Original Article

Comparative Genomic Analysis of Six Mycoplasma Gallisepticum Strains: Insights into Genetic Diversity and Antibiotic Resistance

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ABSTRACT

Mycoplasma gallisepticum (MG) is a significant pathogen that causes respiratory diseases, which have had a substantial economic impact on the poultry industry. Despite the resistance of MG to antibiotics, it is imperative to identify genetic diversity in order to develop countermeasures. In this study, the genomes of six MG strains were examined to gain deeper insights into the mutations. The data pertaining to Variant Annotation and Mutation Analysis using SnpEff, along with the calculation of mutation rates as the ratio of total mutations to the length of the genomic regions analyzed, were thoroughly examined. The comprehensive evaluation yielded a total of 25,942 variants across the six strains, underscoring substantial genetic diversity. Notably, strain S6 exhibited a preponderance of frameshift mutations. A notable finding was the presence of a mutation in the MsbA gene shared by all six strains. Furthermore, five of the six strains, with the exception of strain F99 Lab, exhibited a mutation at position 5158, which impacts a multidrug transport system. Notably, strain ATCC exhibits a distinctive mutation at position 942, while strain S6 displays a unique mutation at position 6855, which is linked to efflux ABC transporter components. Furthermore, a substantial degree of genetic variation was observed among the CrmA, GapA, and vlhA genes among the various strains. High-impact changes, such as insertions and deletions, exhibited a higher frequency in CrmA, particularly in strain S6. Conversely, nonsynonymous variations demonstrated a heightened prevalence in GapA, particularly in strain F99 Lab. The vlhA gene exhibited a spectrum of effects, ranging from synonymous mutations to high-impact mutations such as stop-gains and frameshifts, particularly in strains k5111a and k4602. The functional variations observed among the strains can be attributed to these mutations, which have the potential to alter gene expression or protein function. Furthermore, substantial mutations in the dxr and rpoC genes were associated with antibiotic resistance. These mutations underscore the ongoing evolutionary adaptations of M. gallisepticum. Consequently, there is an imperative for the revision of treatment protocols and the formulation of targeted vaccines to regulate resistance within the poultry industry.

Keywords: *Mycoplasma Gallisepticum,* Genetic Variation, Antibiotic Resistance, Poultry Industry.

1. Introduction

Mycoplasma gallisepticum (MG) is a pathogenic bacterium that poses significant challenges to the poultry industry (1). The bacterium's impact on chickens, turkeys, and pigeons is characterized by chronic respiratory infections, which have been demonstrated to result in diminished productivity, elevated mortality rates, and augmented expenditures related to treatment and prevention measures (2). Respiratory diseases in poultry are frequently multifactorial in nature, involving intricate interactions between viral and bacterial pathogens. For instance, avian metapneumovirus (APMV) has been identified as a causative agent of highly contagious respiratory infections in poultry, with variable mortality rates that can be significantly elevated in cases of co-infection (3,4). Similarly, the Newcastle disease virus (NDV) is a primary pathogen that can exacerbate respiratory outcomes and render birds susceptible to secondary bacterial infections, thereby exacerbating the severity of respiratory disease (5). This synergistic relationship between MG and viral pathogens underscores the complex etiology of respiratory diseases in poultry, highlighting the need for comprehensive management strategies to mitigate CRD-associated losses (6). This bacterium has the capacity to evade the host's immune system and resist antibiotics due to its multiple mutations (7). The outbreaks necessitate large-scale culling and prolonged use of antibiotics, which predominantly lead to heightened antimicrobial resistance (7). Research has demonstrated that M. gallisepticum can evade the host immune system and develop resistance to antibiotics. (8). The pivotal mechanism underlying bacterial resistance to antibiotics is mutation, encompassing the adaptation of plasmids and mobile genetic elements to antibiotics and environmental stress (9, 10). However, the existing literature on antibiotic resistance has been predominantly focused on specific strains or on isolated facets of its pathogenicity (8). However, a paucity of literature exists on a comprehensive analysis of the genetic diversity and mutation patterns across multiple strains. It is imperative to develop a profound comprehension of the genetic variation to identify mutations that influence the pathogen's capacity to resist treatment and augment its virulence (11,12). It is imperative to consider the interplay between virulence factors and antibiotic resistance mutations in MG, given their symbiotic relationship. The development of antibiotic resistance in pathogens enables their persistence within treated hosts, resulting in more severe or protracted infections. Furthermore, virulent strains have the potential to manifest more severe infections that are resistant to standard treatment regimens. Consequently, a comprehensive investigation into virulence and resistance mutations is imperative to develop efficacious control measures, such as vaccines and antibiotics, to target the evolving genetic landscape of MG (13). In light of the mounting demand for poultry products, there is an imperative for the effective management of M. gallisepticum. In recent years, a variety of bioinformatics methodologies have been proposed for the analysis of genetic diversity. The aligned reads were processed using SAMtools to detect genomic variants, which converts the Sequence Alignment/Map (SAM) files into binary alignment/map (BAM) format and identifies single nucleotide polymorphisms (14). However, despite the importance of this issue, information is not yet complete in the treatment and resistance section, and further bioinformatic investigations are needed. This study constitutes the inaugural comparative genomic analysis of six distinct strains of MG, thereby offering novel insights into the genetic diversity of this significant poultry pathogen. This study addresses a significant gap in the existing literature by providing novel insights into the genetic diversity of MG, particularly with regard to antibiotic resistance and virulence-related mutations. These findings are crucial for the development of vaccines, the design of therapeutic interventions, and the implementation of prevention strategies.

