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Whole genome sequencing and analysis of a goose-derived *Mycoplasma gallisepticum* in Guangdong Province, China

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Abstract

Introduction: *Mycoplasma gallisepticum* (MG) infection is a primary cause of chronic respiratory disease in poultry, threatening the economic viability of China's goose-farming industry. This study investigated the pathogenicity and drug resistance of an MG strain isolated from geese and whole-genome sequenced the strain. **Material and Methods:** A strain designated MG-GD01/22 was isolated from the air-sac tissues of five geese with chronic respiratory disease on a Guangdong goose farm. Its pathogenicity was assessed, antimicrobial susceptibility tests were performed using agar dilution, and its total DNA was extracted for whole-genome sequencing and gene function annotation with second- and third-generation sequencing technologies. The homology of the 16S ribosomal RNA (rRNA) region was analysed and a phylogenetic tree was constructed, as was an evolutionary tree of the *mgc2* gene. Gene co-linearity analysis was performed to compare MG-GD01/22 with the strains in the GenBank database. **Results:** The isolate produced "fried egg" colonies and was pathogenic to goslings. It was resistant to enrofloxacin, danofloxacin and spectinomycin and susceptible to valnemulin, tilmicosin, tylosin, acetylisovaleryltylosin tartrate and tiamulin. The genome analysis revealed 1,666 coding genes. Gene database annotation identified 25 virulence-related genes, 22 drug resistance–related genes, 13 pathogen-host–interaction genes and 9 carbohydrate-active enzyme genes. The isolate exhibited 99.9% homology to the MG S6 strain by its 16S rRNA, while the *mgc2* gene typing results indicated that it differed from known MG model strains. The genome of MG-GD01/22 showed high homology but poor co-linearity with MG S6, characterised by numerous gene deletions, inversions and displacements. **Conclusion:** This study offers theoretical references for the diagnosis, prevention and treatment of MG in geese in the Guangdong region.

Keywords: goose-origin *Mycoplasma gallisepticum*, isolation and identification, pathogenicity, antimicrobial susceptibility test, whole-genome sequencing.

Introduction

Mycoplasma gallisepticum (MG), belonging to the Mollicutes class in the *Mycoplasma* genus, is a pathogenic microorganism capable of inducing chronic respiratory diseases (CRD) in poultry. Intermediate in size between bacteria and viruses, MG lacks a cell wall, possesses soft and pleomorphic cells and can permeate a 0.44 μm microporous membrane. Poultry species, including chickens, turkeys, quails, guinea fowls, pigeons, ducks and geese, are all susceptible to MG (11, 36). Infected animals commonly manifest clinical symptoms like coughing, nasal discharge and respiratory distress signs, and some also exhibit conjunctivitis and sinusitis, leading to decreased egg production, hatchability and feed conversion rates. *Mycoplasma gallisepticum* infection therefore poses a substantial threat to the poultry industry in China. It is reported that various mycoplasmas were capable of infecting geese and causing respiratory symptoms, encompassing *Mycoplasma gallisepticum* (38), *Mycoplasma imitans* (MI) (8) and mycoplasmas identified by their host- or tissue-specificity such as those found in geese, ducks, cloacal regions and bursal tissue (4, 9, 18, 35). The diversity of mycoplasmas causing respiratory diseases in geese has posed considerable challenges in diagnosing and treating MG infections in geese.

Because the 16S ribosomal RNA (rRNA) sequences of MG and MI are very similar and differ by only two nucleotides (as indicated by the sequences

logged under GenBank accession Nos M22441 and L24103) (16), primers designed based on the 16S rRNA sequence are not sufficiently specific to reliably distinguish between MG and MI in clinical samples, especially from waterfowl (16). The results of García *et al.* (15) showed that MG intergenic spacer region (IGSR) sequences had significant sequence variability and good discriminability among MG strains, offering a more reliable diagnostic aprroach. Furthermore, the functional proteins encoded by these sequences also present distinctive features. The choice of the protein to distinguish between MG and MI in this study fell on MGC2, a protein encoded by the MG cell adhesion gene discovered by Hnatow *et al.* (17) which is involved in the colonisation and infection of trachea mucosa.

The swift advancement of high-throughput technologies, such as microarrays and second-generation, third-generation and single-cell sequencing, has led to the extensive application of bioinformatics analysis. This application serves to identify pivotal genes and signalling pathways in diverse diseases, contributing to precise diagnoses and personalised treatment strategies. After an extensive literature review, it is evident that comprehensive sequencing and bioinformatics analyses have been conducted on only 26 chicken-origin strains of *Mycoplasma* globally, with the majority of these studies having been reported from the United States. Notably, there is a significant gap in the comprehensive genomic analysis of Chinese strains of MG. Whole-genome sequencing assumes a key role in eliciting biological information about pathogenic microorganisms, specifically contributing to molecular diagnostics, vaccine development, guidance for drug therapies and investigations into pathogenic mechanisms. Consequently, the aim of this study is to employ a combination of second-generation and third-generation whole-genome sequencing on a strain of MG derived from geese. This endeavour is aimed at predicting and annotating its gene functions, thereby establishing a fundamental basis for subsequent investigations into the pathogenicity of MG originating from geese.

Material and Methods

Sample collection. Partial air-sac tissues were obtained from five suspected *Mycoplasma gallisepticum*-infected geese on a meat goose farm located in Sanshui District, Foshan City, Guangdong Province in China.

Clinical symptoms and post-mortem examination. Clinical symptoms in diseased geese were systematically observed, and post-mortem examinations were conducted on deceased geese. Detailed records of the findings were made.

Isolation, purification and colony morphology observation of goose-origin MG. Air-sac tissues from affected geese were collected for the isolation of MG. A small amount of air-sac tissue was added to modified Frey's liquid medium (Qingdao Haibo Biotechnology Co., Qingdao, China), vortexed and then incubated at 37°C. When the medium colour changed from red to yellow, the sample was plated. Colonies exhibiting clear *Mycoplasma* characteristics were selected for cultivation and sub-culturing. The purified second-generation bacterial fluid was inoculated onto modified Frey's solid medium and incubated at 37°C in a 5% CO_2 incubator for 7 d to observe colony morphology under a microscope (Nikon, Tokyo, Japan). To rule out the possibility of erroneously studying L-form bacteria, which have certain similarities to mycoplasma, the isolated pathogen was cultured and was observed conditionally on its displaying a stable and typical "fried-egg" appearance.

Animal pathogenicity test of isolated strains. Twenty healthy 7-day-old Yangzhou geese, obtained from a goose hatching facility (Qingyuan Hongxing, Qingyuan, China), were used in the study. The geese were evenly distributed, with an equal representation of males and females. The criteria for selection included the absence of respiratory symptoms and negative results in a plate agglutination test and tracheal swab PCR. The geese were randomly divided into two groups of 10: one for inoculation and the other as a control group. A 0.5 mL sample of the third-generation bacterial fluid in the logarithmic growth phase was inoculated twice daily into the trachea of goslings in the inoculation group. Continuous monitoring of clinical symptoms was performed in the inoculation group and the control group. After 15 d, post-mortem examinations were conducted to observe pathological changes.

PCR identification. A 10 μL reaction system was prepared according to Table 1, and amplification was carried out using a PCR machine (Touch T960; JingGe Scientific Instrument, Hangzhou, China). The selection of primers was based on a thorough literature review. The intergenic spacer region primers (5'16S-IGSR-23S-3') (31), designed to differentiate between *Mycoplasma* and *Mycoplasma gallisepticum*, were synthesised by Sangon Biotech Co. (Shanghai, China)). The details of the primers are provided in Table 2. The identification of amplification products was accomplished through agarose gel electrophoresis.

