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Genomic analysis and experimental pathogenic characterization of *Riemerella anatipestifer* isolates from chickens in China

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ABSTRACT Waterfowl have a high likelihood of being infected with *Riemerella anatipestifer*. Although the pathogen is found in domestic ducks, turkeys, geese, and wild birds, there is little information available about the consequences of infection during egg laying and hatching in chickens. Here, we present the first report of a novel sequence type of *R. anatipestifer* S63 isolated from chickens in China. On the basis of pan-genome analysis, we showed S63′s genome occupies a distinct branch with other *R. anatipestifer* isolates from other hosts. *Galleria mellonella* larval tests indicated that S63 is less virulent than *R. anatipestifer* Ra36 isolated from ducks. Ducks and hens are susceptible to S63 infection. There is no mortality rate for chickens or ducks, but adult chickens experience neurological symptoms that reduce egg production and hatching rates. In chickens, S63 might be passed vertically from parents to offspring, resulting in "jelly-like" lifeless embryos. Using quantitative PCR, S63 was detected in the brain, liver, reproductive organs, and embryos. As far as we know, this is the first report of *R. anatipestifer* in hens, a disease that can reduce egg productivity, lower hatching rates, and produce jelly-like lifeless embryos, and the first report to raise the possibility that hens can be infected by roosters via semen.

Key words: *R. anatipestifer,* genome analysis, infection chicken, jelly-like, vertically

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INTRODUCTION

The Gram-negative bacterium *Riemerella anatipestifer* is known to cause acute or chronic sepsis in birds, including ducks, geese, and turkeys (Gyuris et al., 2017). This disease affects most countries and regions worldwide with intensive duck production, resulting in enormous economic losses (Sun et al., 2012).

Infections with *R. anatipestifer* often result in substantial mortality and morbidity among ducklings, and subclinical or even asymptomatic diseases among adults (Gao et al., 2021). A clinical case of *R. anatipestifer* infection in commercial broilers in Greece was reported in 2021 (Tzora et al., 2021). Another two typical cases of *R. anatipestifer* infections were reported in China and Australia (Omaleki et al., 2021). However, little is known about the pathogen's effects on egg laying in chickens carrying this pathogen.

An outbreak of *R. anatipestifer* infection was reported in March 2019 at a large chicken farm in Shandong Province, China. Compared with cases in previous years, the adult chickens observed in 2019 displayed neurological symptoms, a drop in egg production by 5 to 8%, and an increase in dead embryos in eggs by 8 to 10% (data obtained from the large chicken farm located in Shandong Province, China). In this study, we isolated the *R. anatipestifer* S63 strain from chicken brain tissue. The pathogenicity of the S63 strain was evaluated in *Galleria mellonella* larvae, chicken, and duck models. Our results suggested that hens may be infected by roosters via semen.

MATERIALS AND METHODS

Strain isolation and Identification

The animal tissue and dead embryos samples were immediately coated on sheep blood agar containing 20% sheep blood, also on McConkey agar, then incubator at 37°C with 5% CO_2 (Vo et al., 2022). 16S rDNA primers (27F:AGAGTTTGATCMTGGCTCAG; 1492R:AAGG

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AGGTGATCCAGCC) was used to identify species. The specific primers in this study (dnaB-F: CCTTCAATTCTTTTATGTACTTTCGC; dnaB-R: GATGCCTACAATTTATGAAGGATAAT) were used to detect the presence of *R. anatipestifer* S63, *R. anatipestifer* Ra37 (isolated from duck, kept in our lab), and *R. anatipestifer* ATCC 11845. Minimal inhibitory concentrations (**MICs**) of different antibiotics (including Florfenicol, Gentamicin, Amoxicillin-clavulanate etc.,) (Microbial Reagent Co., Ltd, Hangzhou, China) were determined by microdilution according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) 2018 (Fig. 2C).