2. Materials and Methods

2.1. Genome Collection and Preparation

The genomic data for six MG strains and the reference strain VA94 were retrieved from the database of NCBI (accessed on 18 September 2024). The strains encompassed a broad geographic diversity and pathogenic background to capture a comprehensive spectrum of genetic diversity within M. gallisepticum. The selection of these strains was guided by their relevance to previous outbreaks and their clinical significance. The study thus encompassed both commonly encountered strains and those showing distinct resistance patterns. Utilizing the VA94 reference strain facilitated consistent comparative analysis, establishing it as a standard for evaluating genetic variations in the other strains (15).

2.2. Sequence Alignment

The next-generation sequencing platform of Illumina was selected for its high throughput and accuracy. The quality of the raw reads was assessed using the FastQC tool, which guided the subsequent trimming and filtering of low-quality sequences to enhance data accuracy. The raw sequencing data was then subjected to a series of pre-processing steps, including adapter removal and duplicate sequence elimination. These steps were carried out using Picard tools configured to identify and remove low-quality reads, adapters, and duplicate sequences. The resultant, refined reads were then aligned to the reference genome (VA94) using Bowtie2. Bowtie2 is a widely used tool for short-read alignment, exhibiting high efficiency in handling gapped reads with high accuracy (15). The quality of the alignment was assessed by evaluating coverage depth and mapping accuracy across all genomes (Table 1).

2.3. Variant Calling and Filtering

The aligned reads were processed using SAMtools, a software that retains unique reads and detects genomic variants. This process converts the Sequence Alignment/Map (SAM) files into binary alignment/map

Table 1. The sequencing libraries and Id for six strains of MG.

Library	Reads	Strain
PE1	SRR8647642_	*Mycoplasma gallisepticum F99 Lab*
PE2	SRR16232599	*Mycoplasma gallisepticum S6*
PE3	SRR10165019_	*Mycoplasma gallisepticum 6/85*
PE4	SRR891945	*Mycoplasma gallisepticum ATCC 19610*
PE5	SRR26702964	*Mycoplasma gallisepticum k5111a*
PE6	SRR26702970_	*Mycoplasma gallisepticum k4602*

(BAM) format. It also identifies single nucleotide polymorphisms (SNPs), indels, and other genomic variants (14). Coverage depth calculations were conducted using SAMtools, which reported on total reference bases, median and mean coverage, as well as zero-coverage regions for each library. To minimize the occurrence of false positives, the variant was named based on the guidelines, including the removal of low-confidence regions and the utilization of base quality score recalibration. Subsequent filtering was implemented to eliminate variants with a depth of coverage lower than 10 reads, ensuring the inclusion of only high-confidence variants. Finally, variant calling accuracy was verified by comparing detected variants across multiple strains.

2.4. Variant Annotation and Mutation Analysis

Given that SAMtools alone does not perform variant calling, FreeBayes, a robust Bayesian-based variant caller, was utilized in conjunction with SAMtools to identify high-quality variants and its capacity to detect both single-nucleotide polymorphisms (SNPs) and small indels effectively. Variant effect annotation was carried out using snpEff, providing gene-level impact predictions that were further refined for clarity and relevance in the context of the study (16). The mutations were subsequently categorized into three distinct types: synonymous, non-synonymous, and frameshift. Synonymous mutations were defined as those that did not alter the amino acid sequence, while non-synonymous mutations were those that resulted in amino acid substitutions, which potentially affected protein function. Frameshift mutations were of particular interest given their potential impact on pathogenicity and resistance mechanisms. The functional effects of the mutations were classified on the basis of their predicted severity (high, moderate, or low). This approach enabled the identification of key mutations that may influence the virulence or antibiotic resistance of the strains.