Table 1. PCR reaction system for amplification of *Mycoplasma* isolates from air-sac tissue of Chinese geese inoculated with *Mycoplasma gallisepticum*

Component	Volume (μL)
TaKaRa Taq Version 2.0 plus dye	5
Forward primer (10μM)	0.3
Reverse primer (10μM)	0.3
ddH_2O	3.4
Bacteria solution	1

dd – double-distilled

Table 2. Primers used for PCR analysis of amplicons of *Mycoplasma* from air-sac tissue of Chinese geese inoculated with *Mycoplasma gallisepticum*

Species	Primer sequences 5'→3'	Fragment length (bp)	Annealing temperature (°C)
Mycoplasma gallisepticum	IGSR-F : GTAGGGCCGGTGATTGGAGTTA IGSR-R : CCCGTAGCATTTCGCAGGTTTG	792	57

bp – base pairs; IGSR – intergenic spacer region; F – forward; R – reverse

Table 3. The bioinformatics software used for the prediction and annotation of *Mycoplasma gallisepticum* strain MG-GD01/22 isolated from inoculated Chinese geese

Database	Website
NR	https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins/
SwissProt	http://www.ebi.ac.uk/swissprot/
COG	https://www.ncbi.nlm.nih.gov/research/cog-project/
GO	http://www.geneontology.org
KEGG	http://www.kegg.jp/orhttp://www.genome.jp/kegg/
VFDB	http://www.mgc.ac.cn/VFs/
CARD	http://arpcard.mcmaster.ca
PHI-base	http://www.phi-base.org/index.jsp
CAZy	http://www.cazy.org/

NR – Non-redundant Protein Database; COG – Clusters of Orthologous Groups (of proteins); GO – Gene Ontology; KEGG – Kyoto Encyclopedia of Genes and Genomes; VFDB – Virulence Factor Database; CARD – Comprehensive Antibiotic Resistance Database; PHI-base – Pathogen Host Interactions; CAZy – Carbohydrate-Active enZYmes

Strain preservation information. The identified clinical isolates were purified again, and part of the samples were sent to the Chinese Center for Type Culture Collection (CCTCC, Wuhan, China). The isolates were named *Mycoplasma gallisepticum* MG-GD01/22 according to their species classification and assigned preservation number CCTCC M 20232686.

Antimicrobial susceptibility test. A minimal inhibitory concentration (MIC) test was performed utilising an agar dilution method with slight modifications incorporating blank controls and quality controls (10). Modified Frey's solid medium with an appropriate drug concentration was prepared. The MIC of eight antibiotics were determined against the goose-origin MG-GD01/22, the antibiotics spanning four classes: enrofloxacin and danofloxacin as quinolones, spectinomycin as aminoglycosides, tilmicosin, tylosin, and acetylisovaleryltylosin tartrate as macrolides, and tiamulin and valnemulin as pleuromutilins. Bacterial fluid containing the isolate was diluted to 10^6 colony-forming units (CFU)/mL, and 100 µL of the bacterial fluid was inoculated on the drug-containing solid plates, ensuring even distribution. The plates were then incubated at 37°C in a 5% CO_2 incubator for 7 d.

Prediction and annotation of MG-GD01/22 gene functions. A 5-mL volume of MG-GD01/22 bacterial fluid was inoculated into 45 mL of modified Frey's liquid medium and cultured at 37°C to the logarithmic phase. After centrifugation at 12,000 rpm and 4°C for 30 min, the culture medium was discarded. The white precipitate was washed with ultrapure water, and the liquid was discarded after centrifugation. The collected precipitate was resuspended in 120 µL of ultrapure water and vortexed evenly. Extraction of total DNA from the MG-GD01/22 strain was achieved with a Mag-Bind Bacterial DNA 96 Kit (Omega Bio-tek, Norcross, GA, USA) as stated in the manufacturer's manual. The DNA concentration was determined by micro-UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The qualified DNA was stored at −20°C and then sent to Sangon Biotech (Shanghai, China) for third-generation whole-genome sequencing. The whole genome of MG-GD01/22 was sequenced using PacBio combined with Illumina technology, and sequence splicing was performed using Canu 1.3 (23), GapFiller v. 1.11 (7) and PrlnSeS-G 1.0.0 (29). The Prokka tool (32) was utilised to predict basic gene information from the MG-GD01/22 whole-genome sequencing results. Clustered, regularly interspaced short palindromic repeats (CRISPR) were predicted using the CRISPR Recognition Tool (6), gene islands were predicted using IslandPath-DIMOB (5), and prophage prediction analysis using PhiSpy (1). Gene function annotation was performed for the isolate. The bioinformatics software used in this study is listed in Table 3.

Comparative genomic analysis. The 16S rRNA and *mgc2* gene sequences of MG-GD01/22 were subjected to comparative analysis with those of various representative strains of MG and a single strain of MI archived in the GenBank database. The phylogenetic tree and the nucleotide sequence homology assessment of 16S rRNA and the evolutionary tree for the *mgc2* gene

were constructed by MEGA XI; FigTree and MegAlign Pro software (DNASTAR, Madison, WI, USA). A gene collinearity analysis between MG-GD01/22 and evolutionarily proximate MG representative strains was also performed using MUMmer3.1 software (25).

Results

Clinical symptoms and post-mortem findings. The affected geese manifested lethargy, unkempt feathers and decreased food consumption. Some animals presented symptoms including nasal discharge, head shaking, neck extension, open-mouth breathing and elevated body temperature. Auscultation revealed wet rales in the lungs.

Post-mortem findings. Initial dissection revealed mucus accumulation in the trachea and bronchi, pinpoint bleeding and congestion in lung tissues. A minor fibrous exudate between the thoracic and abdominal membranes and the air sac membranes was also found. Subsequent observations indicated patchy deposits on the air sacs accompanied by a degree of air-sac thickening and turbidity. In advanced stages of the disease, the air sacs contained cheesy white or yellowish substances, while the lungs and bronchi were filled with purulent secretions. Localised congestion or parenchymal consolidation was evident in lung tissues (Fig. 1A and B).

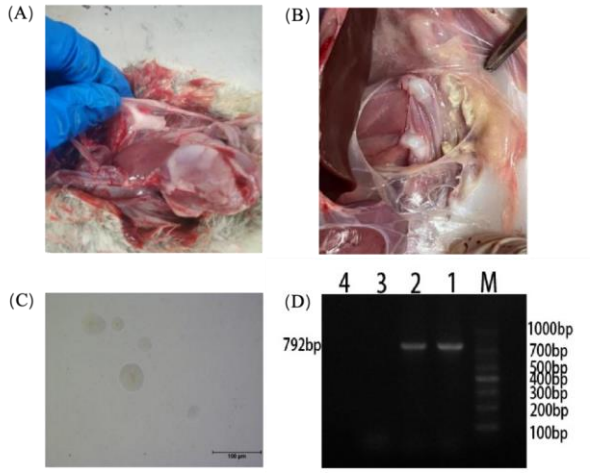

Fig. 1. Isolation and identification of goose-origin *Mycoplasma gallisepticum*. A – thickened air-sac wall of an affected goose: wall with white viscous liquid; B – blood in statis in the lung tissue of an affected goose, and large amounts of yellow and white cheese-like material in the air sacs; C – colonies of clinical MG from goose isolates (100×); D – the PCR identification result of goose-origin MG. M – DL1000 Marker; 1 – MG-GD01/22; 2 – MG S6; 3 – *Mycoplasma synoviae* strain MS wvu1853; 4 – control

Colony morphology observation results of goose-origin MG. The inoculation of the purified bacterial fluid on modified Frey's solid medium yielded mycoplasma colonies exhibiting characteristic of "fried-egg" morphology under microscopic examination (Fig. 1C). None of the bacteria identified as L-form, and the viable cell count surpassed 10^8 CFU/mL.

Identification of goose-origin MG in PCR. A distinctive band aligned with the MG S6 standard strain manifested at around 800 bp (Fig. 1D). This and the colony morphology identification methods collectively confirmed the goose-origin nature of this clinical isolate. The strain was designated as MG-GD01/22.

Pathogenic results of goose-origin MG. Animals in the infected group exhibited clinical manifestations, including open-mouthed breathing, head shaking, nasal discharge, depression and decreased food intake over a 15-day period. Necropsies revealed the nasal cavity to be filled with yellow purulent secretions, the air sacs to be slightly thickened and the existence of point-shaped and patchy light-yellow cheesy exudates. Contrastingly, animals in the control group displayed neither evident clinical symptoms nor pathological alterations (Fig. 2).

