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RESEARCH ARTICLE

Genomic Analysis and Evaluation of Pathogenicity and Immunogenicity of *Riemerella anatipestifer* Isolates from Chickens in China

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ABSTRACT

Riemerella anatipestifer (*R. anatipestifer*) infection is a significant cause of duck serositis, characterized by high mortality and resulting in substantial economic losses globally. Waterfowl, turkeys and wild birds have traditionally been recognized as reservoirs for *R. anatipestifer*. However, recent reports indicate an increasing incidence of infections in chickens. This study presents the whole-genome analysis of the RA-C01 strain isolated from broiler chickens in China. Phylogenetic analysis revealed that this strain clusters within a small branch alongside the HXb2 strain of duck origin and within a larger branch with the 20190604J2-1 strain of chicken origin. The results of the antibiotic susceptibility test (K-B method) showed that the RA-C01 strain was susceptible to Ceftiofur sodium, Florfenicol and Levofloxacin. Animal infection experiments demonstrated that the RA-C01 strain induces lethargy and lameness in SPF chickens without causing mortality, while pathological examination revealed pericarditis, perihepatitis, air sacculitis, peritonitis, purulent exudation in joint and keel bone. The pathogenicity of the RA-C01 strain in ducks was found to be mild. SPF chickens immunized with an inactivated vaccine derived from the RA-C01 strain were challenged 14 days post-immunization. Results indicated that the immunized group exhibited no clinical or pathological symptoms upon challenge. This study establishes a stable *R. anatipestifer* infection model in chickens and demonstrates its immunogenic potential for the first time, providing valuable insights for future vaccine development.

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INTRODUCTION

Riemerella anatipestifer infection, also referred to as duck infectious serositis, is a highly contagious bacterial disease caused by *Riemerella anatipestifer* (*R. anatipestifer*) (Eleazer *et al.*, 1973). This pathogen induces characteristic lesions such as pericarditis, perihepatitis, air sacculitis, and meningitis in infected ducks (Sawicka-Durkalec *et al.*, 2023). The disease is widely distributed in the worldwide, exhibiting high infection and mortality rates, making it one of the most significant bacterial diseases threatening the waterfowl farming industry (Pathanasophon *et al.*, 1995).

R. anatipestifer is a Gram-negative bacterium classified under the genus *Riemerella* within the family *Flavobacteriaceae*. Based on antigenic differences, *R. anatipestifer* can be divided into distinct serotypes. To date, a total of 21 serotypes has been officially recognized globally (Liu *et al.*, 2013; Pathanasophon *et al.*, 1995). *R. anatipestifer* primarily affects waterfowl, turkeys, and wild birds (Glunder and Hinz, 1989; Gyuris *et al.*, 2017). In recent years, some cases of *R. anatipestifer* infections in chickens have been reported in China (Chen *et al.*, 2024; Li *et al.*, 2011), Australia (Omaleki *et al.*, 2021) and Greece (Tzora *et al.*, 2021).

Currently, the primary approach for controlling *R. anatipestifer* infection in chickens involves the combined use of multiple antibiotics. However, the frequent application of antibiotics may elevate the risk of developing drug-resistant strains (Xihui *et al.*, 2023). Therefore, vaccination is currently regarded as the most effective strategy for controlling the disease in ducks. Inactivated bacterins (Liang *et al.*, 2024; Liu *et al.*, 2013), live attenuated vaccines (Kang *et al.*, 2018; Yang *et al.*, 2023) and egg yolk immunoglobulin Y (Yang *et al.*, 2020) have all been reported to effectively prevent the disease.

In this study, we clinically extracted a substantial quantity of pus from the leg joints of adult white-feathered broilers exhibiting limping symptoms and isolated a strain of *R. anatipestifer*. We conducted comprehensive genome-wide characterization, analyzed its drug resistance profile, and evaluated its pathogenicity in specific pathogen-free (SPF) chickens and ducks. Additionally, we investigated the immune efficacy of an inactivated vaccine derived from this strain when used to immunize SPF chickens, thereby providing insights and references for the prevention and control of *R. anatipestifer* infections in chicken flocks.

MATERIALS AND METHODS

Bacterial isolation and identification: Pus samples from infected chicken joints were aseptically streaked onto tryptic soy agar (TSA) supplemented with 5% serum and incubated anaerobically at 37°C. Colonies were screened via Gram's staining and PCR amplification of the 16S rRNA gene using primers 16s-F (ACGAATTTCCGTTCCGAGA) and 16s-R (TCGGCAAAACTAACCGTCCCA). Antibiotic susceptibility (Ceftiofur, Amikacin, Gentamicin, Florfenicol, Spectinomycin etc.) (Microbial Reagent Co., Ltd, Hangzhou, China) was assessed via disk diffusion test (K-B method).