DNA Extraction and Analysis of Genome Sequences

Genomic DNA was extracted S63 by means of a commercial extraction kit (Roche, Basel, Switzerland). The S63 genome was sequenced using a combined strategy of long-read Nanopore platform (Oxford Nanopore Technologies, Oxford, UK) and short-read Illumina NovaSeq 6000 platforms (Illumina, San Diego, CA). Briefly, base calling with the Guppy v3.2.6 software in the MinKNOW software package converts fast5 format data into fastq format. Further filtering of reads with adapters, low quality, and short fragments (<2,000 bp) is performed. The filtered reads were assembled by Canu v1.5 software (Koren et al., 2017), the assembly results were corrected by Racon v3.4.3 software with the third-generations of reads, and the Circlator v1.5.5 software was utilized to cyclize and adjust the starting sites. Pilon v1.22 software was utilized to further correct errors with next generation data and obtain genomes with higher accuracy for subsequent analysis. Non-redundant protein databases were analyzed to predict gene functions (Fu et al., 2012). Rfam databases were used to predict rRNA and Trna (Kalvari et al., 2018). Genomic visualization was performed using Circos software (Krzywinski et al., 2009). The genome islands of S63 were predicted by using IslandPath-DiMob v0.2 software (Bertelli and Brinkman, 2018). The prophages were predicted by using PhiSpy (Akhter et al., 2012) and Phaster software (Arndt et al., 2016). The CARD database was used for drug resistance gene prediction (Jia et al., 2017). The multilocus sequence typing (**MLST**) of S63 was performed by using MLST database website (https://pubmlat.org/). In pan-genome analysis, all genome sequences used were annotated or reannotated by Prokka to ensure consistent (Seemann, 2014), pan-genome analysis was performed with Roary(Page et al., 2015), the core-genome alignment phylogenic analysis was done with Fasttree (Price et al., 2010), and the phylogenic tree was beautified with iTOL (Letunic and Bork, 2019).

Experiments on Infected Animals

Experimental Infection of* G. mellonella *Larvae *R. anatipestifer* S63 was tested for virulence using *G. mellonella* larvae that were 2 to 2.2 cm in length, as previously described (Liu et al., 2019). To inoculate, 20-μL aliquots of varying doses of S63 (from 1×10^1 CFU to 1×10^8 CFU) were injected into the worms (n = 20 per group). Equal doses of *R. anatipestifer* ATCC 11845 and *R. anatipestifer* Ra37 were used to challenge the worms. An equal volume of PBS was used to inoculate the control group. The survival of larvae was determined 24 h after infection. The LD_{50} of *G. mellonella* was calculated with SPSS software (version 19.0) and the bliss algorithm.

Experimental Infection of Chicken Embryos Chicken embryos were used to determine the virulence of *R. anatipestifer,* as previously described with minor modifications (Seo et al., 2013). Briefly, 40 specific pathogen-free (**SPF**) chicken embryos were randomly divided into four groups (n = 10 per group). The air chamber of each chicken embryo was injected with 20 μL of the S63 strain at different concentrations (1×10^5 CFU, 1×10^6 CFU, and 1×10^7 CFU). The control group was injected with the same volume of PBS, then all eggs were placed into an incubator (37°C). The hatched chickens were killed 21 d later. Quantitative PCR (**qPCR**) was used to detect S63 bacteria in the brain, liver, reproductive organs, and dead embryos. Other pathogenic bacteria, including *Escherichia coli, Salmonella, Enterococcus faecalis, Staphylococcus aureus*, and *Pseudomonas aeruginosa*, were also detected with the same method.

Experimental Infection of Chicks Forty-eight 10-day-old SPF chicks were randomly divided into eight groups (n = 6 per group). Every chick was injected with 100 μL of S63 or Ra37 strain at different concentrations (1×10^5 CFU, 1×10^6 CFU, or 1×10^7 CFU) via the intramuscular route. Four groups (n = 6 per group) received injections of S63 strain or an equal volume of PBS; the other 4 groups (n = 6 per group) received injections of Ra37 strain or an equal volume of PBS.

Experimental Infection of Ducklings In the pre-experiment, twelve 10-day-old commercial ducklings were randomly divided into 3 groups (n = 6 per group). Each duckling was injected via the intramuscular route with 100 μL of Ra37 strain (1×10^7 CFU), or an equal volume of PBS. The results showed that Ra37-infected group resulted in a 100% death rate (data not shown). To study the effect of S63 on ducklings, we used eighteen 10-day-old ducklings, as previously described with minor modifications (Flores et al., 2021), each of which was inoculated via the intramuscular route with 100 μL of S63 (1×10^7 CFU). On the 3rd, 7th, 14th, and 42nd d after the challenge, 3 ducklings were randomly selected for sacrifice. S63 DNA was detected in the brain, liver, and reproductive organs by qPCR methods.