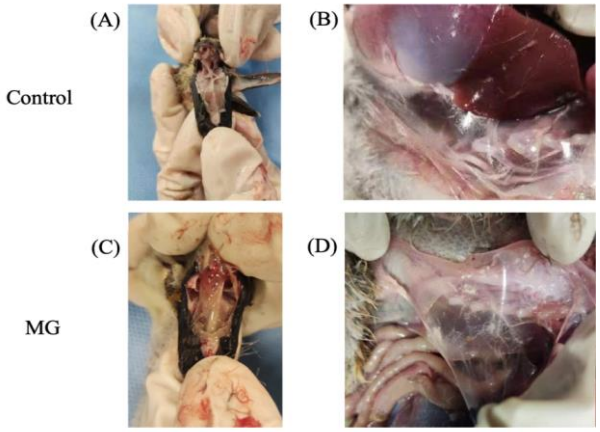

Fig. 2. Changes caused by *Mycoplasma gallisepticum* in geese. A – clean nasal cavity; B – normal, thin and transparent air sacs in a control-group goose; C – yellow, viscous secretion in the nasal cavity of an infected-group goose; D – slightly thickened air sacs withs cloudy, light-yellow cheese-like exudation in an infected-group goose

Minimum inhibitory concentration sensitivity test of MG-GD01/22. The findings suggested that MG-GD01/22 had resistance to quinolones (levofloxacin and enrofloxacin) and aminoglycosides (florfenicol) but susceptibility to macrolides (tilmicosin, tylosin and tiamulin) and pleuromutilins (thiamphenicol and valnemulin) (Table 4).

Table 4. Minimum inhibitory concentrations (MICs) of antimicrobials against a *Mycoplasma gallisepticum* reference strain (MG S6) and the isolate obtained in the experiment (MG-GD01)

Drug name	MG S6 MIC	MG-GD01 MIC
Danofloxacin	0.8	3.2
Enrofloxacin	0.4	3.2
Tilmicosin	0.2	0.4
Tylosin	0.2	0.4
Acetylisovalerylтylosin tartrate	0.1	0.3
Tiamulin	0.00625	0.0125
Valnemulin	0.00625	0.0125
Spectinomycin	0.4	3.2

Database analysis for basic information and bioinformatics prediction of MG-GD01/22. The concentration of total DNA extracted from MG-GD01/22 was measured at 147.5 ng/μL. The sample purity was evaluated by means of the OD 260 nm:OD 280 nm ratios as 1.91 and OD 260 nm:OD 230 nm ratios as 2.00, which met the prescribed standards for whole-genome sequencing. Following first-generation sequencing validation, the species origin was conclusively identified as *Mycoplasma gallisepticum*. Utilising the third-generation gene sequencing technology, the comprehensive genome length of MG-GD01/22 was determined to be 1,024,330 bp, featuring a guanine + cytosine content of 31.56%. A total of 1,666 coding genes were predicted, constituting 72.25% of the genome, complemented by 33 transfer RNAs and 6 ribosomal RNAs (Fig. 3A).

There were three genomic islands and three prophage structures were anticipated within MG-GD01/22. According to the CRISPR prediction, the repetitive sequence in MG-GD01/22 began at position 867,297 and concluded at position 869,120. The repetitive sequence spanned 36 bp, featuring an interspace of 30 bp between consecutive repetitive sequences. A total of 28 repetitive sequence structures were identified in this study (Fig. 3B).

Clusters of orthologous groups of proteins (COG) functional classification annotation. The COG system was established by the National Center for Biotechnology Information (NCBI) to annotate genes in prokaryotes for the direct homologous relationships among them. It categorises genes into three primary functional divisions. The system's evolutionary relationship clusters genes with homologous traits from diverse species into distinct groups, wherein genes within the same group exhibit like functionalities. This enables the prognostication and elucidation of the acquired gene sequences. In this study, the predicted protein sequences of genes were aligned with the COG database for annotation, and the annotated genes were subsequently classified into specific groups (Fig. 4). Successfully annotated genes were classified within the MG-GD01/22 genome. The largest gene cluster was predicted to be associated with translation, ribosomal structure and biogenesis and to comprise 174 genes involved in cellular processes and signal transduction. The next-largest category within information storage and processing genes was replication, recombination and repair, encompassing 115 genes. The most prevalent category predicted was the metabolic cluster, which included 49 genes specifically involved in energy production and conversion. Separately, another subset comprised 45 genes associated with carbohydrate transport and metabolism. Additionally, the study identified two other distinct groups of 36 genes each, one involved in nucleotide transport and metabolism, and the other in the transport and metabolism of inorganic ions. The synthesis, transport and metabolism of secondary metabolites had the fewest predicted genes with only one of each. There remained 22 genes with unknown functions (Fig. 4).

Gene ontology functional classification annotation. Gene ontology serves as an internationally standardised gene function classification system, offering a dynamically updated standard vocabulary for a thorough depiction of the characteristics of genes and their products within an organism. It encompasses three principal categories: molecular function of genes, cellular location and participation in biological processes. In this study, genes were categorised based on the GO system, and the outcomes are delineated in Fig. 5. We conducted GO classification on the acquired genes and identified the predominant gene category associated with biological processes. Among these genes, 724 were linked to metabolic processes, 694 to cellular processes, 103 to localisation, 71 to response to stimulus, 51 to biological regulation processes and 17 to biological adhesion. The next most numerous category comprised molecular function genes, predominantly catalytic activity genes (682) and binding factor genes (664). There were 122 transporter protein activity genes and 82 structural molecular activity genes, while 10 genes were associated with nucleic acid binding transcription factor activity. The category with the lowest predicted gene count was that of cell components, encompassing 577 genes predicted to be involved in cell-related processes, 244 genes related to membranes and 124 genes associated with cellular organelles (Fig. 5).

Specific function annotation. The Pathogen Host Interactions database (PHI-base), dedicated to cataloguing pathogen–host interactions, was used in this study. Annotation efforts revealed ten genes associated with loss of pathogenicity, seven genes increased the pathogen's virulence, seven genes acted as effector, three genes with negligible impact on pathogenicity, and two genes reduced virulence (Table 5).

Table 5. Pathogen Host Interactions database analysis of the *Mycoplasma gallisepticum* strain MG-GD01 isolated from inoculated Chinese geese

Taxon	Gene name	Gene number
Loss of pathogenicity	*pmc1*, *cls*	10
Increased virulence	*algT*, *gyrA*, *glyA*	7
Effector	*pstB*, *groEL*	7
Unaffected pathogenicity	*FTT0673p/prsAp*, *secA2*	3
Reduced virulence	*secA*, Calcium-transporting	2

Virulence Factor Database. The Virulence Factor Database (VFDB) collates pathogenic factors in bacteria, chlamydiae and mycoplasmas. The *setA* gene is its core, symbolising experimentally verified virulence genes. The comparison of gene sequences of this study with the VFDB led to the annotation of 25 virulence genes in MG-GD01.