Genomic sequencing and comparative analysis: Strain RA-C01 was sequenced by Tsingke Biotech (Beijing, China) using a combined strategy of Illumina NovaSeq 6000 (Illumina, San Diego, CA) and Oxford Nanopore PromethION (Oxford Nanopore Technologies, Oxford, UK). Reads were assembled using Canu v1.5 and circularized with Circlator v1.5.5. Comparative genomic against strains S63 and 20190904J2-1 utilized progressive mauve alignment (Darling *et al.*, 2004). Resistance genes were predicted via the CARD database (Alcock *et al.*, 2023). The multilocus sequence typing (MLST) was performed by using MLST database website (pubMLST.org) (Jolley *et al.*, 2018) and minimum spanning tree construction by Phyloviz 2.0 (Nascimento *et al.*, 2017) defined phylogenetic relationships. In pan-genome analysis, all genome sequences used were annotated or reannotated by Prokka (Seemann, 2014) to ensure consistent, pan-genome analysis was performed with Roary (Page *et al.*, 2015). The core genome was sequenced by Seqkit2 (Shen *et al.*, 2024) and aligned by MAFFT (Katoh *et al.*, 2019; Kuraku *et al.*, 2013), then Fasttree (Price *et al.*, 2009) was used for alignment phylogenic analysis, and the phylogenic tree was beautified with iTOL (Letunic and Bork, 2019).

Animals and housing: Specific pathogen-free (SPF) chickens (Beijing Boehringer Ingelheim, Beijing, China) and ducklings (Shandong Haotai, Jinan, Shandong, China) were housed in isolated facilities with ad libitum feed/water. Protocols adhered to Chinese National Standard GB/T 42011-2022 and approved by the Institutional Ethics Committee of The National Animal Health Products for Engineering Technology Research Center (GCZX RD YF046-1.0, 16 October 2024). Humane endpoints (inability to eat/drink) were enforced, with euthanasia via injectable agents.

Experimental infection of SPF chickens and ducklings: Twenty 21-day-old SPF chickens/ducklings were randomized into infection (n=10) and PBS control (n=10) groups. Infection groups received 0.5 mL RA-C01 (1×10^9 cfu, i.m.). Clinical symptoms and lesions were monitored for 7 days post-challenge.

Immunogenicity test of the RA-C01 strain on SPF chickens: The inactivated vaccine was prepared by emulsifying the inactivated RA-C01 strain(1×10^{10}CFU) with a Marcol 52 white oils (ExxonMobil, USA) adjuvant at a 1:2 ratio. And administered subcutaneously (0.3 mL, s.c.) to 7-day-old SPF chickens (n=10). Controls (n=10) received no vaccine. Fourteen days post-vaccination, all chickens were challenged with 0.5 mL of RA-C01 (1×10^9 cfu, i.m.). Clinical symptoms and lesions were evaluated over 7 days.

RESULTS

Isolation and genomic characterization of R. anatipestifer RA-C01: *R. anatipestifer* strains were isolated from the joint pus of adult broilers exhibiting symptoms of joint swelling and cultured on TSA plates supplemented with 5% serum (Fig. 1A). PCR analysis confirmed that all selected monoclonal isolates were positive (Fig. 1B) and Gram's staining identified a gram-negative brevibacterium (Fig. 1C), designated as strain RA-C01, which was subsequently utilized for further investigation.

Genome sequencing revealed that the chicken RA-C01 strain has a genome length of 2,276,804 bp with a GC content of 34.99% (Fig. 2). Using Prodigal v2.6.3 software, 2,133 functional genes were predicted, totaling 2,041,611 bp in length with an average gene length of 957 bp (Table S1). Additionally, 49 non-coding RNAs were predicted, including 40 tRNAs and 9 rRNAs. Eighteen clustered regularly interspaced palindromic repeats (CRISPR) regions were identified using CRT v1.2 software (Table S2). The RA-C01 genome contains three genomic islands (Table S3) and three prophages (Table S4).

RanA, RanB, aadS, vanT, FloR, tetX and ErmF were the 7 antibiotic-resistance genes predicted by CARD database. The results of antibiotic susceptibility test (K-B method) showed that the RA-C01 strain was Susceptible to Ceftiofur sodium, Florfenicol and Levofloxacin, intermediate to Spectinomycin and Doxycycline, resistant to Amikacin, Neomycin, Erythromycin, Polymyxin, Amoxicillin, Lincomycin, Enrofloxacin, Trimethoprim.

Complete genome comparison and evolutionary analysis of R. anatipestifer RA-C01: To identify homologous sequences, we retrieved the complete genomes of 56 R. anatipestifer strains from the NCBI database (Table S5). Phylogenetic analysis revealed that R. anatipestifer RA-C01 forms a small clade with the duck-origin HXb2 strain and clusters within a larger clade containing the chicken-origin 20190604J2-1 strain, while exhibiting a distant phylogenetic relationship with the S63 strain of another chicken origin (Fig. 3). Based on the analysis of MLST data, three R. anatipestifer strains isolated from chickens belonged to distinct sequence types, while 12 strains, including R-3 in the MLST database of the RA-C01 strain, shared the same sequence type (Fig. 4).