The Growth Cycle of Chicks Infected With S63 To determine the effect of infection on chick growth, twenty-four 10-day-old commercial roosters and forty-eight 10-day-old commercial hens, were randomly divided into 2 groups (Figure 7). Every chick was inoculated with 100 μL of 1×10^7 CFU of S63 strain via the intramuscular route. On d 14 postinfection (**p.i.**), 201 p.i., and 231 p.i., 2 hens and 2 roosters were randomly

killed (Fig. 7). On d 7 p.i., 14 p.i., 203 p.i., and 231 p.i., the body weight of the chickens was determined. On d 231 p.i., 6 hens and 18 males in Group I were randomly divided into 2 groups (each groups included 3 positive roosters and 9 positive hens); 6 hens and 18 males in Group IV were randomly divided into two groups (each groups included 3 negative roosters and 9 negative hens). Group II was combined with 3 positive roosters from Group I and 9 negative hens from Group IV, and Group III was combined with 3 negative roosters (had no S63 infection) from Group IV and 9 positive hens (had no S63 infection) from Group I. The eggs collected from Group II and Group IV on d 203 to 231 p.i and 238 to 266 p.i, respectively, and then incubated. The hatched chickens were killed on d 21. qPCR methods were used to detect S63 bacteria in the brain, liver, reproductive organs, and dead embryos.

The Score of Health Status The health status of each group of chicks was given a score between 0 and 5 as previously described with minor modifications (Chen et al., 2018), as follows: 5: normal health, condition unremarkable; 4: low spirits; 3: diarrhea symptoms; 2: neurological symptoms; 1: near death; 0: death. The survival of chicks at 42 d was recorded. All chickens were killed on d 42 after infection, and the different tissues of the chickens infected with S63 were measured by qPCR methods.

Quantitative PCR Analysis Bacteria S63 DNA in Different Tissues The concentration of the extracted bacterial DNA was determined using an ultramicro spectrophotometer (Denovix,Wilmington, DE) as previously described (Chen et al., 2019). Briefly, absolute quantification of S63 was performed using a Touch Real-Time PCR Detection System (Roche, Basel, Switzerland). Reaction mixtures contained 10 ng of DNA, SuperReal PreMix (probe) (Sangon Biotech, Shanghai, China), TaqMan probe (Sangon Biotech, Shanghai, China), sense and reverse primers (20 μmol/L) (Sangon Biotech, Shanghai, China), and RNase-free water in a total volume of 20 μL. The primer sequences were as follows: sense primer, 5'-GATAATCTTAATGCTATGGAGC-3'; reverse primer, 5'-CACTAGGAACACCTATGGTC-3'; probe 5'-(FAM) GCTATGGGTAAACTAGGCAGCTCATTCC (TAMRA)-3'. The qPCR reaction protocol was as follows: denaturation at 95°C for 30 s, followed by 40 cycles at 95° C for 15 s, 56°C for 30 s, and 72°C for 30 s.

Animals and Ethics Statements Ten-day-old specfic-pathogen-free (**SPF**) and commercial chicks, 10-day-old commercial ducklings, and SPF eggs were purchased from Huahong Biology Co., Ltd. All commercial animals are free of *R. anatipestifer*. The animal-use procedures were approved by Shandong Academy of Agricultural Sciences SAAS-2020-018.

Statistical Analysis

The data were analyzed using GraphPad Prism 8.0 software (San Diego, CA). Comparisons were performed through the "two-way ANOVA" or "multipe *t*-test" strategy. The significance level was set at $P < 0.05$ (*).

RESULTS

Isolation and Genomic Characterization of R. anatipestifer *S63*

R. anatipestifer strains were isolated from adult chickens (28−30 wk) with neurological symptoms and dead embryos (18−20 d). The DNA and RNA of the possible viruses in the dead embryos were extracted, but second-sequencing analysis showed no evidence of these viruses. The strains were isolated from chicken brain, liver, and embryo samples (Figures 1A and B), and there was no bacterial growth after 24 h or 48 h on McConkey agar. PCR analysis revealed the same bands in the positive samples as the 12 single clones selected from the blood plate (Figure 1A). Examination of strains for the 16S ribosomal RNA gene of *R. anatipestifer* SDAU-1 produced a 100% identification rate (data not shown). A *R. anatipestifer* strain, named S63, isolated from chicken brain tissue was chosen for further study.

Genome sequencing confirmed that the *R. anatipestifer* S63 strain of chicken origin was 2,243,639 bp in length with 35.42% G+C. There were 2,123 functional genes predicted using non-redundant protein databases. The total length of genes is 2,028,195 bp, and the average gene length is 955 bp. The genome of S63 has 9 rRNAs and 40 tRNAs in total (Figure 1C). Six genomic islands are harbored in the S63 genome according to gene function analysis using IslandPath-DiMob v0.2 software (Table S2). To find homologous sequences, the complete genomes of the 32 *R. anatipestifer* strains were downloaded from the NCBI database (Table S1). The analysis suggested there is a distant phylogenetic relationship between the chicken origin *R. anatipestifer* 20190604J2-1 and S63 (Figure 1D).