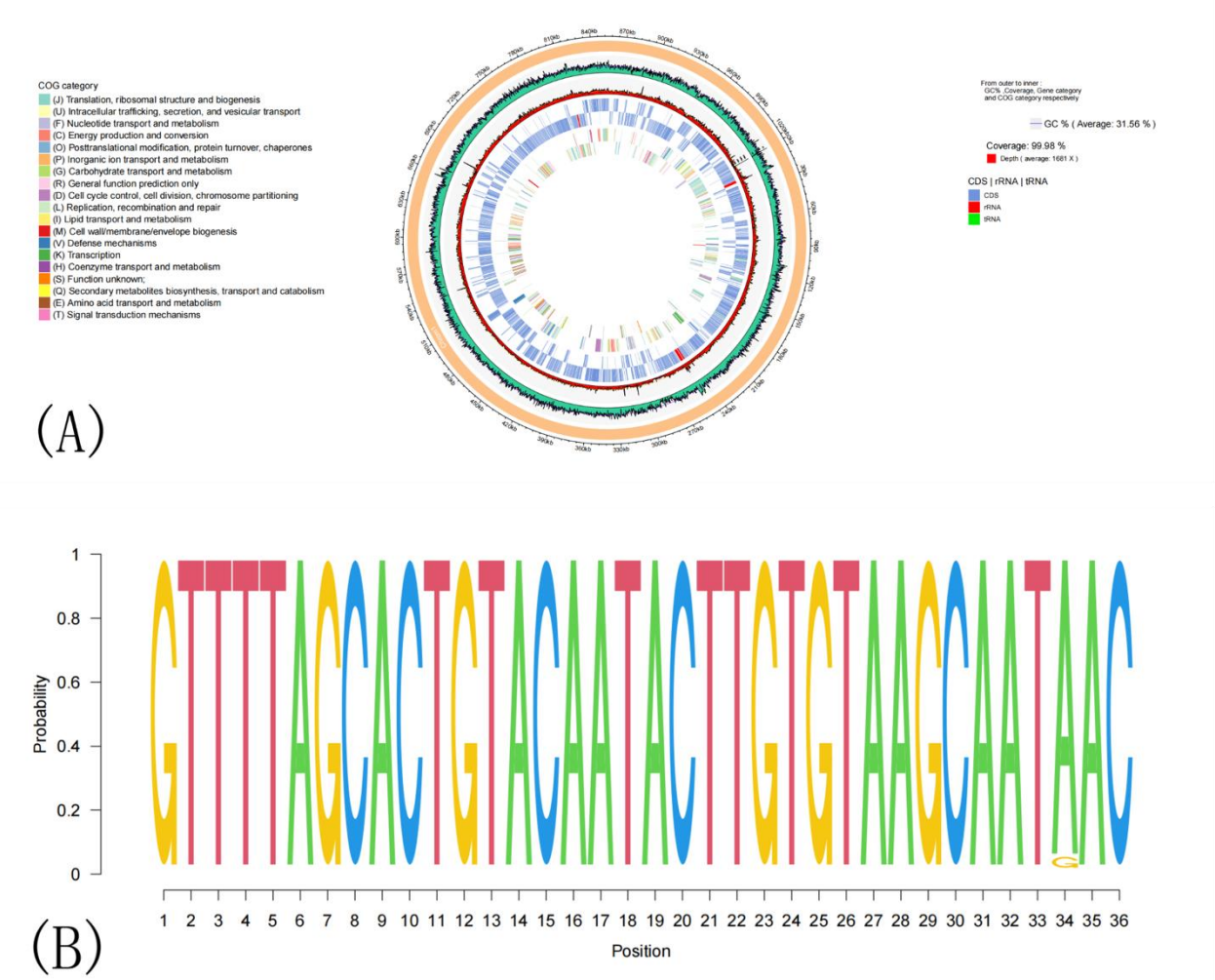

Fig. 3. Basic information and bioinformatics prediction for *Mycoplasma gallisepticum* strain MG-GD01/22 isolated from inoculated Chinese geese. A – visualisation of the MG-GD01/22 genome; B – clustered, regularly interspaced short palindromic repeat fragment SeqLogo of MG-GD01/22. COG – clusters of orthologous groups; GC – guanine + cytosine; CDS – coding sequence; rRNA – ribosomal RNA; tRNA – transfer RNA

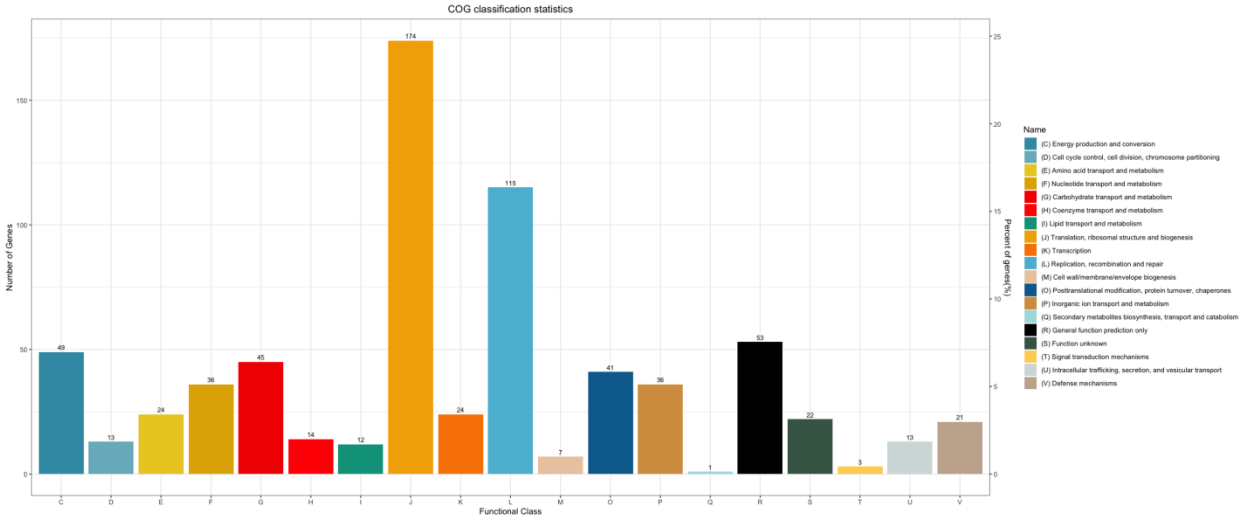

Fig. 4. Functional analysis of the *Mycoplasma gallisepticum* strain MG-GD01/22 isolated from inoculated Chinese geese based on Cluster of Orthologous Groups (of proteins) (COG) database annotation results

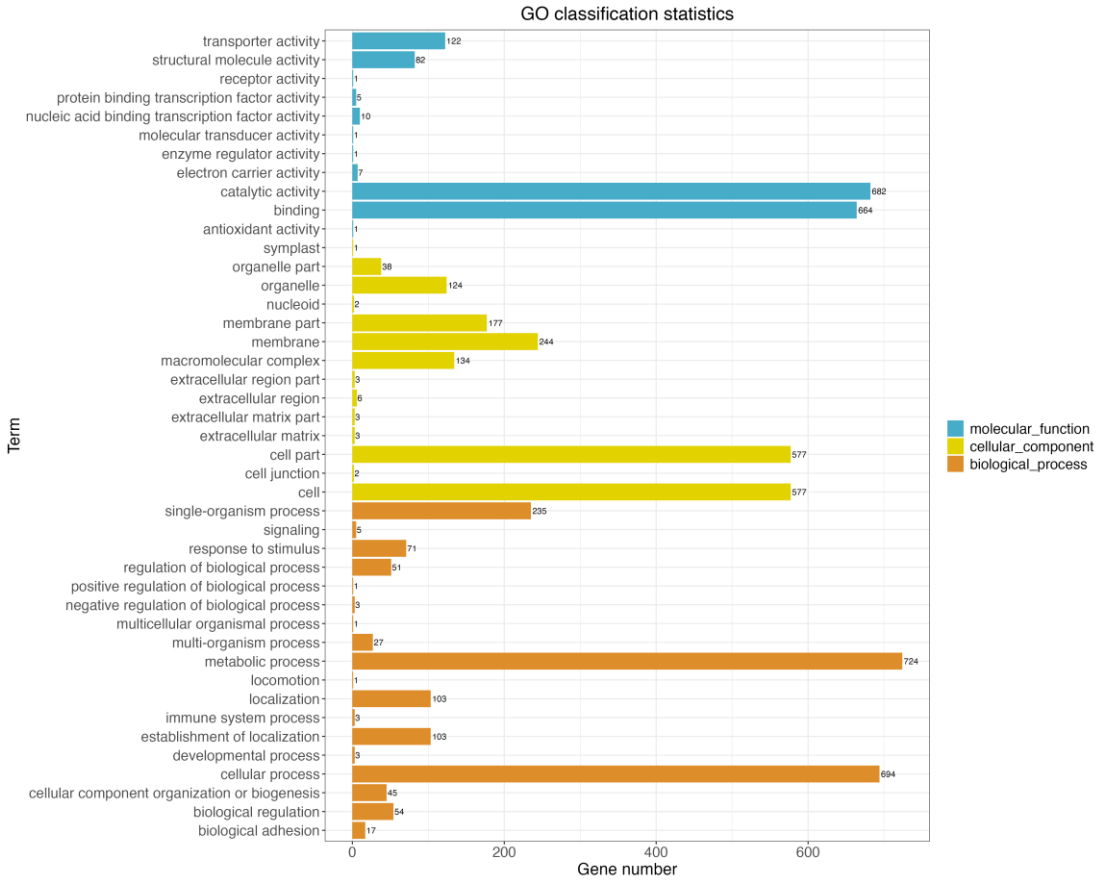

Fig. 5. Functional analysis of the *Mycoplasma gallisepticum* strain MG-GD01 isolated from inoculated Chinese geese based on the Gene Ontology (GO) database annotation results