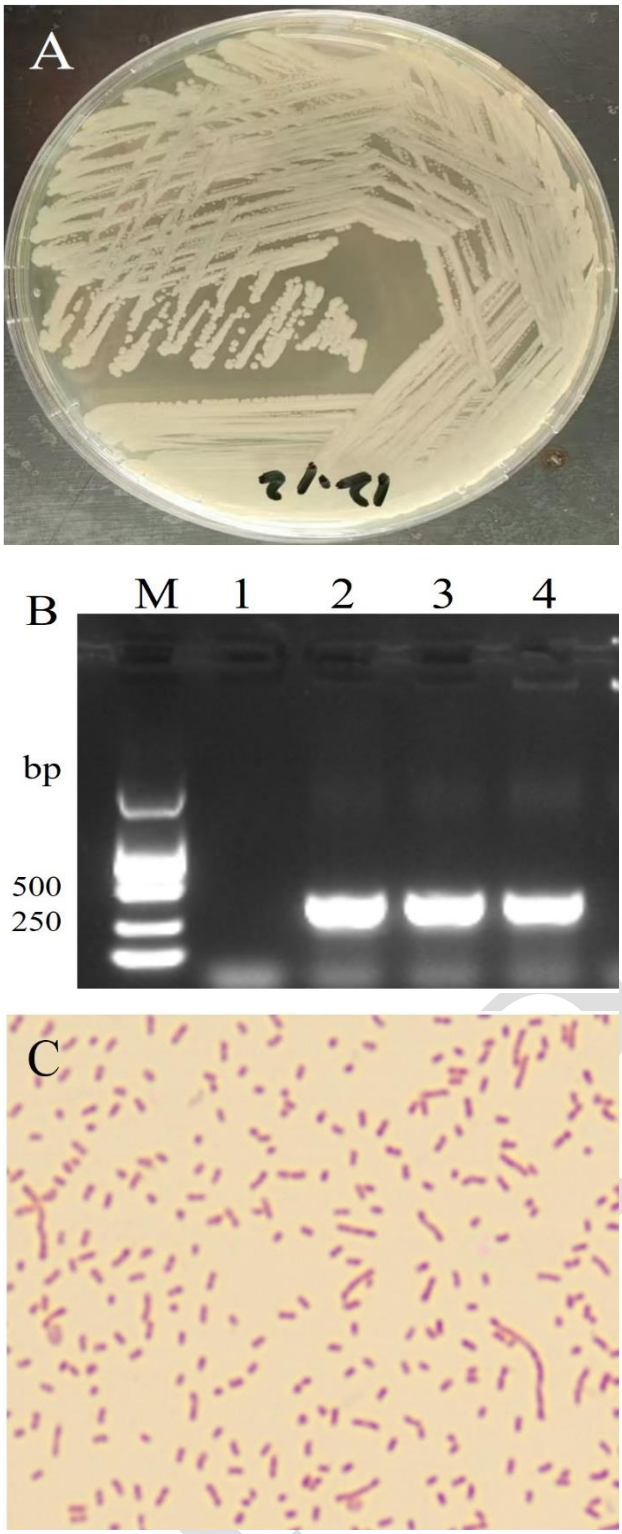

Fig. 1: Isolated *R. anatipestifer* RA-C01. (A) Colony formation of RA-C01.(B) *R. anatipestifer* was identified with PCR methods. Lane 1 is control group, lane 2 represents positive DNA sample. (C) Gram staining of RA-C01.

The genome of R.anatipestifer RA-C01 、20190604J2-1 and S63, which were isolated from chickens, were compared by mauve software, and results indicated that S63 and 20190604J2-1 shared nine local collinear blocks; however, RA-C01 was deficient in one of these blocks, which spans approximately 18,000bp (Fig. 5A). Easyfig software (Sullivan et al., 2011) was used to compare this region, there are 15 predicted protein fragments, including YecR-like lipoprotein, DUF805 domain-containing protein, helix-turn-helix domain, AAA domain-containing protein, ATP-dependent endonuclease, AAA family ATPase, ATP-dependent helicase, DUF3871 family protein, ATP-binding protein, and site-specific integrase (Fig. 5B).