2.5. Statistical Analysis

The utilization of statistical methodologies in Python libraries such as Pandas, Matplotlib, and Seaborn enabled a comparative analysis of mutation rates, thereby facilitating an assessment of evolutionary pressures. The mutation rates were calculated by dividing the total number of mutations by the length of the genomic regions that were analyzed. Furthermore, these rates were compared to the mutation rate of the VA94 reference strain. The statistical significance of the observed differences in mutation rates was determined using one-way analysis of variance (ANOVA; $p\text{-value}<0.05$). Furthermore, the impact of mutations on antibiotic resistance and virulence-related genes was analyzed through the use of descriptive statistics, including mutation intensity scores and their associated 95% confidence intervals. To illuminate the distribution and prevalence of mutations across strains, heatmaps and bar charts were created, offering profound insights into the genetic landscape of *M. gallisepticum.*

2.5 Data Handling and Analysis

Subsequent to a series of calls and annotations, the data were analyzed using Python-based tools to ensure the efficient management and comprehensive analysis of large datasets. The Pandas library was employed for data manipulation and the creation of a structured framework for the categorization, filtration, and organization of mutations in the six MG strains. Descriptive statistics, encompassing mutation counts, types, and frequencies, were computed to ascertain the genetic diversity in the dataset. The creation of graphical representations of the mutation data was achieved through the utilization of Matplotlib and Seaborn. These visualizations, including pie charts, bar graphs, and heatmaps, were employed to depict the distribution of synonymous, non-synonymous, frameshift mutations, and other variant types across the strains. The utilization of these figures has facilitated a more nuanced interpretation of the functional impacts and evolutionary trends.

3. Results

3.1 Comprehensive Mutation Analysis

The genetic landscape of MG across multiple strains was evaluated, including the following: strain F99 Lab, strain S6, strain 6/85, strain ATCC 19610, strain k5111a, and strain k4602. Each strain manifested distinctive sequencing characteristics. The coverage depth for each isolate is delineated in Table 2. A total of 25,942 variants were identified in six MG strains. These variants were subsequently classified into various categories, including synonymous, non-synonymous, insertions, deletions, and frameshift mutations. The proportion of synonymous mutations ranged from 53% to 58%, while non-synonymous mutations accounted for 37% to 41% of the total variants. Insertions and deletions accounted for approximately 2–3% of the total mutations (Figure 1).

Table 2. The most important mutations, including the gene, position, mutation type, and predicted impact.

Strain	Median Coverage	Mean Coverage
F99 Lab	3399	3214.8
S6	319	312.2
6/85	2393	2315.5
ATCC 19610	1124	1111.8
k5111a	2859	2748.5
k4602	353	337.5

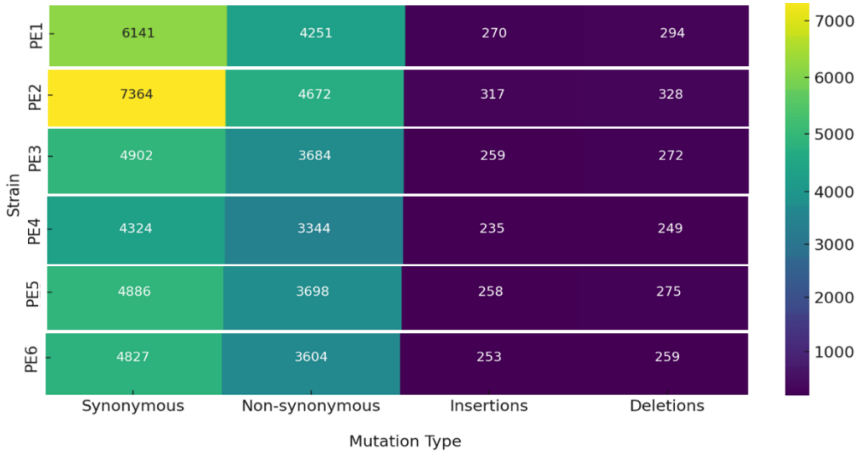

Figure 1. The heatmap illustrates the distribution of mutation types (synonymous, non-synonymous, deletions, and insertions) in different strains of *MG*.

3.2 Mutation Distribution and Frameshift Mutation Analysis

The figure illustrates the total number of frameshift mutations identified in each of the six strains of MG. The strain designated S6 exhibited the highest number of frameshift mutations, predominantly affecting genes associated with antibiotic resistance and virulence, including the MsbA gene, which is involved in lipid transport. It is noteworthy that other strains exhibited a significant number of frameshift mutations, particularly in the rpoC gene, as illustrated in Figure 2. A comparison of the mutation rates revealed a substantial genetic diversity. Mutation in the gene encoding the Lipid A export ATP-binding and permease protein MsbA, observed at position 5059 on Contig CP003506, indicated an extensive evolutionary and present in all six strains. Furthermore, mutations were identified within a multidrug transport system, at position 5158 on Contig CP003506, in five strains, with the exception of strain F99 Lab. A mutation at position 6855 in a gene that plays a role in the efflux of ABC transporter permease components is only observed in strain S6. A mutation at position 942 in a gene encoding for a hypothetical protein is only presented in strain ATCC 19610. Furthermore, a multitude of frameshift mutations were identified across MG strains. As illustrated in Figure 3, the atpD and ach1 genes exhibited the highest number of frameshift variants, with eight and seven mutations, respectively. The remaining genes, including glpK, dppD/oppD, uvrB, maoC, hlp2, and beta-pgm, exhibited two to three frameshift variants.