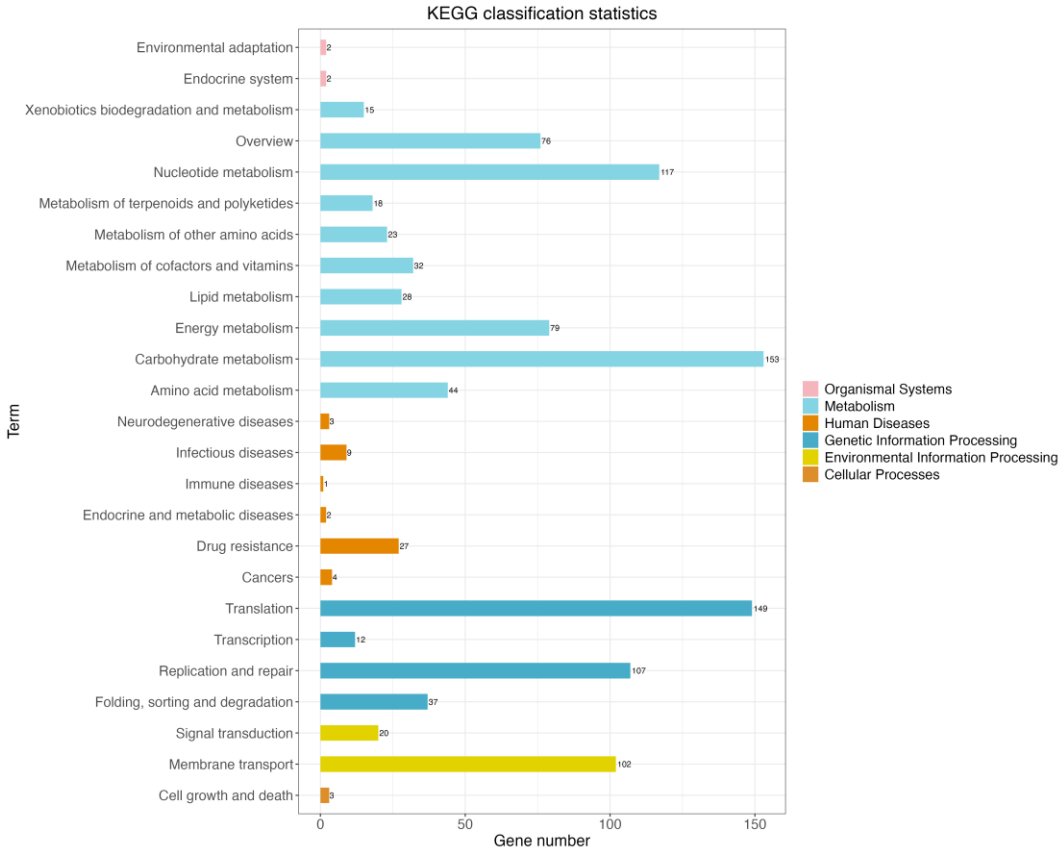

Fig. 6. Functional analysis of the *Mycoplasma gallisepticum* strain MG-GD01 isolated from inoculated Chinese geese based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database annotation results

Predominantly, these genes belonged to the category of adenosine triphosphate synthase–binding cassette transporters (48%). Additional predictions encompassed *vlhA (pMGA)*, *pvpA*, *mgc2*, *gapA/crmA*, *groEL*, *tuf*, *relA* encoding (p)ppGpp, *msrA*, *msrB* and various other virulence-related genes.

Comprehensive Antibiotic Resistance Database. The Comprehensive Antibiotic Resistance Database (CARD) is a database of bacterial antibiotic resistance genes. A total of 22 antibiotic resistance genes in MG-GD01 were identified in this study. Notably, the highest number of genes conferred resistance to quinolone antibiotics (10 genes), a lower number were associated with aminocoumarin resistance (7 genes), and a single gene was noted to confer streptomycin resistance (Table 6).

Table 6. The Comprehensive Antibiotic Resistance Database analysis of the *Mycoplasma gallisepticum* strain MG-GD01 isolated from inoculated Chinese geese

Antibiotic type	Gene name	Gene number
Fluoroquinolone	*parC, gyrB, parE, gyrA*	10
Streptomycin	*rpsL*	1
Aminocoumarin	*alaS, gyrB, parE, parY*	7
Daptomycin	*rpoB, rpoC*	3
Rifamycin	*rpoB*	2
Other antibiotics	*macB, dfrE*	2

Carbohydrate-Active enzymes database. The Carbohydrate-Active enzymes (CAZy) database encompasses six major enzyme categories: auxiliary oxidoreductases, glycoside hydrolases, glycosyltransferases, polysaccharide lyases and carbohydrate esterases. A search of the database for genes in the MG-GD01/22 strain found nine encoding proteins related to carbohydrate enzymes, including four proteins from the glycosyltransferase family 2 (carbohydrate structure domains) and two components of the pyruvate dehydrogenase complex dihydrolipoamide dehydrogenase (E3) (glycolipase). There were annotations for one gene each in polysaccharide lyases, auxiliary oxidoreductases and glycosyltransferases, with the highest proportion being proteins containing the found-in-various-architecture domain. No genes encoding proteins related to the glycoside hydrolase superfamily were annotated.

Comparative genomics analysis based on 16S rRNA. Thirty-two MG strains and one MI strain with high similarity to the 16S rRNA gene sequence (246,487 bp–247,991 bp; 1,505 bp) of MG-GD01/22 were selected by the NCBI basic local alignment search tool (BLAST) as reference strains for nucleotide sequence homology and phylogenetic tree analysis. The current isolate MG-GD01/22 differed minimally in nucleotide sequence from MG reference strains, demonstrating homology ranging from 99.4% to 99.9%. Its closest phylogenetic relationship was identified with MG S6 at 99.9%, whereas the homology with MI was comparatively lower at 95.2% (Fig. 7).

Recovery sequencing confirmed that the sequencing outcomes were entirely consistent with those of the 16S rRNA and *mgc2* gene fragments initially isolated from the strain. The evolutionary tree topology of the current strain alongside reference strains indicated MG-GD01/22's closest relationship being to the MG S6 strain and its remoteness from MI 4229 (Fig. 8).

Sequence of the *mgc2* gene. A meticulous comparative analysis was executed on the *mgc2* gene sequence (155,060 bp–156,418 bp; 1,359 bp) of MG-GD01/22. Twenty-five MG strains exhibiting high similarity were chosen through NCBI BLAST to serve as reference strains for comprehensive comparison and systematic evolutionary tree construction. The resultant evolutionary tree topology indicated the distinctive nature of MG-GD01/22's *mgc2* gene typing in comparison to the 25 established strains, albeit with only minimal differences to 3 of them (Fig. 9).

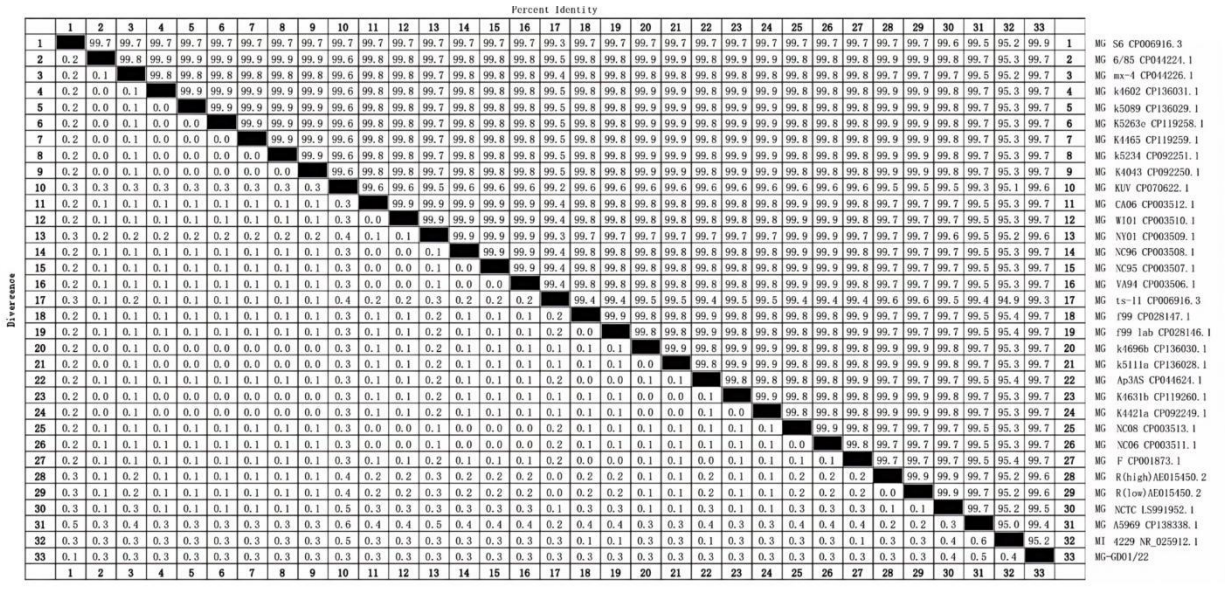

Fig. 7. Nucleotide sequence homology analysis of the 16S ribosomal RNA of the *Mycoplasma gallisepticum* strain MG-GD01/22 isolated from inoculated Chinese geese