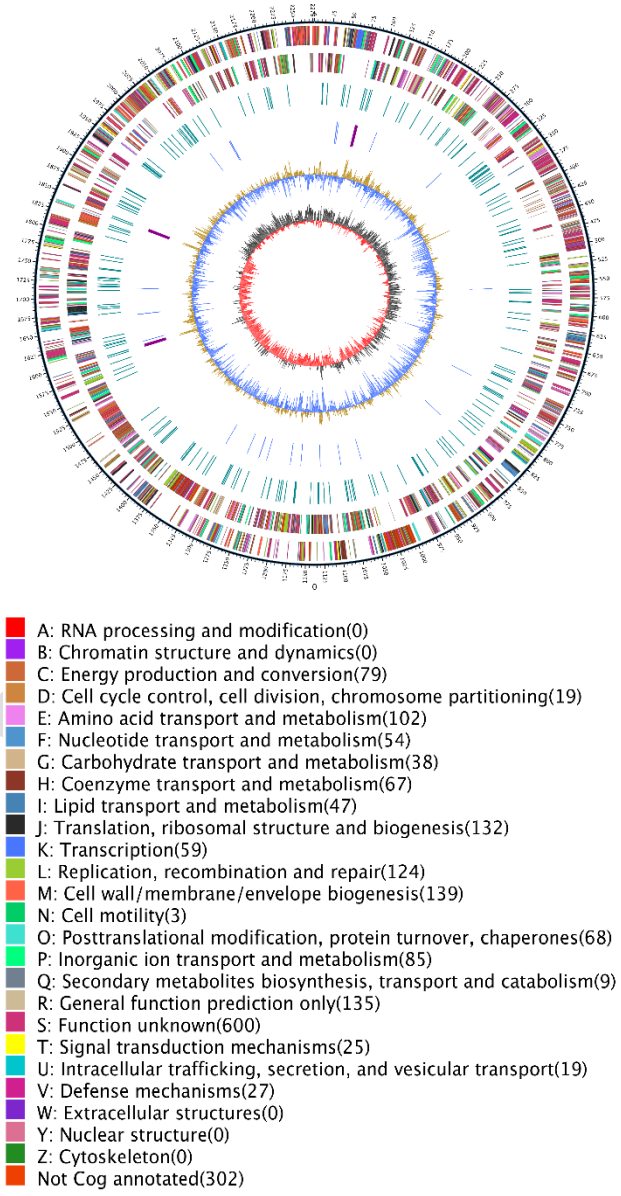

Fig. 2: The complete genome of RA-C01. The outermost circle indicates the size of the genome; each scale is 5 Kb.

Pathogenicity of R. anatipestifer RA-C01 to SPF chickens: Twenty-four hours after SPF chickens were infected with *R. anatipestifer* RA-C01, they exhibited clinical signs including lying on the ground, ruffled feathers, and lethargy. These symptoms persisted until the fifth day post-infection and subsequently subsided. No mortalities were observed in the SPF chickens. A pathological autopsy conducted seven days post-infection revealed the following lesions in the SPF chickens: severe pericarditis (90%), air sacculitis (60%), purulent exudates in leg joints (60%), severe peritonitis (50%), purulent exudates in the keel bone (20%), and hepatic fibrinous exudation (10%) (Fig. 6A-F).