3.3 Antibiotic Resistance Gene Mutations and Virulence-Related Genes

A substantial degree of genetic diversity was identified in the six MG strains, with a particular focus on mutations that likely influenced pathogenicity and antibiotic resistance. Of particular note are the high-impact mutations observed in the rpoC and dxr genes, which are essential for fundamental cellular processes (Table 3). The analysis of mutations in the CrmA and GapA genes across strains revealed varying degrees of effect from the mutations. The CrmA gene has been found to contain numerous high-impact mutations. Notably, insertions were identified at positions 246707 (in strain F99 Lab) and 246699, 246755, and 247089 (in strain S6). Moreover, deletions were identified at positions 246706 (strain ATCC 19610) and 248530 (strain 6/85, strain k5111a). Furthermore, nonsynonymous changes were identified in several locations within strain F99 Lab and strain S6. These variations exhibit functional diversity amongst strains, with strain S6 displaying a significantly greater number of insertions than the other strains. Conversely, a comprehensive array of mutations, predominantly nonsynonymous, was identified across all strains of the GapA gene. Notably, the majority of nonsynonymous changes were observed in strain F99 Lab, predominantly between positions 242055 and 242210. A limited number of high-impact mutations, predominantly insertions and deletions in strains S6 and ATCC 19610, were identified. Each strain exhibited a distinct mutation pattern for the vlhA gene. For instance, strain S6 and strain k5111a exhibited a greater prevalence of disruptive changes

compared to other strains, which predominantly exhibited missense mutations. Position 801088 has been identified as a region of particular mutation frequency across multiple strains, exhibiting alterations such as Val119Ala, which are deemed to have a moderate impact. Furthermore, there is a notable prevalence of mutations across various strains within specific regions, particularly those located at 800k. (Figure 4).

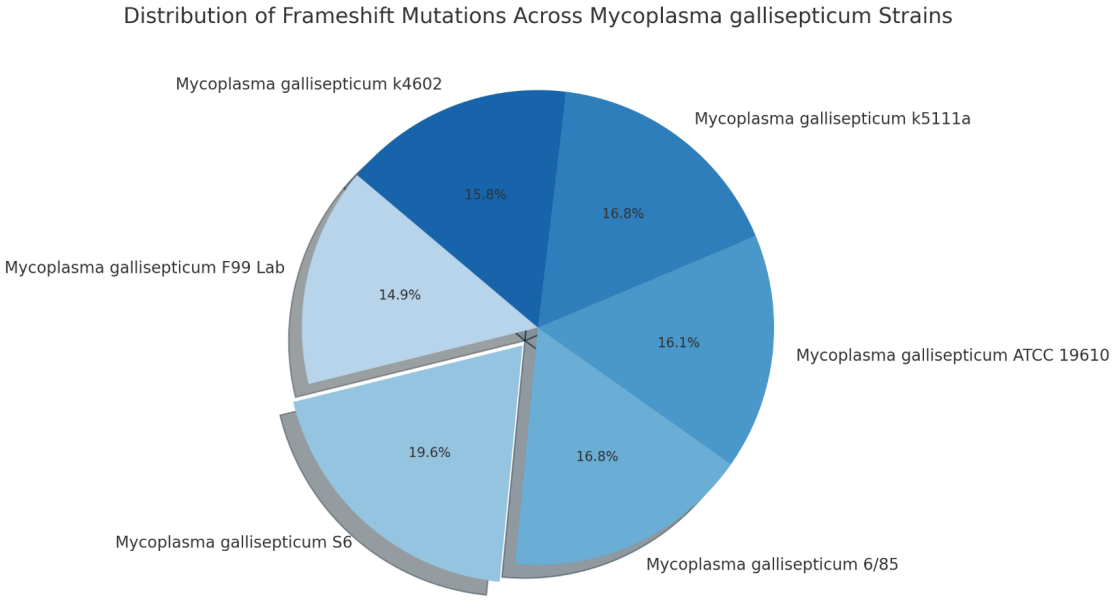

Figure 2. Pie chart illustrating the distribution of frameshift mutations among six MG strains.