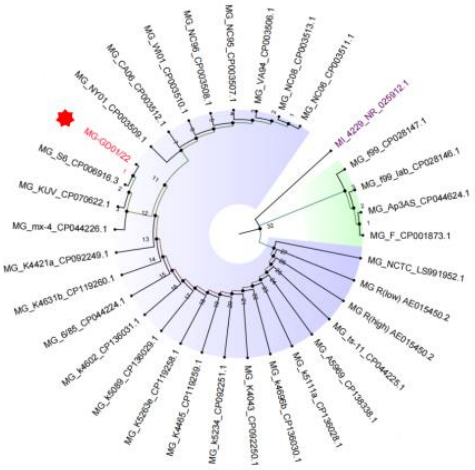

Fig. 8. Phylogenetic tree of 16S ribosomal RNA of the *Mycoplasma gallisepticum* strain MG-GD01/22 (indicated by the red star) isolated from inoculated Chinese geese

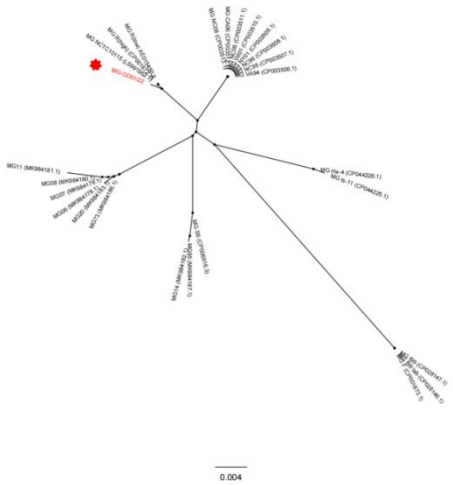

Fig. 9. Phylogenetic tree of *mgc2* of MG-GD01/22 (indicated by the red star) isolated from inoculated Chinese geese

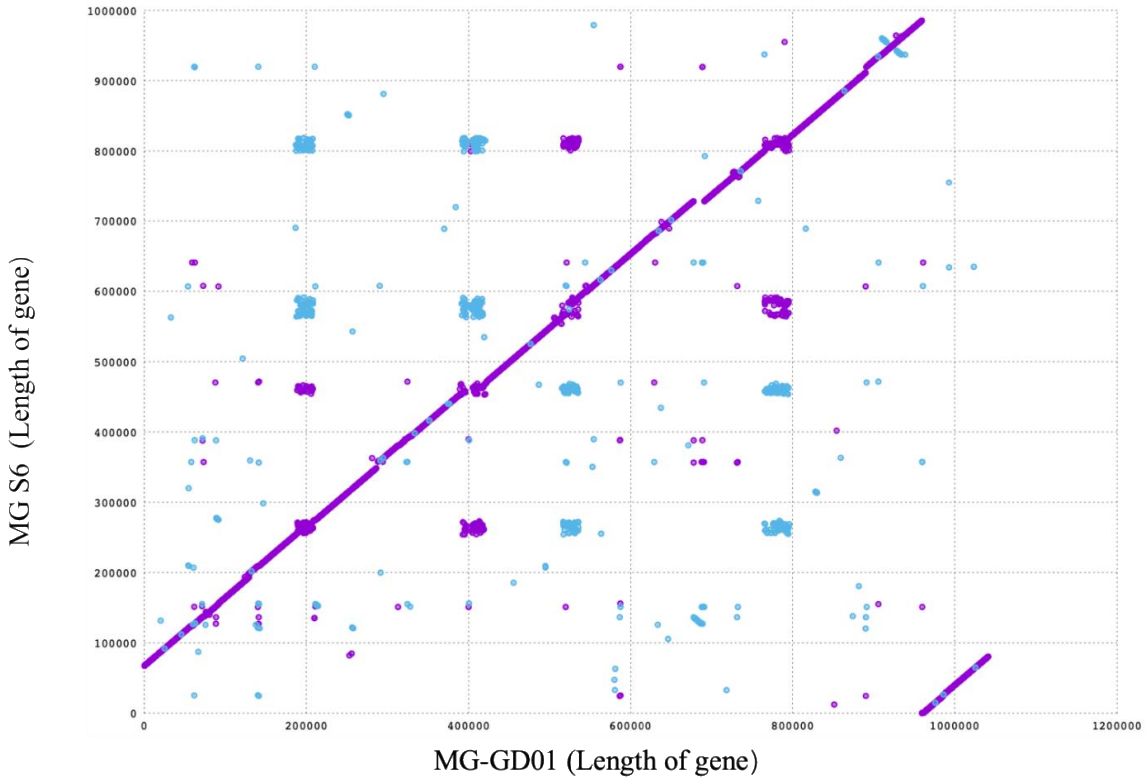

Fig. 10. Collinearity analysis of the genomes of the *Mycoplasma gallisepticum* strain MG-GD01/22 (x-axis) isolated from inoculated Chinese geese and the MG S6 strain (y-axis). Forward maximal unique matches (MUMs) are plotted as red lines/dots while reverse MUMs are plotted as blue lines/dots. A line of dots with slope = 1 represents an undisturbed segment of conservation between the two sequences, while a line of slope = −1 represents an inverted segment of conservation between the two sequences

Analysis of gene collinearity between MG-GD01/22 and MG S6. MUMmer is a suffix tree algorithm designed to find exact maximal unique matches (MUMs) of some minimum length between two input sequences. The match lists produced by MUMmer can be used alone to generate alignment dot plots, or can be passed on to the clustering algorithms for the identification of longer non-exact regions of conservation. The outcomes unveiled a substantial genomic homology between the clinical isolate and MG S6, although the collinearity was comparatively deficient. Noteworthy modifications were observed in the distribution of numerous genes, encompassing instances of gene loss and inversion (Fig. 10).

Discussion

Mycoplasma infections in poultry, notably with MG and *Mycoplasma synoviae* (MS), are significant pathogens. Among the 25 recognised species of *Mycoplasma* affecting poultry, four – MG, MS, *M. meleagridis* (MM), and *M. imitans* (MI) – are primarily considered pathogenic (13). The first two of these, which are listed by the World Organisation for Animal Health, are critical respiratory pathogens with substantial clinical and economic impacts globally (38). *Mycoplasma gallisepticum* is linked to respiratory disease, reductions in egg production and hatchability and the devaluation of carcasses at slaughter because of its systemic effects (27, 34). *Mycoplasma synoviae* causes infectious synovitis, respiratory disease, egg production losses and eggshell abnormalities in commercial poultry (12).

In 2022, research focusing on the Guangdong region reported a 42% infection rate of MG among local geese. The open-air farming conditions, complex environments and stringent growth requirements for MG complicate the pathogen's isolation, identification and detection. Consequently, epidemiological studies on *Mycoplasma* causing diseases in geese and chickens in this region lag behind. Field observations indicate that geese of all ages are susceptible, with younger geese exhibiting more severe symptoms and higher mortality rates. Adult geese often suffer chronic infections, and the disease spreads both horizontally and vertically.

Considering the high prevalence of CRD, susceptibility to other respiratory pathogens, reduced egg production, prolonged treatment durations and frequent recurrences, which collectively make for significant economic losses, it is imperative for the goose farming industry to prioritise this issue. The sector must foster the conditions for further experimental research to enhance prevention and control strategies for *Mycoplasma* infections.

