment of *M. gallisepticum* proteins range from 30 kDa to 140 kDa [47]. In the present study, significant polypeptide bands were observed on SDS-PAGE analysis, with common protein bands at 120, 100, 70, 64, 67, 56, 53, 45, 43 and 26 kDa formed in all *M. gallisepticum* field isolates from group 1 and group 2. However, isolates MG68 and MG95 from group 1 and MG79 and MG66 from group 2 formed an additional band at 28 kDa. Group 2 isolates also produced a band at 15 kDa. Emmam et al. [48] analysed the protein profiles of *M. gallisepticum* field isolates using SDS-PAGE and reported that 60% of isolates (5/9 isolates) formed bands at 73, 72, 56, 53, 47, 46 and 24 kDa, 70% (6/9 isolates) at 54 and 20 kDa, 80% (7/9 isolates) at 70 kDa, 90% (8/9 isolates) at 19 kDa and all isolates shared a common protein band at 55 kDa. In this study, all MG field isolates showed protein bands at 70 kDa. In the study, the WB technique was used to evaluate the different bands formed by antibodies reacting against the proteins analysed from the field isolates, *M. gallisepticum* 6/85 vaccine strain and *M. gallisepticum* S6 strain on the NM. Ellakany et al. [49] experimentally exposed chickens of different age groups to *M. gallisepticum* infection and analysed the antibodies produced at different stages of the humoral immune response against the pathogen using the WB technique. They reported the detection of antibodies against proteins at p200, p120, p98, p80, p75, p72, p60, p50, p45, p40, p35, p33, p31, p28, p26, p24 and p22 kDa. In this study, as in other analyses, the field isolates in Group 1, and Group 2 showed high similarity in the WB analysis and most of them produced similar antibody profiles against the species-specific proteins. Antibody bands against proteins at p200, p120, p100, p98, p67, p64, p40, p35 and p26 kDa were observed in all field isolates from group 1, and group 2. In addition, group 1 isolates produced antibodies against p56. Similar to the findings of Ellakany et al. [49], antibodies against the *M. gallisepticum* species-specific p67 and p64 kDa proteins were produced by all field isolates, and antibodies against p200, p120, p98, p56, p40, p35 and p26 kDa proteins were also observed. Avakian et al. [50] stated in their study that the most immunodominant species-specific proteins with the greatest potential for use as antigens in serological tests were those at p64 and p56 kDa, based on WB analysis of the humoral immune response against *M. gallisepticum* S6 and variant strains.

After SDS-PAGE, the *M. gallisepticum* proteins transferred to NM reacted with serum from infected poultry, producing reactions with most species-specific immunogenic *M. gallisepticum* polypeptides,

such as p39, p20, p76, and p69 or p85, p64, p56, and p26. The species-specific p64 protein was detectable in most *M. gallisepticum* strains when hyperimmune serum was used, but its detection rate decreased by half when serum from the post-disease recovery period was used. The p56 protein is consistently present in most *M. gallisepticum* strains and can induce a strong immune response in chickens and turkeys even during variant *M. gallisepticum* infections [47]. In this study, species-specific immunogenic proteins such as p120, p64, p26 and p56 were observed in the antibody bands of both groups and the control strains. However, in group 2 there was no reaction against the p56 protein observed by SDS-PAGE. This suggests that the *M. gallisepticum* isolates in group 2 are phylogenetically different from those in group 1. In this study, by using polyclonal serum for WB analysis of the common and distinct bands observed on SDS-PAGE (p120, 100, p67, p64, p56, p26 and p56), antigenic differentiation between some *M. gallisepticum* isolates was observed. However, it is speculated that the use of monoclonal antibodies in WB analysis may provide more specific differentiation in terms of antigenicity and immunogenicity.

Conclusion

At the end of the study, we obtained valuable preliminary epidemiologic data on the presence of *M. gallisepticum* infections in domestic poultry with respiratory problems in region and its surroundings. We concluded that molecular tools can be used to discriminate between field and vaccine strains and that these techniques can also be used to reveal some distinguishing features in field isolates. Further work on different genes of *M. gallisepticum* for more specific discrimination between field isolates is needed based on the available data. Our results will help to improve the diagnosis and surveillance of CRD in the region. In addition, naturally infected flocks can be distinguished from vaccinated flocks, which is a very effective method to control and monitor the infection.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Author contributions

AGY, OK were responsible for conception and design of the study. AGY, OK participated in data collection. Laboratory work was conducted by AGY and the data were analysed by AGY, OK. AGY, OK coordinated the study. AGY, OK were responsible for drafting the manuscript and preparation of the figures. All authors participated in revising the draft manuscript. All authors read and approved the final version of the manuscript.