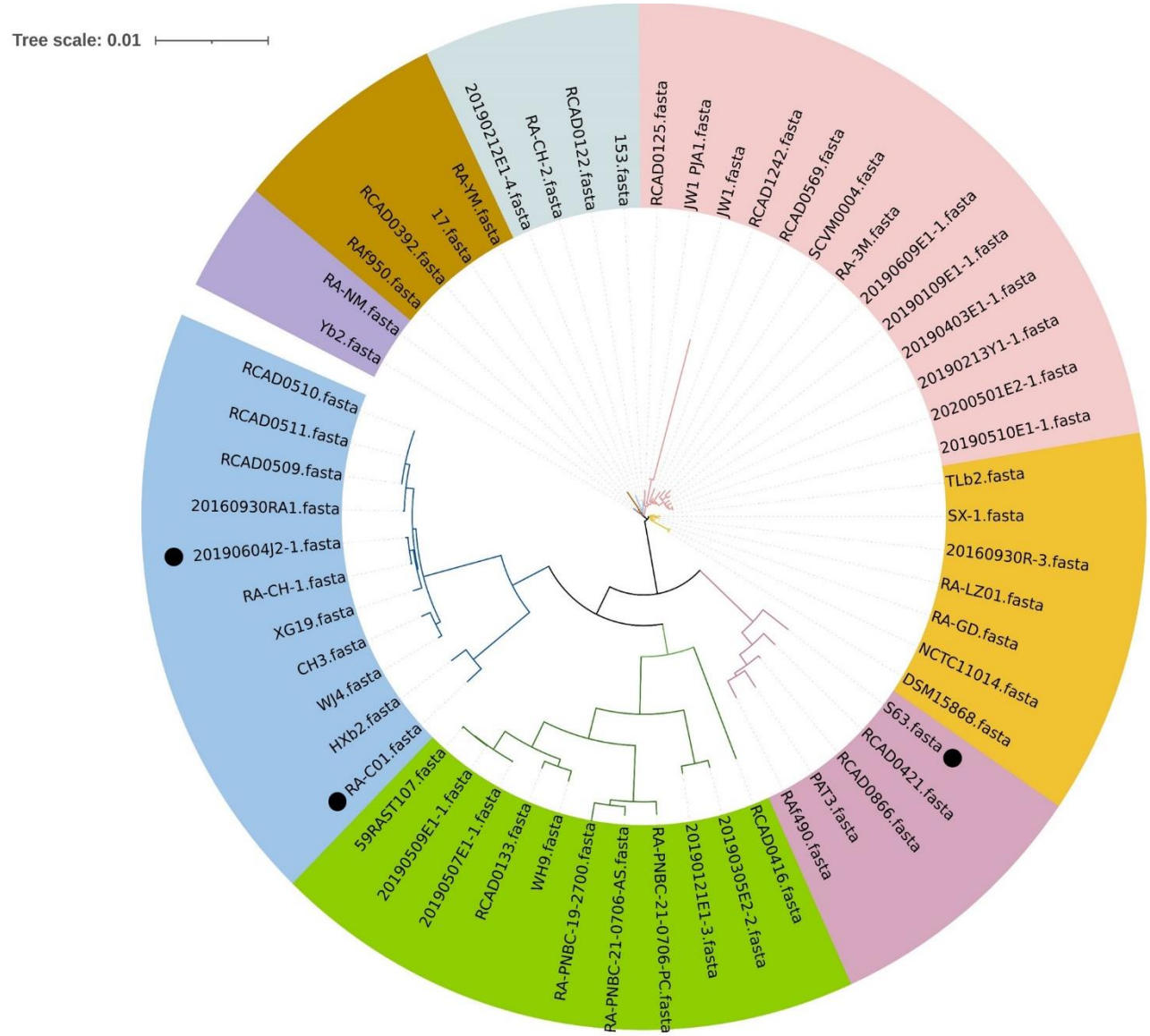

Fig. 3: Phylogenetic tree based on complete sequences of 56 *R. anatipestifer* strains in the NCBI database.

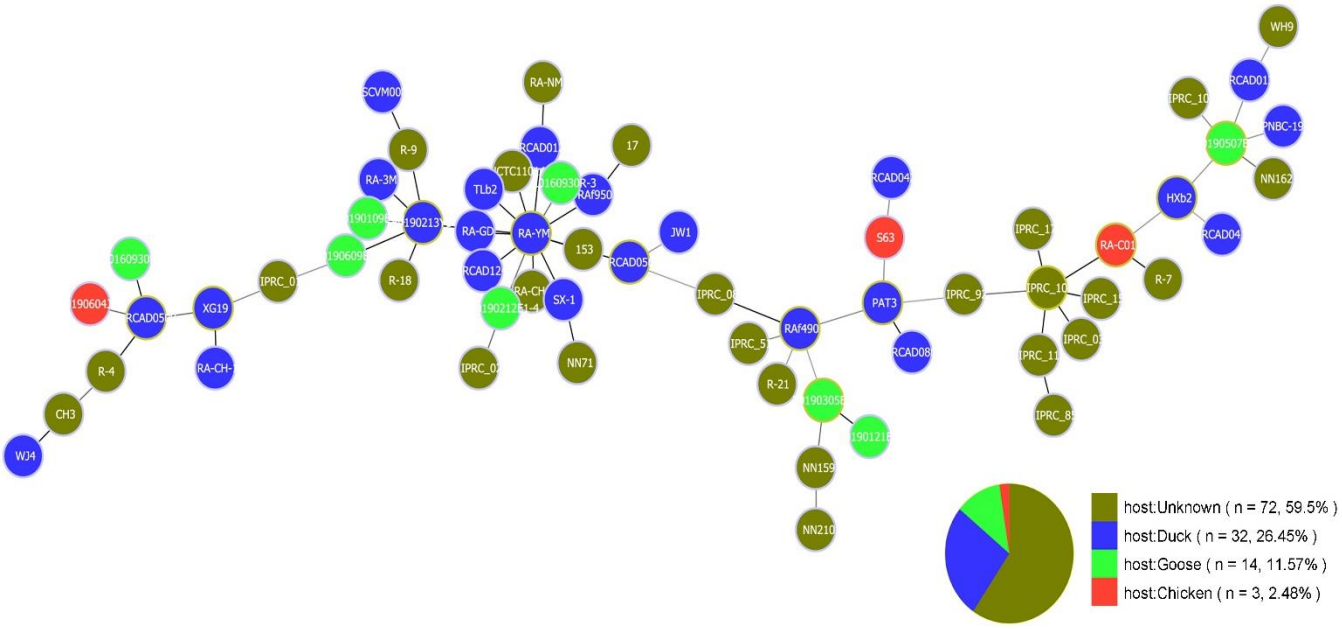

Fig. 4: Minimum spanning tree based on the MLST data.