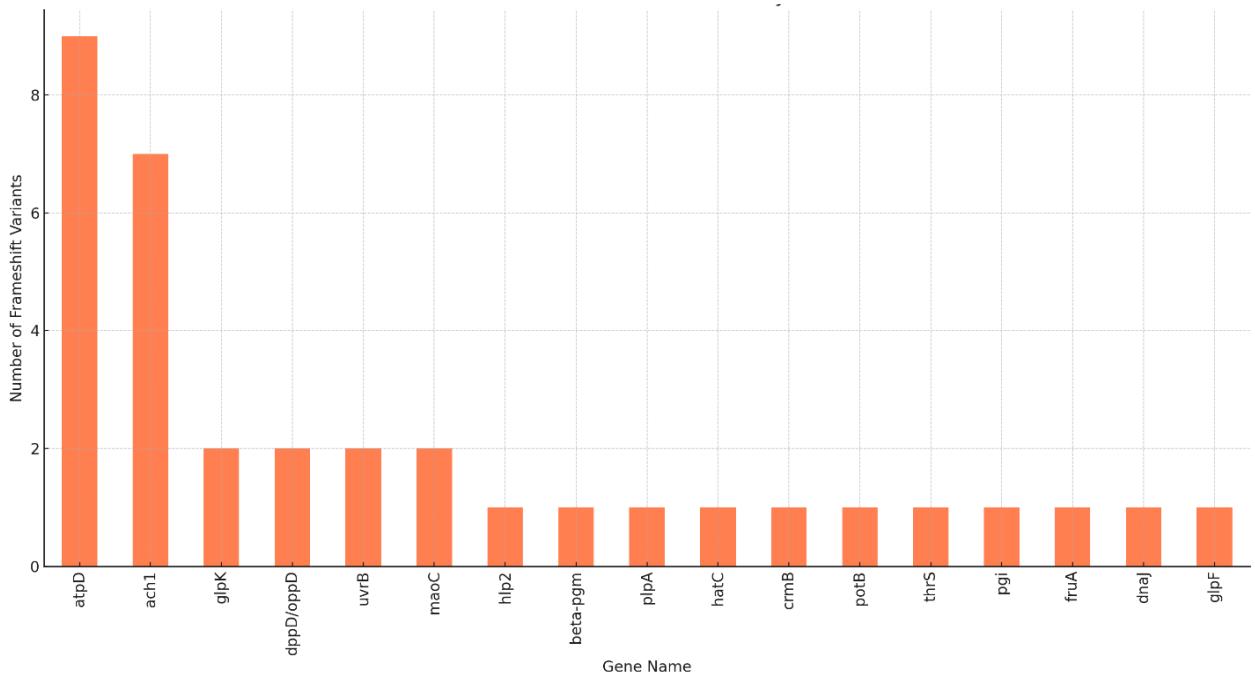

Figure 3. The overall impact of mutations is represented by mutation intensity.

Table 3: The most important mutations, including the gene, position, mutation type, and predicted impact.

Gene	Position	Type	Predicted Impact
dxr	109331	High Impact	Affects isoprenoid synthesis
rpoC	305216	High Impact	Affects transcription
glpF	15027	Frameshift	Disrupts glycerol transport
gyrA	60001	Moderate Impact	Affects DNA gyrase
rpoB	120056	Moderate Impact	Affects RNA polymerase