Ethical of approval

Ethical permission of the study was obtained from "Harran University Animal Experiments Local Ethics Committee (No: 2021/001).

TABLE 1. Primer sequences used for amplification of the *mgc2* and 16S rRNA gene regions in the PCR analysis

Method	Positive	Negative
	Tracheal swab	
PCR	42 (50%)	42 (50%)
Culture	3 (3.6%)	81 (96.4%)
	Tissue homogenate after necropsy	
PCR	23 (63.9%)	13 (36.1%)
Culture	4 (11.1%)	32 (88.9%)
Total		
PCR	65 (54.2%)	55 (45.8%)
Culture	7 (5.8%)	113 (94.2%)

TABLE 2. Culture and PCR results of the samples used in the study

Target Microorganism	Primers	Gene region	Amplicon size	Reference
M. gallisepticum	14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3' 13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'	16S rRNA	185 bp	[5]
M. gallisepticum	F: 5'-GGAGTTAGCTTTGTGTTCTCGG-3' R: 5'-AAGTTCATGCGGTTTGGACC-3'	*mgc2*	750-800 bp	[18]

TABLE 3. Fragment sizes of the field isolates and reference strains of *M. gallisepticum* after digestion with *AluI, HaeII*, and *HinfI* restriction enzymes

RE	*Alu*I	*Hae*II	*Hinf*I
	Base intervals cut by REs (bp		
MG_4	352,294,62,6	637,77	413,189,67,45
MG_9	365,294,62	644,77	413,156,67,45,40
MG_68	360,294,62	639,77	413,191,67,45
MG_95	360,294,62	640,76	412,192,67,45
MG_120	311,294,59,31,17	638,74	410,190,67,45
MG_66	368,357,57	-	516,185,67,14
MG_79	360,357,65	777,7	524,191,67
MG_6/85	360,359,68,	638,80	416,190,67,45
MG_S6	360,359,68	-	356,170,129,67,45,20

TABLE 4. Percentage similarity of isolated *M. gallisepticum* field isolates with *M. gallisepticum* 6/85 and TS-11 vaccine strains and M. gallisepticum S6 standard strain

	Field isolates	MG 6/85	MG TS11	MG S6
Group 1	**MG_120**	99.0%	89.6%	96.0%
	MG_4	99.5%	90.2%	96.5%
	MG_9	99.5%	90.2%	96.5%
	MG68	99.5%	90.2%	96.5%
	MG_95	99.5%	90.2%	96.5%
Group 2	**MG_66**	97.3%	100.0%	97.2%
	MG_79	97.3%	100.0%	97.2%

TABLE 5. Table showing the percentage similarity of *M. gallisepticum* field isolates to each other and to *M. gallisepticum* 6/85 and TS-11 vaccine strains, the standard *M. gallisepticum* S6 strain and isolates/strains from GenBank