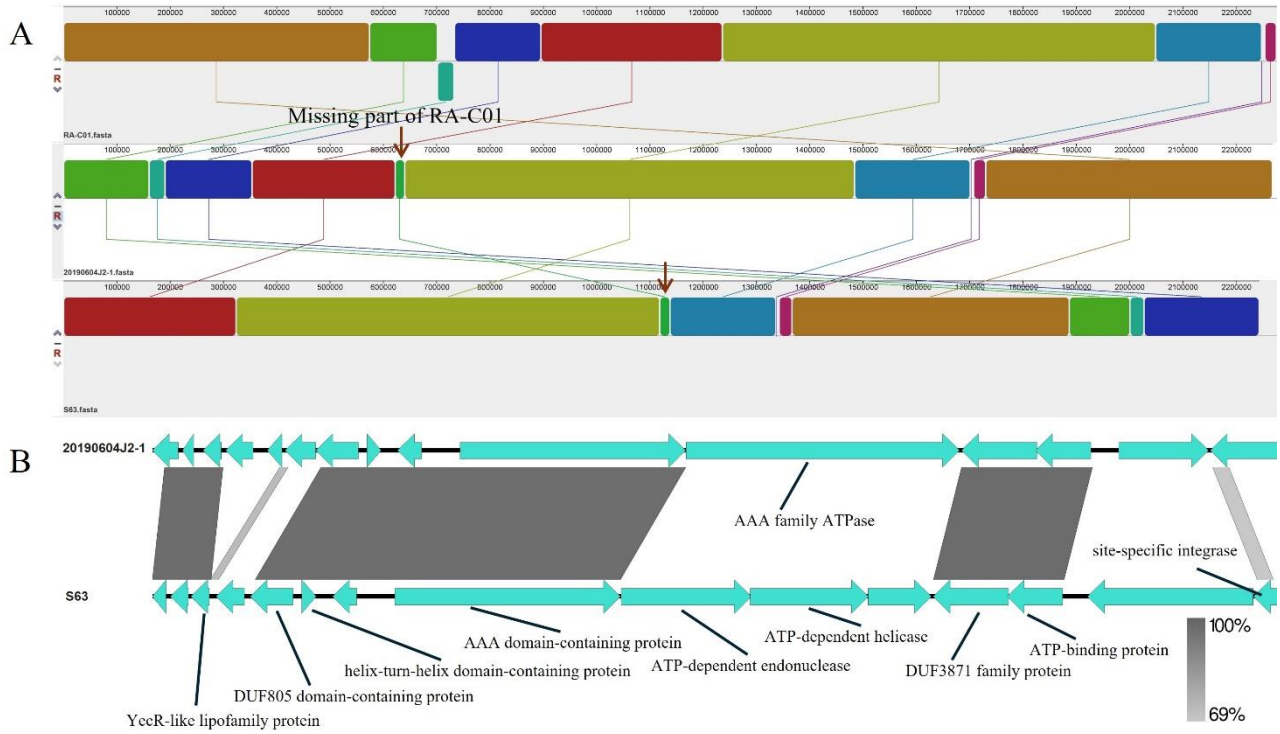

Fig. 5: Genome comparison of Riemerella anatipestifer from chicken. (A) Comparative genomic analyses of chicken-origin RA-C01、S63 and 20190904J2-1 using progressive Mauve alignment. (B) The missing part of RA-C01 genome using Easyfig software.

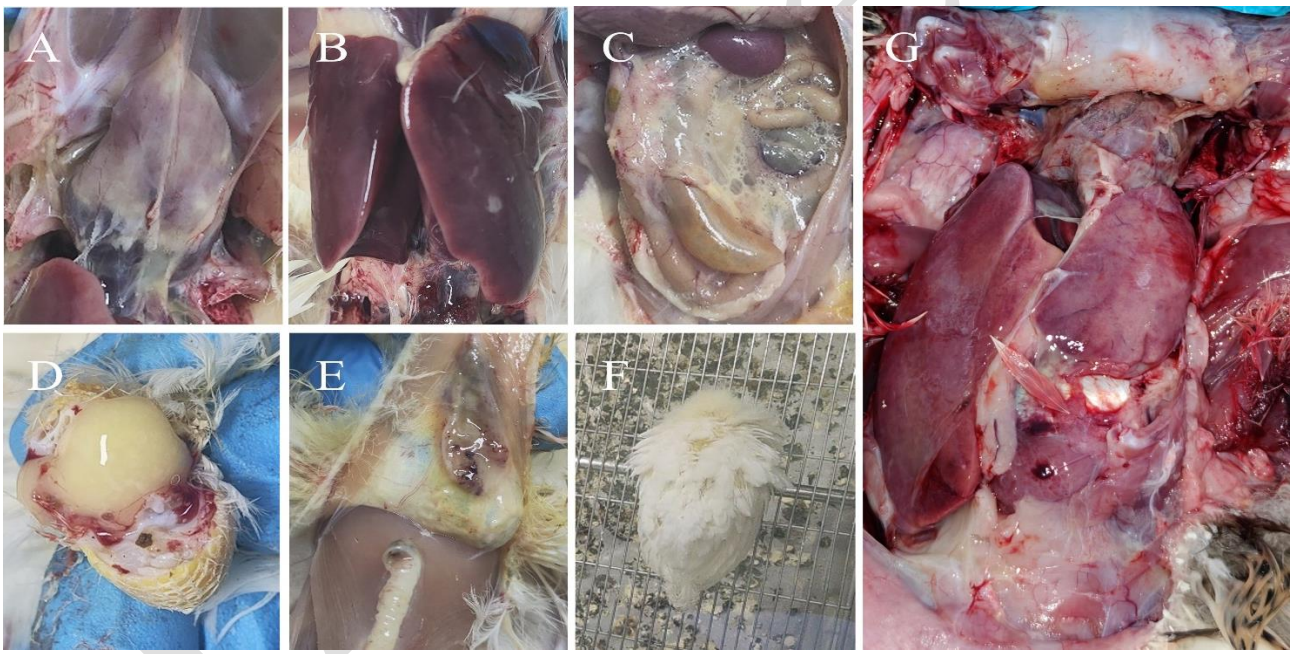

Fig. 6: Clinical signs and gross lesionsin *R. anatipestifer* RA-C01 infected SPF chickens (A-F) and ducks (G). (A) heart, pericarditis; (B) liver, hepatic fibrinous exudation; (C) abdominal cavity, severe peritonitis; (D) Joint, purulent exudates ;(E) Keel bone, purulent exudates; (F) clinical symptoms, lying on the ground, ruffled feathers, and lethargy (G) Severe pericarditis and hepatic fibrinous exudation.