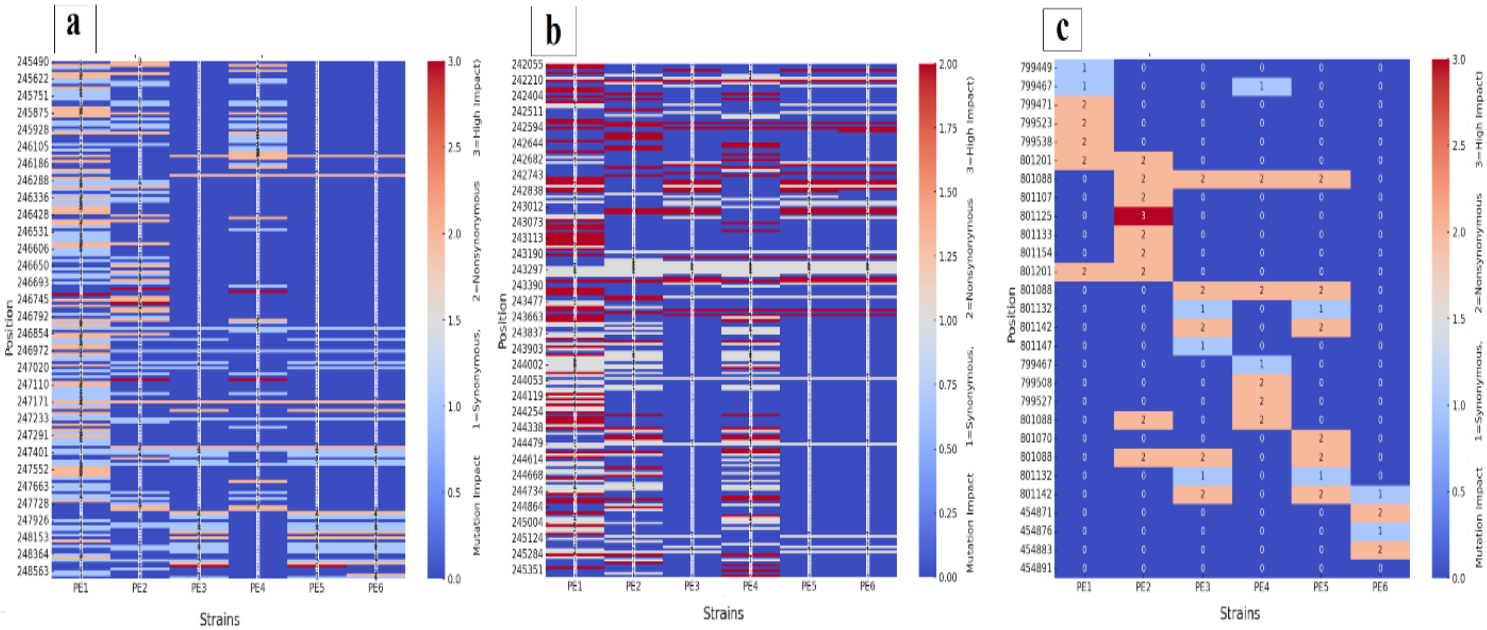

Figure 4. Heatmap of mutations in the genes across strains. a) *CrmA* mutation impact b) *GapA* mutation impact c) *vlhA* mutation impact.

4. Discussion

The 25,942 variants observed among six distinct strains of MG are indicative of a high degree of genetic diversity. The majority of these mutations were found to be synonymous, while a significant number involved frameshift and non-synonymous variations. Notably, strain S6 demonstrated a particularly high frequency of frameshift mutations, which likely contributes to its distinctive adaptive characteristics. The findings were consistent with the general understanding of the evolutionary pressures on pathogen genomes, particularly in the context of selective pressures from antibiotic use and genes like vlhA, CrmA, and GapA. The significant mutations in genes such as glpF, dxr, and rpoC underscore the adaptive mechanisms of M. gallisepticum and the potential for disease control. As was the case in the present study, previous studies have determined that genetic diversity in MG isolates from Iran, particularly in the pvpA gene and nucleotide similarities of 94.5%–99.9% among isolates, is linked to environmental factors. It was determined that M. gallisepticum isolates exhibit substantial genetic diversity, particularly in the genes associated with virulence and resistance. However, the number of variants observed in this study was marginally lower than that reported in the present study due to the limited number of analyzed strains (12,17). However, the scope of genetic diversity in our study surpasses that of Kabiri et al. (2019) and Limsatanun et al. (2022), as we focused on a wider range of genes across different functional categories, including those linked to antibiotic resistance and virulence. The presence of frameshift mutations in genes such as rpoC and MsbA in the S6 strain underscores the substantial evolutionary pressure exerted by antibiotics, a factor that received less emphasis in earlier studies that primarily focused on surface proteins like