In this study, IGSR primers were applied to differentiate MG-GD01/22 from other causative agents successfully, confirming the 99.9% homology of its 16S rRNA with that of MG S6, and establishing it as goose-origin MG. Because of its good specificity, *mgc2* has been widely used as target for MG diagnosis in recent years. In this study, the *mgc2* gene sequence of MG-GD01/22 was compared with that of the MG model strain in GenBank. Despite the high similarity of MG and MI, the specificity based on *mgc2* makes it possible to distinguish the two strains completely, highlighting its potential as a diagnostic target.

In this investigation, goslings were tracheally inoculated with purified MG-GD01/22. Within a 15-day period, all the birds manifested conspicuous respiratory symptoms, and post-mortem examinations exhibited substantial pathological alterations in the respiratory system, aligning with the natural MG infection observed in afflicted geese and substantiating the pathogenicity of MG-GD01/22 in geese. Numerous virulence-related genes in MG-GD01/22 were annotated by VFDB database analysis. A pivotal stage in the MG invasion process involves adhesion, succeeded by colonisation and infection. Substances such as the elongation factor heat shock protein (GroEL), adhesin (*Mycoplasma gallisepticum* cytadhesin 2, PVPA and GapA/CrmA) and haemagglutinin (VlhA) have been definitively identified as virulence factors in respiratory mycoplasmas. These components play crucial roles in host invasion and immune escape (14, 39). The study also predicted three novel virulence-related proteins in MG that have not been previously documented: elongation factor Tu (EF-Tu), methionine sulfoxide reductase (Msr), stringent response factor (RelA) and (p)ppGpp (guanosine 5'-diphosphate, 3'-diphosphate). Elongation factor-Tu is the most abundant conserved protein in bacteria (21, 30). Current studies have shown that EF-Tu is a virulence factor of bacteria which can assist *Mycoplasma pneumonia* and *Pseudomonas aeruginosa* in achieving immune escape from host cells, so that they can better infect the host (2, 3, 24). The gene expressing EF-Tu has been confirmed to encode a protein of *Mycoplasma ovipneumoniae* with strong immunogenicity (20). Methione sulphoxide reductase is a widely distributed repair enzyme in organisms, which plays a vital role in survival under oxidative stress conditions. Type A or B is the dominant reductase in most organisms. Existing research suggests that MsrA/B in *Neisseria gonorrhoeae* functions as a significant virulence factor associated with adhesion and biofilm formation and displays notable immunogenicity (19, 22, 26, 28). RelA serves as a synthetase for (p)ppGpp, catalysing the addition of pyrophosphate to another GTP or GDP at the 3′ position, which enables *Mycobacterium tuberculosis* to withstand survival pressure imposed by host macrophages, maintaining a non-proliferate state within macrophages; this state drives a gradual recovery from or relapse of tuberculosis (33). This regulatory pathway has been verified in most pathogens and is proposed as a potential antibacterial target (37). Consequently, the identified genes coding for these enzymes require further exploration and may yield novel insights into the diagnosis, prevention, and treatment of goose-origin MG.

In vitro MIC tests demonstrated MG-GD01/22's susceptibility to valnemulin, tilmicosin, tylosin, acetylisovaleryltylosin tartrate and tiamulin, but resistance to enrofloxacin, danofloxacin and spectinomycin. According to the annotation results of the CARD database search, multiple quinolone resistance genes and one streptomycin resistance gene were predicted in MG-GD01/22, which is consistent with the *in vitro* MIC results, suggesting the preferable selection of macrolides and truncated *Pleurotus* antibiotics in the clinical treatment of goose origin MG infection. The MG S6 strain, along with MG R (high) and MG R (low), are reference strains listed in the NCBI database. Comparative genomics revealed the closest relationship of MG-GD01/22 to be with MG S6 based on 16S rRNA gene typing. In contrast, based on *mgc2* gene typing, the closest relationships were with MG strain R (high), and

MG strain R (low). Gene collinearity analysis indicated poor collinearity of MG-GD01/22 with MG S6, suggesting potential structural changes due to geographical or host variations. This implied the independent evolution of goose-origin MG, warranting further comprehensive research in this domain.

Conclusion

This investigation encompassed a comprehensive exploration into the pathogenicity, drug susceptibility and whole-genome sequencing analysis of a clinical isolate, MG-GD01/22, belonging to goose-origin *Mycoplasma gallisepticum* in the Guangdong region in China. Employing a combination of second-generation and third-generation gene sequencing technologies, we found MG-GD01/22 to manifest pathogenicity in goslings and to have heightened susceptibility to lincosamides and resistance to enrofloxacin, danofloxacin and doxycycline. The complete genome of MG-GD01/22 spanned 1,024,330 bp with a GC content of 31.56%. Gene annotations conducted in the COG, GO, and KEGG databases identified 703, 1,012, and 530 genes, respectively.

Virulence Factor Database analysis hinted at the presence of various virulence-related factors in MG-GD01/22, potentially influencing invasion mechanisms and inducing host damage. The findings from the CARD annotation aligned with the antimicrobial susceptibility test. Notably, the study pinpointed the *mgc2* gene in MG-GD01/22 as a promising molecular diagnostic target. Furthermore, comparative genomics analysis with the MG S6 genome showed significant distinctions, which deserve further scrutiny and exploration.

Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this article.

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References

1. Akhter S., Aziz R.K., Edwards R.A.: PhiSpy: a novel algorithm for finding prophages in bacterial genomes that combines similarity- and composition-based strategies. Nucl Acids Res 2012, 40, e126, doi: 10.1093/nar/gks406.
2. Balasubramanian S., Kannan T.R., Baseman J.B.: The surface-exposed carboxyl region of *Mycoplasma pneumoniae* elongation factor Tu interacts with fibronectin. Infec Immun 2008, 76, 3116–3123, doi: 10.1128/iai.00173-08.
3. Barel M., Hovanessian A.G., Meibom K., Briand J.-P., Dupuis M., Charbit A.: A novel receptor - ligand pathway for entry of *Francisella tularensis* in monocyte-like THP-1 cells: interaction between surface nucleolin and bacterial elongation factor Tu. BMC Microbiology 2008, 8, doi: 10.1186/1471-2180-8-145.
4. Benoina D., Tadina T., Dorrer D.: Natural infection of geese with *Mycoplasma gallisepticum* and *Mycoplasma synoviae* and egg transmission of the mycoplasmas. Avian Pathol 1988, 17, 925–928, doi: 10.1080/03079458808436514.
5. Bertelli C., Brinkman F.S.L.: Improved genomic island predictions with IslandPath-DIMOB. Bioinformatics 2018, 34, 2161–2167, doi: 10.1093/bioinformatics/bty095.
6. Bland C., Ramsey T.L., Sabree F., Lowe M., Brown K., Kyrpides N.C., Hugenholtz P.: CRISPR Recognition Tool (CRT): a tool for automatic detection of clustered regularly interspaced palindromic repeats. BMC Bioinformatics 2007, 8, 209, doi: 10.1186/1471-2105-8-209.
7. Boetzer M., Pirovano W.: Toward almost closed genomes with GapFiller. Genome Biol 2012, 13, doi: 10.1186/gb-2012-13-6-r56.
8. Bradbury J.M., Abdul-Wahab O.M., Yavari C.A., Dupiellet J.P., Bove J.M.: *Mycoplasma imitans* sp. nov. is related to *Mycoplasma gallisepticum* and found in birds. Int J Syst Bacteriol 1993, 43, 721–728, doi: 10.1099/00207713-43-4-721.
9. Carnaccini S., Ferguson-Noel N.M., Chin R.P., Santoro T., Black P., Bland M., Bickford A.A., Senties-Cué C.G.: A Novel *Mycoplasma* sp Associated with Phallus Disease in Goose Breeders: Pathological and Bacteriological Findings. Avian Dis 2016, 60, 437–443, doi: 10.1637/11309-102315-RegR.
10. Clinical and Laboratory Standards Institute: M100-S25: Performance Standards for Antimicrobial Susceptibility Testing; 25th Informational Supplement. Wayne, PA, USA, 2015.
11. Çelebi O., Özdemir U., Büyük F., Ünsal Baca A., Erpek S.H., Karahan M., Otlu S., Şahin M., Coskun M.R., Celik E., Sağlam G.A., Büyük E., Akca D.: Isolation of *Mycoplasma* spp. from Geese with Pneumonia and Identification of Microbial Isolates via Molecular Methods. Braz J Poultry Sci 2022, 24, doi: 10.1590/1806-9061-2021-1522.
12. Feberwee A., de Wit S., Dijkman R.: Clinical expression, epidemiology, and monitoring of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*: an update. Avian Pathol 2022, 51, 2–18, doi: 10.1080/03079457.2021.1944605.
13. Ferguson-Noel N., Armour N.K., Noormohammadi A.H., El Gazzar M., Bradbury J.M.: Chapter21, Mycoplasmosis. In: *Diseases of Poultry*, Volume 2, 14th Edition, edited by D.E. Swayne, M. Boulianne, C.M. Logue, L.R. McDougald, N. Venugopal, D.L. Suarez, S. de Wit, T. Grimes, D. Johnson, T.Y. Prajitno, I. Rubinoff, G. Zavala, John Wiley & Sons, Hoboken, NY, USA, 2020, pp. 907–965.
14. Fu P., Wang F., Zhang Y., Qiao X., Zhang Y., Zhou W., Yan X., Wu W.: The application of aptamer Apt-236 targeting PvpA protein in the detection of antibodies against *Mycoplasma gallisepticum*. Anal Methods 2021, 13, 3068–3076, doi: 10.1039/d1ay00515d.
15. García M., Ikuta N., Levisohn S., Kleven SH.: Evaluation and Comparison of Various PCR Methods for Detection of *Mycoplasma gallisepticum* Infection in Chickens. Avian Dis 2005, 49, 125–132, doi: 10.1637/7261-0812204r1.
16. Harasawa R., Pitcher D.G., Ramírez A.S., Bradbury J.M.: A putative transposase gene in the 16S-23S rRNA intergenic spacer region of *Mycoplasma imitans*. Microbiology-Sgm 2004, 150, 1023–1029, doi: 10.1099/mic.0.26629-0.
17. Hnatow L.L., Keeler C.L., Jr., Tessmer L.L., Czymmek K., Dohms J.E.: Characterization of MGC2, a *Mycoplasma gallisepticum* cytadhesin with homology to the *Mycoplasma pneumoniae* 30-kilodalton protein P30 and *Mycoplasma genitalium* P32. Infect Immun 1998, 66, 3436–3442, doi: 10.1128/iai.66.7.3436-3442.1998.