1	KY362287.1	MG_6/85	MG_120	362210.1_137-606	MG_4	KP318741.1	MG_9	MG_68	CP044224.1	MG_95	MG_66	MG_79	MG_s6	5577607.1_89-714	NY01_2001.047-2	362217.1_137-762	KP300754.1	MG_ts11	JQ770175.1	KJ019174.1	KY362288.1	KY362210.1
2	KY362287.1																					
3	MG_6/85	97,1%																				
4	MG_120	96,4%	99,0%																			
5	KY362210.1_137-606	97,3%	99,4%	99,4%																		
6	MG_4	97,1%	99,5%	99,5%	99,8%																	
7	KP318741.1	97,1%	99,5%	99,5%	99,8%	100,0%																
8	MG_9	97,1%	99,5%	99,5%	99,8%	100,0%	100,0%															
9	MG_68	97,1%	99,5%	99,5%	99,8%	100,0%	100,0%	100,0%														
10	CP044224.1	97,1%	99,5%	99,5%	99,8%	100,0%	100,0%	100,0%	100,0%													
11	MG_95	97,1%	99,5%	99,5%	99,8%	100,0%	100,0%	100,0%	100,0%	100,0%												
12	MG_66	98,2%	97,3%	98,7%	97,1%	97,3%	97,3%	97,3%	97,3%	97,3%	97,3%											
13	MG_79	98,2%	97,3%	98,7%	97,1%	97,3%	97,3%	97,3%	97,3%	97,3%	97,3%	100,0%										
14	MG_s6	98,7%	94,7%	98,0%	98,4%	98,5%	98,5%	98,5%	98,5%	98,5%	98,5%	97,2%	97,2%									
15	KU577607.1_89-714	98,2%	97,3%	98,7%	97,1%	97,3%	97,3%	97,3%	97,3%	97,3%	97,3%	100,0%	100,0%	97,2%								
16	MG_NY01_2001.047-5-1	97,3%	95,2%	98,7%	97,0%	97,2%	97,2%	97,2%	97,2%	97,2%	97,2%	97,6%	97,6%	98,8%	97,6%							
17	KY362217.1_137-762	98,4%	97,1%	98,5%	97,3%	97,1%	97,1%	97,1%	97,1%	97,1%	97,1%	99,8%	99,8%	97,1%	99,8%	97,4%						
18	KP300754.1	97,8%	96,7%	98,7%	97,1%	97,2%	97,2%	97,2%	97,2%	97,2%	97,2%	97,6%	97,6%	98,0%	97,6%	98,7%	97,5%					
19	MG_ts11	96,2%	89,1%	89,6%	90,0%	90,2%	90,2%	90,2%	90,2%	90,2%	90,2%	100,0%	100,0%	91,4%	100,0%	91,3%	99,8%	90,2%				
20	JQ770175.1	96,2%	96,9%	96,2%	96,6%	96,9%	96,9%	96,9%	96,9%	96,9%	96,9%	100,0%	100,0%	96,9%	100,0%	97,3%	99,8%	97,5%	100,0%			
21	KJ019174.1	96,4%	96,9%	96,2%	96,6%	96,9%	96,9%	96,9%	96,9%	96,9%	96,9%	99,6%	99,6%	96,9%	99,6%	97,3%	99,4%	97,5%	99,6%	99,6%		
22	KY362288.1	97,1%	99,6%	98,9%	99,8%	99,6%	99,6%	99,6%	99,6%	99,6%	99,6%	96,5%	96,5%	96,2%	96,5%	96,7%	96,7%	97,1%	96,5%	96,5%	96,7%	
23	KY362210.1	97,3%	99,8%	99,1%	100,0%	99,8%	99,8%	99,8%	99,8%	99,8%	99,8%	96,7%	96,7%	96,5%	96,7%	96,9%	97,0%	97,4%	96,7%	96,7%	97,0%	99,8%

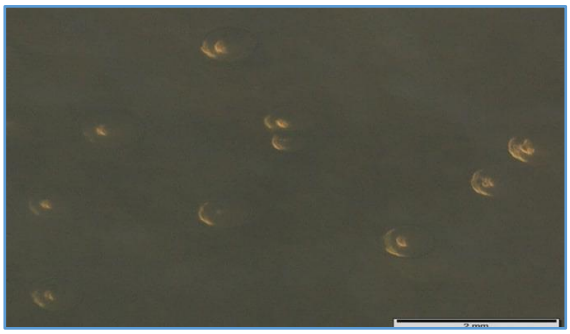

Fig. 1. Typical fried egg-like colony structures of *M. gallisepticum* observed under a stereo microscope (Olympus SZX7,Japan).

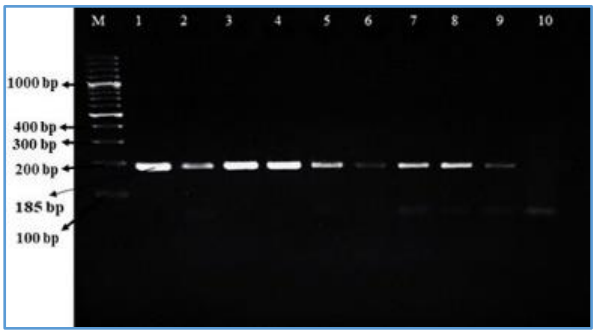

Fig. 2. Gel electrophoresis image of the samples as a result of the PCR test. M; Marker (100 bp ladder) 1-*M. gallisepticum* S6 (Positive control), 2- *M. gallisepticum* 6/85 vaccine strain, 3–9-*M. gallisepticum* field strains, 10-Negative control.

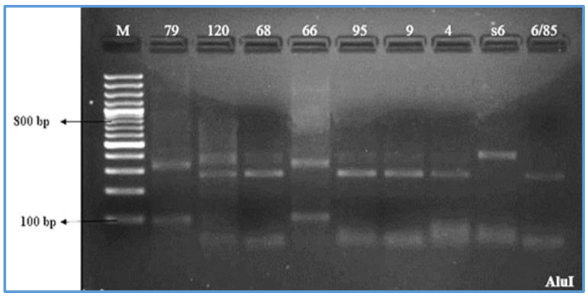

Fig. 3. Image of 2% agarose gel electrophoresis for the restriction enzyme *AluI* used in PCR-RFLP. The samples are arranged as follows: M: marker (100 bp DNA ladder), 79, 120, 68, 66, 95, 9, 4 (isolated *M. gallisepticum* field strains), *M. gallisepticum* S6, *M. gallisepticum* 6/85 vaccine strain.