Pathogenicity of R. anatipestifer RA-C01 to ducks: The ducks infected with *R. anatipestifer* strain RA-C01 exhibited no apparent clinical symptoms. On the 7th day post-infection, pathological examination revealed that 40% of the ducks had pericarditis and 30% exhibited hepatic fibrinous exudation (Fig. 6G).

Immunogenicity of inactivated vaccine of R. anatipestifer RA-C01: The SPF chickens in the control group exhibited clinical signs of lying on the ground, ruffled feathers, and lethargy following infection. These signs gradually subsided 5 days post-infection, and one chicken died 2 days post-infection. No clinical signs were observed in the inactivated vaccine group. Pathological examination of the control group SPF chickens revealed severe pericarditis (90%), air sacculitis (50%), purulent exudates in leg joints (40%), severe peritonitis (60%), purulent exudates in the keel bone (10%), and hepatic fibrinous exudation (50%). In contrast, all organs (100%) in the inactivated vaccine group were normal (Fig. 7).

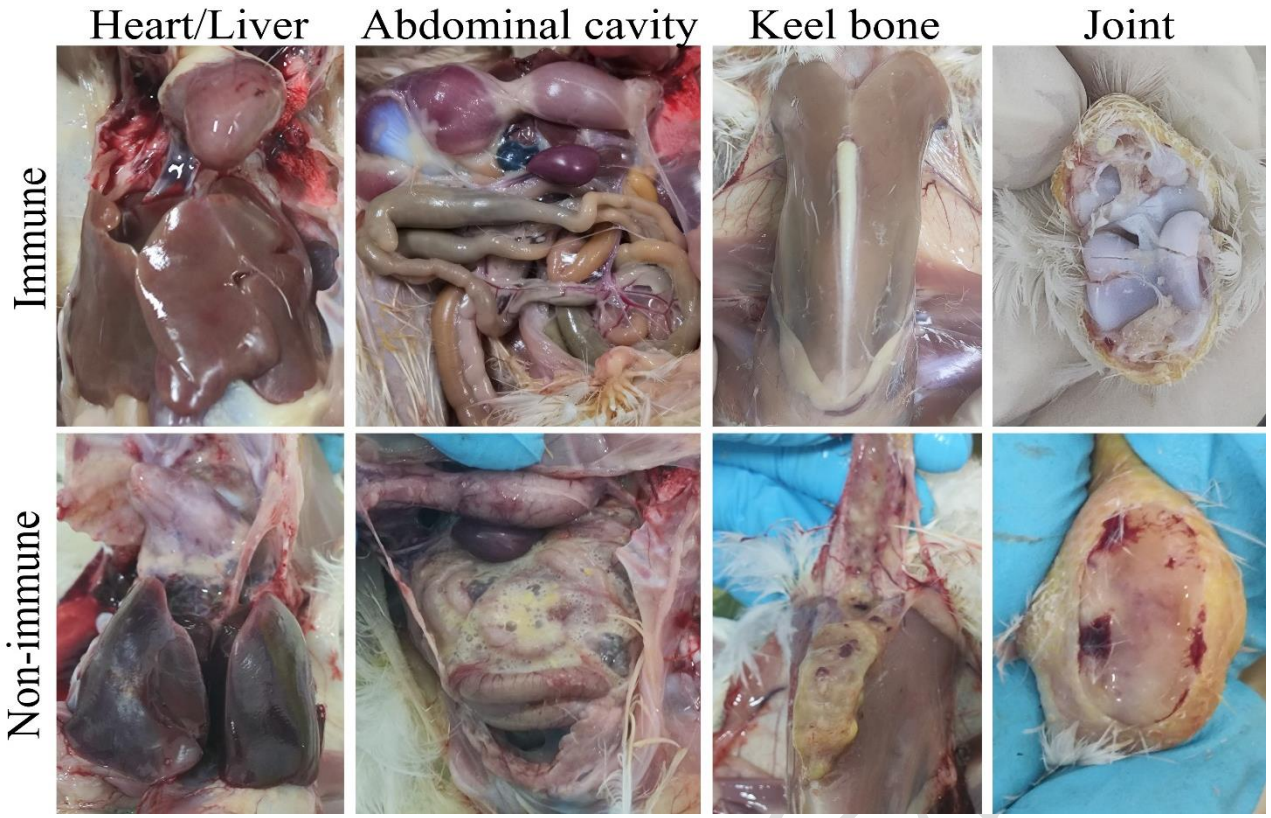

Fig. 7: Gross lesions of immunized and control SPF chickens after challenge.