pvpA. This expanded analysis provides a more comprehensive view of how various selective pressures contribute to the adaptive evolution of MG across different regions and environments (17,18). It has been documented that mutations in membrane proteins affect the structure of the ABC-type multidrug transport system (19). Frameshift mutations have the potential to significantly impact gene function. Furthermore, frameshift mutations in glpF have been shown to modify glycerol transport, thereby enhancing the survival and virulence of pathogens. The ability of *MG* to adhere to host tissues is necessary for colonization and pathogenicity, and this requires the co-expression of *GapA* and *CrmA*. mutations in these genes cause cytoadherence to be disrupted, which results in markedly decreased virulence, especially harming the respiratory system. This is consistent with our research, which shows that changes in the *CrmA* and *GapA* genes may affect the bacterium's ability to adhere to host cells and propagate infections, particularly in the respiratory system (25). Furthermore, strains lacking both GapA and *CrmA* proteins showed reduced ability to colonize the respiratory tract and internal organs in infected birds, as noted by Indiková et al. (2013). The lower virulence and systemic dissemination observed in mutant strains lacking these proteins provide evidence for the involvement of these genes in host-pathogen interactions (21). According to a previous study, *GapA* and *CrmA* are important not only for initial attachment but also for the infection's progression, which includes the production of biofilms and the development of respiratory diseases. Further highlighting the crucial role these cytoadhering play in pathogenicity, the mutations found in our investigation probably contribute to the variations in virulence across the strains (20). We found several high-impact mutations, including insertions and deletions, in *F99 Lab* and *S6*, the immune-evading *CrmA* gene displayed notable variety. This finding lends more credence to the theory that the genetic variety of these genes allows the pathogen to adapt to different host immunological responses (8). The capacity of MG to adhere to host tissues is imperative for colonization and pathogenicity, a process that necessitates the coordinated expression of GapA and CrmA. Mutations in these genes disrupt cytoadherence, leading to a substantial reduction in virulence, particularly with regard to its impact on the respiratory system. This observation is consistent with the findings of our research, which demonstrates that alterations in the CrmA and GapA genes can influence the bacterium's capacity to adhere to host cells and propagate infections, particularly within the respiratory system (25). Moreover, strains devoid of both GapA and CrmA proteins exhibited diminished capacity to colonize the respiratory tract and internal organs in infected birds, as observed by Indiková et al. (2013). The reduced virulence and systemic dissemination observed in mutant strains lacking these proteins provides evidence for the involvement of these genes in host-pathogen interactions (21). According to a previous study, GapA and CrmA are not only important for initial attachment but also for the infection's progression, which includes the production of biofilms and the development of respiratory diseases. The mutations identified in this investigation likely contribute to the observed variations in virulence among the strains (20). Our investigation identified numerous high-impact mutations, including insertions and deletions, in F99 Lab and S6. Notably, the immune-evading CrmA gene exhibited a considerable degree of variability. This finding lends further credence to the theory that the genetic variability of these genes enables the pathogen to adapt to different host immunological responses (8). An evaluation of the vlhA gene revealed distinctive mutation patterns for each of the strains. A notable observation is that, in contrast to other strains that predominantly exhibit missense mutations, S6 and k5111a demonstrate a greater prevalence of disruptive alterations, including nonsense and frameshift mutations. These disruptive mutations may lead to substantial changes in the way proteins function, which may have an impact on the pathogen's capacity to elude the immune system. A notable discovery is the identification of Val119Ala as a hotspot for mutations across multiple strains, despite its predicted mild effect on protein structure. Distinct environmental or immunological stressors give rise to varying gene expression profiles. The disruptive mutations observed in the S6 and k5111a strains may be indicative of similar selection forces that drive host-pathogen adaptations. Pflaum et al. (2020). Furthermore, Pflaum et al. (2018) observed that several vlhA genes exhibited significant expression in chickens during the early stages of infection, with a discernible change in the dominant genes over time. This dynamic expression of vlhA is consistent with the mutation hotspots that were identified, particularly in the 800k region (22, 23). This finding suggests that the pathogen may possess the capacity to modify its surface proteins, thereby evading immune detection. This ability may be facilitated by structural changes in the vlhA gene, which could confer a selective advantage. It has been documented that phase variation in the expression of distinct vlhA genes at varying stages of infection plays a pivotal role in immune evasion by the vlhA gene family. The study identified two missense variants, Leu3Ser and Thr13Ser, which may alter the surface characteristics of the VlhA protein, thereby impeding the host immune system's ability to identify and neutralize the infection. The significance of vlhA in immune evasion and pathogenicity during chronic infection was emphasized by Pflaum et al. (23). The hypothesis that these mutations could be a component of a larger mechanism for adaptation, enabling M. gallisepticum to persist in its host by changing its surface proteins and evading immune responses, is supported by our findings, especially the recurrent mutations in important regions like the 800k region. In the present study, frameshift mutations in the glpF gene (S6 strain) were identified as a potential virulence factor in M. gallisepticum. These changes are expected to improve the metabolic flexibility of the bacteria, particularly in