The morphology of S63 was also studied, and scanning electron microscopy showed that S63 has a rod-shaped structure without flagella (Figure 2A). The growth curve indicated S63 grew more slowly than the Ra37 and ATCC11845 strains (Figure 2B). TetQ and ErmF are 2 antibiotic-resistance genes that were predicted by blasting sequences against the CARD database. The minimal inhibitory concentrations (MICs) of several antibiotics, such as florfenicol, gentamicin, and amoxicillin-clavulanate, are shown in Figure 2C. The aforementioned findings demonstrated that S63 grows more slowly than Ra37 and ATCC11845 and is sensitive to most antibiotics.

Complete Genome Comparison and Evolutionary Analysis of R. anatipestifer *S63*

The genome of *R. anatipestifer* S63 and 20190604J2-1, which were isolated from chickens, were compared, and the results showed that the S63 genome and 20190604J2-1 genome had extremely consistent mosaic arrangements. Ten locally colinear blocks are displayed in Figure 3A. Prophage 3 and putative prophage regions, together with 2 sizable unique sequences, found

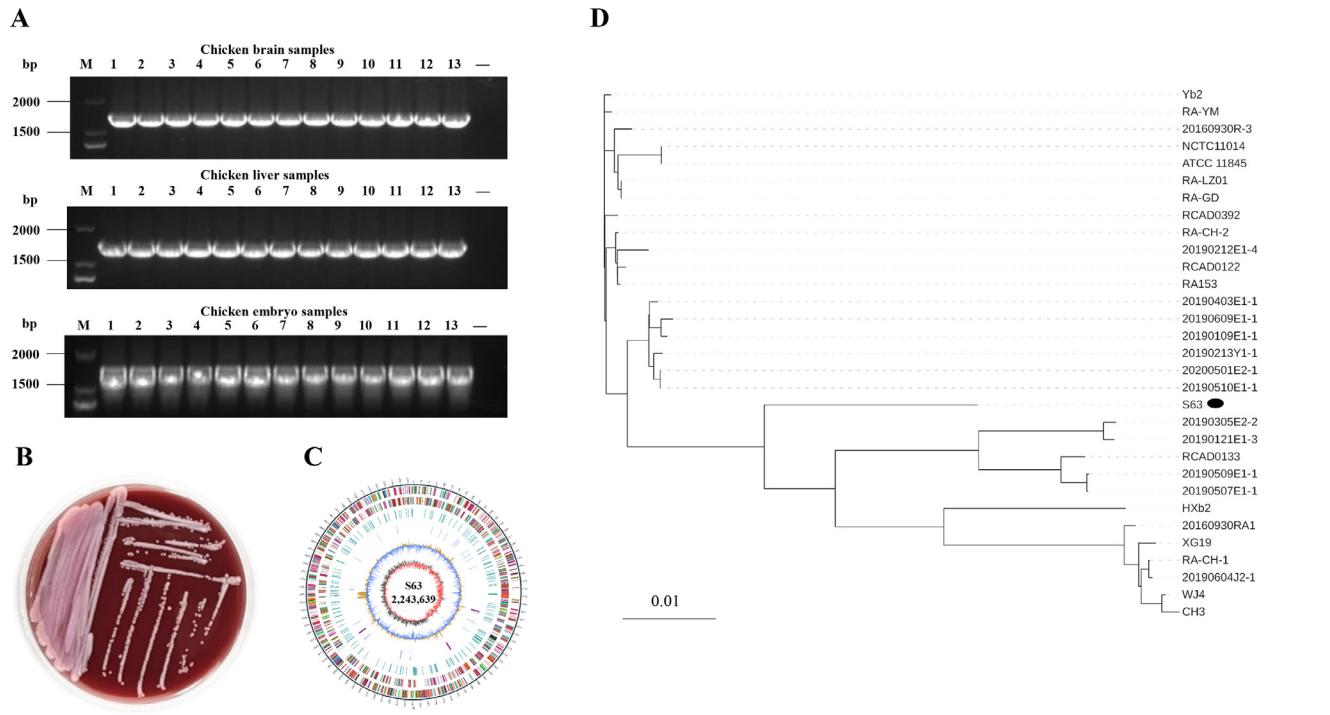


Figure 1. Isolated *Riemerella anatipestifer* S63 and pan-genome analysis. (A) *R. anatipestifer* was identified in chicken brain, liver, and embryo samples using PCR methods. Lane 13 represents positive DNA sample; "-" is control group. (B) Colony formation of S63. S63 strain was grown in agar containing 20% sheep blood at 37°C for 48 h with 5% CO_2. (C) The complete genome of S63. The outermost circle indicates the size of the S63 genome; each scale is 5 Kb. (D) Phylogenetic tree based on complete sequences of 32 representative *R. anatipestifer* strains in the NCBI database.