18. Ivanics E., Glavitis R., Takacs G., Molnar E., Bitay Z., Meder M.: An outbreak of *Mycoplasma anatis* infection associated with nervous symptoms in large-scale duck flocks. Zentralbl Veterinaermed B 1988, 35, 368–378, doi: 10.1111/j.1439-0450.1988.tb00509.x.
19. Jen F.E.C., Semchenko E.A., Day C.J., Seib K.L., Jennings M.P.: The *Neisseria gonorrhoeae* Methionine Sulfoxide Reductase (MsrA/B) Is a Surface Exposed, Immunogenic, Vaccine Candidate. Front Immunol 2019, 10, 137, doi: 10.3389/fimmu.2019.00137.
20. Jiang F., He J., Navarro-Alvarez N., Xu J., Li X., Li P., Wu W.: Elongation Factor Tu and Heat Shock Protein 70 Are Membrane-Associated Proteins from *Mycoplasma ovipneumoniae* Capable of Inducing Strong Immune Response in Mice. PLoS One 2016, 11, e0161170, doi: 10.1371/journal.pone.0161170.
21. Jonak J.: Bacterial elongation factors EF-Tu, their mutants, chimeric forms, and domains: isolation and purification. J Chromatography B 2007, 849, 141–153, doi: 10.1016/j.jchromb.2006.11.053.
22. Kim S., Lee K., Park S.-H., Kwak G.-H., Kim M.S., Kim H.-Y., Hwang K.Y.: Structural Insights into a Bifunctional Peptide Methionine Sulfoxide Reductase MsrA/B Fusion Protein from *Helicobacter pylori*. Antioxidants 2021, 10, 389, doi: 10.3390/antiox10030389.
23. Koren S., Walenz B.P., Berlin K., Miller J.R., Bergman N.H., Phillippy A.M.: Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res 2017, 27, 722–736, doi: 10.1101/gr.215087.116.
24. Kunert A., Losse J., Gruszin C., Huehn M., Kaendler K., Mikkat S., Volke D., Hoffmann R., Jokiranta T.S., Seeberger H., Moellmann U., Hellwage J., Zipfel P.F.: Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. J Immunol 2007, 179, 2979–2988, doi: 10.4049/jimmunol.179.5.2979.
25. Kurtz S., Phillippy A., Delcher A.L., Smoot M., Shumway M., Antonescu C., Salzberg S.L.: Versatile and open software for comparing large genomes. Genome Biol 2004, 5, R12, doi: 10.1186/gb-2004-5-2-r12.
26. Larribe M., Taha M.K., Topilko A., Marchal C.: Control of Neisseria gonorrhoeae pilin gene expression by environmental factors: Involvement of the *pilA/pilB* regulatory genes. Microbiology 1997, 143, 1757–1764, doi: 10.1099/00221287-143-5-1757.
27. Levisohn S., Kleven S.: Avian mycoplasmosis (*Mycoplasma gallisepticum*). Rev Sci Tech Off Int Epiz 2000, 19, 425–442, doi: 10.20506/rst.19.2.1232.
28. Li T., Li Z., Chen F., Liu X., Ning N., Huang J., Wang H.: Eukaryotic-like Kinase Expression in Enterohemorrhagic *Escherichia coli*: Potential for Enhancing Host Aggressive Inflammatory Response. J Infect Dis 2017, 216, 1150–1158, doi: 10.1093/infdis/jix160.
29. Massouras A., Hens K., Gubelmann C., Uplekar S., Decouttere F., Rougemont J., Cole S.T., Deplancke B.: Primer-initiated sequence synthesis to detect and assemble structural variants. Nat Methods 2010, 7, 485–486, doi.: 10.1038/nmeth.f.308.
30. Mayer F.: Cytoskeletons in prokaryotes. Cell Biol Int 2003, 27, 429–438, doi: 10.1016/s1065-6995(03)00035-0.
31. Raviv Z., Callison S., Ferguson-Noel N., Laibinis V., Wooten R., Kleven S.H.: The *Mycoplasma gallisepticum* 16S-23S rRNA intergenic spacer region sequence as a novel tool for epizootiological studies. Avian Dis 2007, 51, 555–560, doi: 10.1637/0005-2086(2007)51[555:Tmgsri]2.0.Co;2.
32. Seemann T.: Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014, 30, 2068–2069, doi: 10.1093/bioinformatics/btu153.
33. Song T., Fu Y.-R., Yi Z.-J.: Bioinformatics analysis of the structure and function of the regulatory protein RelA of *Mycobacterium tuberculosis*. J Pathogen Biol 2017, 12, 402–406, doi: 10.13350/j.cjpb.170505.
34. Stipkovits L., Kempf I.: Mycoplasmoses in poultry. Rev Sci Tech Off Int Epiz 1996, 15, 1495–1525, doi: 10.20506/rst.15.4.986.
35. Stipkovits L., Szathmary S.: Mycoplasma infection of ducks and geese. Poultry Sci 2012, 91, 2812–2819, doi: 10.3382/ps.2012-02310
36. Türkyılmaz S., Çöven F., Eskiizmirliler S.: Detection of antibodies produced in quails (*Coturnix coturnix japonica*) against *Mycoplasma gallisepticum* with different serological tests. Turk J Vet Ani Sci 2007, 31, 267–270.
37. Wilkinson R.C., Batten L.E., Wells N.J., Oyston P.C.F., Roach P.L.: Biochemical studies on *Francisella tularensis* RelA in (p) ppGpp biosynthesis. Bioscience Rep 2015, 35, e00268, doi: 10.1042/bsr20150229.
38. Yadav J.P., Tomar P., Singh Y., Khurana S.K.: Insights on *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection in poultry: a systematic review. Animal Biotechnol 2022, 33, 1711–1720, doi: 10.1080/10495398.2021.1908316.
39. Zhang. L., Chen Y., Zhu K., Zhao Y., Pan Q., Hao W., Yu Y., Xin J.: NF-κB signaling pathway regulates IL-1β production in DF-1 cells exposed to *Mycoplasma gallisepticum* GroEL protein. Chin J Prev Vet Med 2019, 41, 223–228.