conditions with limited nutrients, enabling more effective uptake and metabolism of glycerol. It has been documented that M. gallisepticum's pathogenicity is predominantly contingent on the glycerol ABC transporter system, which encompasses the glpF gene. As Mahdizadeh et al. (2021) observed, disrupting glycerol absorption through mutations in the glycerol transporter system led to a decline in virulence and metabolic efficiency. This finding underscores the significance of glycerol metabolism in bacterial survival and adaptation (24, 25). Significant high-impact mutations in the rpoC and dxr genes, which are crucial for transcription and isoprenoid biosynthesis, respectively, were identified. These alterations are of particular significance due to their association with resistance to multiple antibiotic classes. The rpoC gene mutation, observed in strains such as S6, has been linked to resistance to peptide antibiotics, including myxopyronins and Corallopyronin, which target RNA polymerase. Changes in rpoC, the gene encoding the RNA polymerase beta subunit, have the potential to modify the structure of the enzyme and lower the antibiotics' binding affinity. Kim et al. (2003) underscored the evolutionary divergence of the rpoC gene across Mycoplasma species and its pivotal function in bacterial transcription (26). Our research, in concordance with the findings of De Vos et al. (2013), suggests that alterations in rpoC, particularly in rifampin-resistant bacteria, are likely a compensatory mechanism to mitigate the fitness cost associated with antibiotic resistance (27). The present study investigates MsbA gene frameshift mutations in the S6 strain, suggesting a comparable evolutionary adaptation in MG, likely resulting from antibiotic use-related selection pressures. The MsbA gene, which encodes a multidrug resistance protein, plays a critical role in antibiotic efflux and contributes to the pathogen's resistance to various antibiotics by limiting intracellular drug accumulation. The MsbA gene, an ABC transporter, facilitates the translocation of lipopolysaccharides (LPS) in Escherichia coli, as demonstrated by Polissi et al. (1996). Mutations in this gene have been shown to enhance antibiotic sensitivity by impairing transporter function. Analogous alterations in the S6 strain have been observed to compromise the efflux mechanism, thereby rendering the pathogen more vulnerable to antibiotic treatment and promoting evolutionary adaptations aimed at preserving resistance, as evidenced by the parallels observed between MsbA in E. coli and M. gallisepticum (28). Furthermore, MsbA has been identified as a pivotal gene in MG biofilm production, as reported by Ma et al. (2023). The formation of biofilms serves as a physical barrier, thereby shielding bacterial cells from external stressors such as antibiotics (29). The high rate of mutations in antibiotic resistance-related genes indicates the necessity of revising available treatment approaches in poultry farming. The WHO has indicated that the excessive use of antibiotics likely accelerates the emergence of resistant strains. The findings underscore the imperative to recalibrate treatment protocols in accordance with the distinct mutation profiles exhibited by circulating M. gallisepticum strains. For instance, strains possessing mutations in the rpoC gene have been observed to demonstrate resistance to transcription-targeting antibiotics, underscoring the need for alternative treatment strategies. Furthermore, the implementation of genetic monitoring as a standard surveillance practice in poultry farming is imperative to identify more efficacious treatment regimens and prevent the propagation of resistant strains. The latest development in reverse vaccinology, based on genomic data, can be used to design the next-generation vaccines for M. gallisepticum. Furthermore, the targeting of conserved virulence factors, such as those affecting glycerol transport or isoprenoid biosynthesis, can enhance the effectiveness of vaccines. By focusing on regions of the genome that are less prone to mutation, the development of vaccines can be directed towards providing broad and long-lasting protection against a wide range of MG strains, which are known to evolve (30). It is imperative to deepen our understanding of M. gallisepticum's genetic adaptation processes. This research endeavors to lay the foundation for long-term disease control in the chicken industry by developing next-generation vaccinations that can target conserved mutations and neutralize various strains. A notable limitation of this study is its reliance on in silico forecasts. Further research in vitro and in vivo is necessary to ascertain whether these mutations directly influence protein function or contribute to antibiotic resistance. Although methods such as SnpEff can reveal possible implications of genetic variants, further study is required to confirm these findings. Additionally, the genetic data utilized in this study is derived from publicly accessible sources, which may not accurately reflect the diversity of circulating strains worldwide. In conclusion, this study provides the first comprehensive genomic comparison of six distinct MG strains, revealing significant genetic diversity. The identification of high-impact mutations, particularly in genes such as dxr and rpoC, and those involved in glycerol transport, underscores the evolutionary pressure exerted by antibiotic use and highlights potential targets for next-generation vaccines. These findings offer valuable insights for both academic research and practical poultry health management. The identification of mutations associated with antibiotic resistance and virulence suggests the potential for the development of strain-specific vaccines that target conserved genomic regions, which may offer more effective and durable protection. Future studies should prioritize the expansion of genetic surveillance to encompass a broader array of geographic locations and ecosystems, with the objective of substantiating these findings.

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Authors' Contribution

Study concept and design: K. K, SA.P
Acquisition of data: K. K
Analysis and interpretation of data: SA. P., K. K and T. ZS
Drafting of the manuscript: K. K.
Statistical analysis: K. K., SA. P. and T. ZS.
Critical revision of the manuscript for important intellectual content: SA. P. and T. ZS.
Administrative, technical, and material support: K. K.

Ethics

The sampling process for the submitted article was conducted in accordance with the ethical guidelines established by the ethics committee of the Science and Research Branch, Islamic Azad University, Tehran. The present study was conducted in accordance with the ethical guidelines established by the Science and Research Branch, Islamic Azad University, Tehran.

Conflict of Interest

The authors of this study have no financial interests or personal relationships that could potentially influence the results. The study was not funded by any company or for-profit organization, and the authors maintain full independence in their research and conclusions.

Data Availability

The data that support the findings of this study are available upon request from the corresponding author.

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