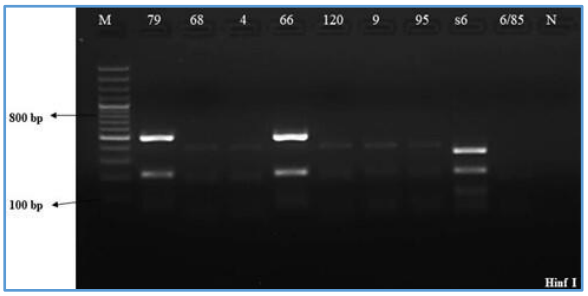

Fig. 4. Image of 2% agarose gel electrophoresis for the restriction enzyme *Hinf*I used in PCR-RFLP. The samples are arranged as follows: M: marker (100 bp DNA ladder), 79, 68, 4, 66, 120, 9, 95 (isolated *M. gallisepticum* field strains), *M. gallisepticum* S6, *M. gallisepticum* 6/85 vaccine strain, N: negative control.

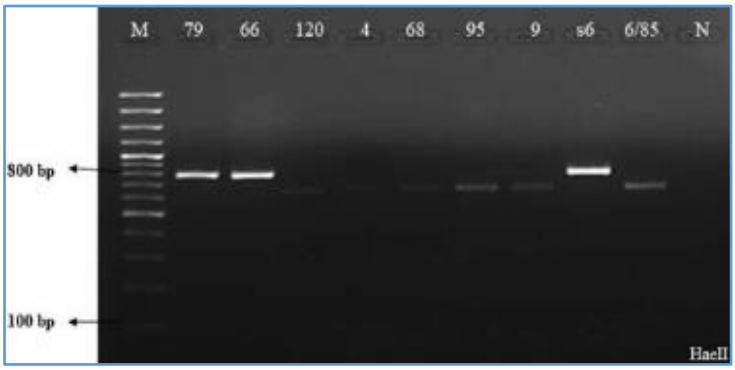

Fig. 5. Image of 2% agarose gel electrophoresis for the restriction enzyme *HaeII* used in PCR-RFLP. The samples are arranged as follows: M: marker (100 bp DNA ladder), 79, 66, 120, 4, 68, 95, 9 (isolated *M. gallisepticum* field strains), *M. gallisepticum* S6, *M. gallisepticum* 6/85 vaccine strain, N: negative control.

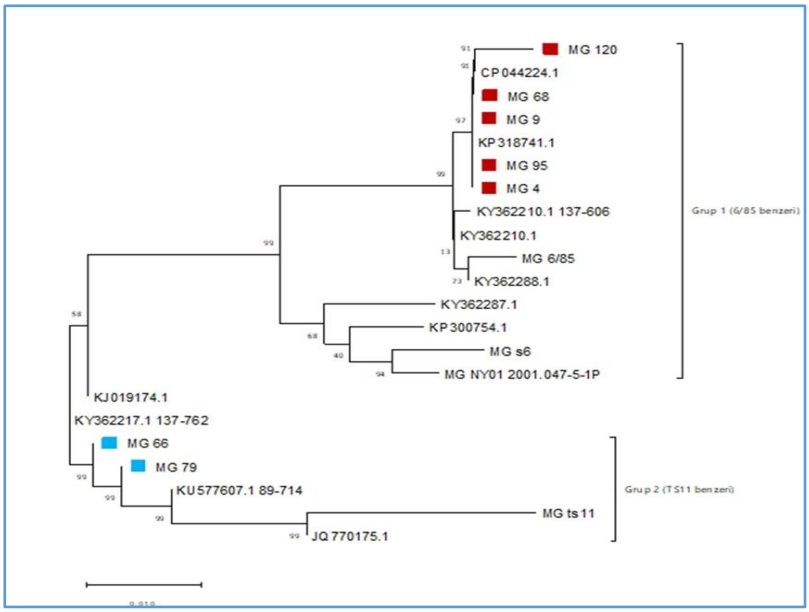

Fig. 6. Phylogenetic tree of 7 *M. gallisepticum* field isolates from poultry from the Şanlıurfa region in Turkey and 15 *M. gallisepticum* strains/isolates from GenBank (NCBI 2023).