DISCUSSION

Currently, the infection of *R. anatipestifer* in chickens has emerged as a significant threat to the poultry farming industry. In China, Li *et al.* (2011) reported the isolation of *R. anatipestifer* from a flock of three-yellow chickens and successfully replicated typical clinical symptoms through artificial inoculation. Subsequently, in 2021, cases of *R. anatipestifer* infection in broiler chickens were documented in both Australia and Greece (Omaleki *et al.*, 2021; Tzora *et al.*, 2021). Chen *et al.* (2024) identified infections in laying hens and highlighted the potential risk of vertical transmission for the first time. Most recently, Morocco reported its first case of *R. anatipestifer* infection in cage-reared laying hens (Bidoudan *et al.*, 2025). In this study, a strain of *R. anatipestifer* was isolated from diseased white-feathered broilers, and typical symptoms were reproduced under laboratory conditions. The increasing diversity and range of chicken hosts infected by *R. anatipestifer* underscore the urgency of addressing this issue. Effective prevention and control strategies are therefore critically needed to mitigate the impact on poultry production.

In this experiment, we performed complete genome sequencing of the RA-C01 strain of *R. anatipestifer* isolated from chickens and compared it with all *R. anatipestifer* genomes available in the GenBank database (including two chicken-derived strains). Our analysis revealed no distinct genetic boundary between chicken-derived *R. anatipestifer* and strains from other sources based on MLST typing and core genome phylogenetic analysis. This suggests that *R. anatipestifer* isolated from chickens may have been directly transmitted from ducks to chickens. The infectivity and pathogenicity of strain RA-C01 in ducks further support this hypothesis. Additionally, in another study conducted by our team, five different serotypes of *R. anatipestifer* originating from ducks were shown to infect SPF chickens, causing varying degrees of morbidity and mortality (unpublished data). Collectively, these findings demonstrate that *R. anatipestifer* from different host origins does not exhibit strict host specificity. Currently, the prevalent mixed breeding and free-range farming practices for chickens and ducks in developing countries undoubtedly heighten the risk of disease transmission. This also presents new challenges for the prevention and control of the diseases.

Despite the significant public health concerns associated with antibiotic abuse and overuse (Innes *et al.*, 2020), antibiotics remain the primary strategy for controlling *R. anatipestifer* infections due to the diversity of bacterial serotypes and the lack of cross-protection among different serotypes (Chu *et al.*, 2015). However, as *R. anatipestifer* continues to evolve, its genome increasingly carries multiple drug resistance genes, complicating infection control (Yang *et al.*, 2024). In this study, the RA-C01 strain was found to harbor up to seven drug resistance genes, rendering it resistant to aminoglycosides, glycopeptide, macrolides, tetracyclines, and penicillins. These findings were corroborated by the disc diffusion drug sensitivity test results. The RA-C01 strain exhibited sensitivity only to Ceftiofur sodium (cephalosporins), Florfenicol (amide alcohols), and Levofloxacin (quinolones). The judicious and appropriate use of these three antibiotics can effectively manage RA-C01 infections in chicken flocks. Nevertheless, vaccination remains the cornerstone for preventing infection and controlling this disease.

At present, numerous studies have been reported on *R. anatipestifer* vaccines (Li *et al.*, 2023; Liang *et al.*, 2024). Compared to inactivated vaccines, attenuated live vaccines are less advanced due to concerns regarding virulence reversion, prolonged development time, and high costs (Hao *et al.*, 2025). Inactivated vaccines exhibit excellent protective efficacy against infection by the corresponding serotype of *R. anatipestifer* (Kang et al., 2018). Consequently, designing multivalent vaccines targeting locally prevalent serotypes represents an effective prevention and control strategy (Hao *et al.*, 2025). In this study, the chicken-derived RA-C01 strain was formulated into an oil emulsion inactivated vaccine, and its efficacy was validated through challenge experiments. Therefore, administering inactivated vaccines at an early age can effectively protect chicken flocks from *R. anatipestifer* infection, facilitating the potential eradication of this disease within the flock. However, this inactivated vaccine also has certain limitations; specifically, it cannot provide protection against new serotypes of *R. anatipestifer* infection. Therefore, it is crucial to continuously monitor, detect and isolate *R. anatipestifer* strains in chickens to better understand the infection dynamics of different serotypes, which will inform the development of future vaccines.

Conclusion: In conclusion, we have for the first time reported the isolation of *R. anatipestifer* from the joint pus of lame chicken in China, clarified its genetic evolution relationship and proposed effective and feasible prevention and control measures, providing a scientific basis for future disease prevention and control as well as vaccine development.

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Author contributions: Conceptualization, DH and YC; methodology, DH and SL; software, DH; validation, RW, SL; formal analysis, DH; investigation, YS; resources, YC and SB; data curation, RW; writing—original draft preparation, DH; writing—review and editing, DH, ZZ and YS; visualization, DH; supervision, YC; project administration, DH; funding acquisition, YC and SB. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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Data availability statement: The original contributions presented in this study are included in the article/supplementary material.

Disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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