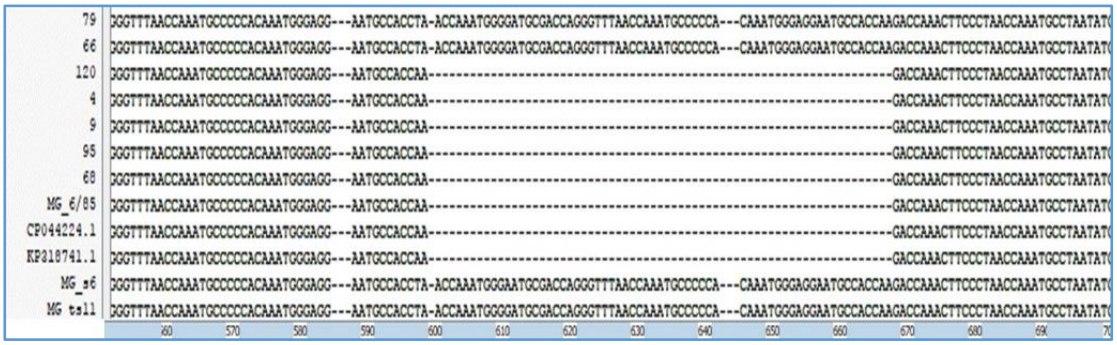

Fig. 7. Section showing the deletion of 63 nucleotides in the *mgc2* gene of group 1 (MG 120, 95, 68, 9, 4) field isolates.

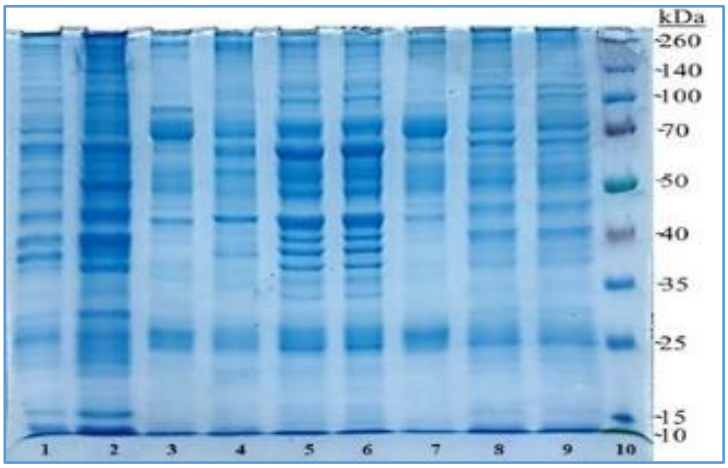

Fig. 8. Gel electrophoresis image of *M. gallisepticum* proteins after SDS-PAGE. 1; *M. gallisepticum* S6, 2; *M. gallisepticum* 6/85, 3-9; isolated *M. gallisepticum* field strains: 3; MG4, 4; MG9, 5; MG68, 6; MG95, 7; MG120 8; MG66 9; MG79 10; M: Spectra Multicolor Broad Range Protein Ladder (Termo, Lot:01161301).

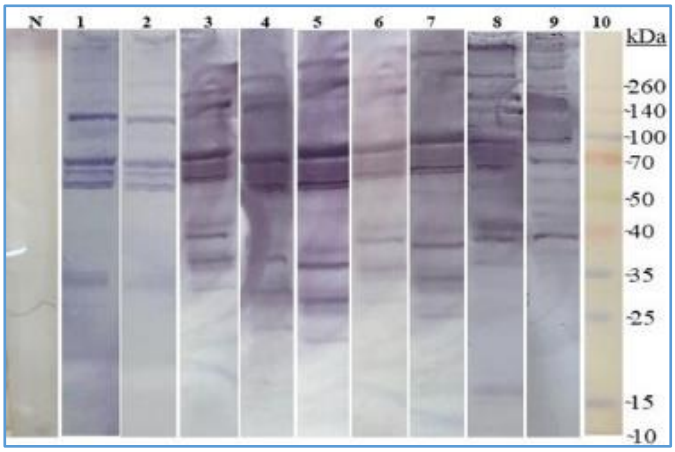

Fig. 9. WB analysis of antibodies raised against *M. gallisepticum* specific proteins. 1-7; Isolated *M. gallisepticum* field strains 1; MG79, 2; MG66, 3; MG4, 4; MG9, 5; MG68, 6; MG95, 7; MG120, 8; *M. gallisepticum* 6/85, 9; *M. gallisepticum* S6, 10; M: Spectra Multicolor Broad Range Protein Ladder (Thermo, Lot: 01161301), N: Negative control.

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