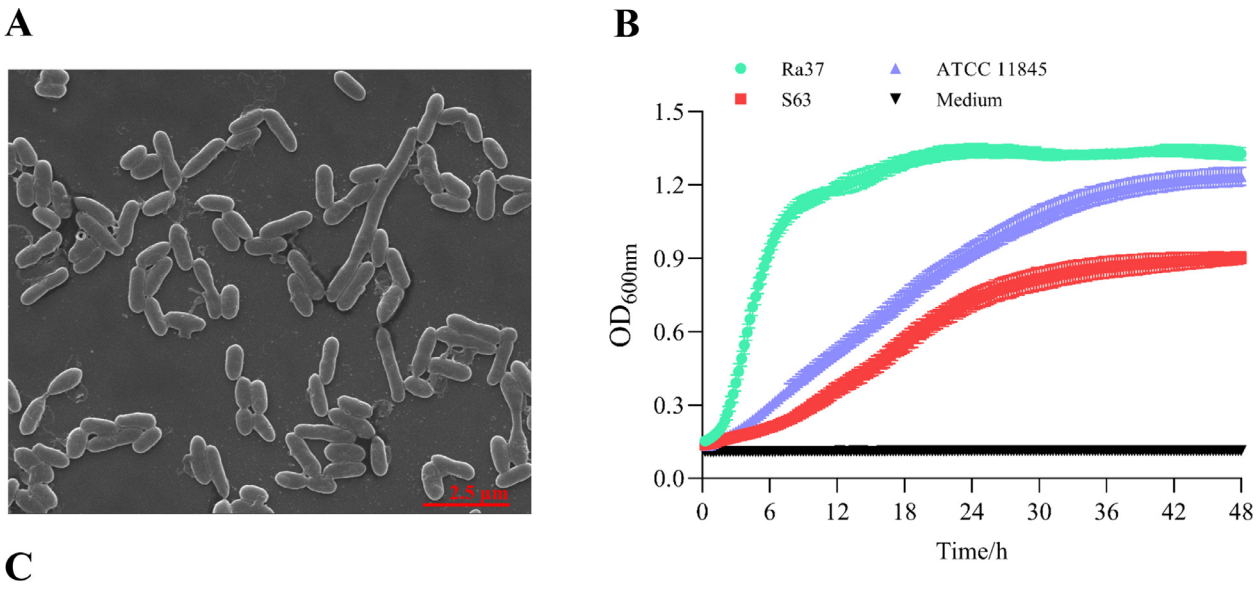
in the S63 genome were absent in the 20190604J2-1 genome (Table S1).

The prophage 3 had a length of 44,184 bp and a G+C content of 50.79%. In total, 49 open reading frames (**ORFs**) were predicted, including integrase, DNA primase, and lysozyme, which are phage-related components. In the prophage 3 genome, no tRNA was predicted (Figure 3B). Additionally, further analysis revealed that a sequence in the genome of S63 had the highest similarity (93% nucleotide sequence identity, 98% coverage) to an element in *Bacteroides fragilis*, CL03T12C07 (GenBank no. CP072257), which is located at nucleotide positions 3,131,916−3,177,941 (Figures 3A and 3B). According to gene annotation and alignment analysis, prophage 3 was found to include a number of integrative conjugative element proteins that may encode a putative type IV secretion system (Fig. 3A&B). The genome of S63 contained a second distinct region between nucleotide locations 2,153,212 −2,210,683. This region's genetic sequence had 80 ORFs that coded for proteins such as the encoded phage tail protein, terminase, morphogenesis protein, lantibiotic efflux ABC transporter, and the tetracycline resistance element mobilization regulatory protein RteC, among others (Figures 3A and B). However, PhiSpy software did not forecast this region to be a prophage; instead, the Phaster program predicted it to be an incomplete prophage (nucleotide positions 2,153,212−2,210,683). According to an analysis of the MLST data, no sequence types (**STs**) were identified for the S63 isolates (Figure 4). The above results indicated that S63 has a distant relationship with the strain 20190604J2-1 isolated from chickens in China, and a close relationship with ST N28, N14, and 31 strains.

R. anatipestifer *S63 Has Less Virulence and Lethality in* G. mellonella *and Chicken Embryos But Can Cause Dead Embryos to Have a "Jelly-Like" Rather Than Chick-Shaped Appearance*

The pathogenicity of S63 against the *G. mellonella* model was evaluated (Figure 5A). The analysis showed that 20 μL of ATCC11845 and S63 at 1×10^7 CFU or 1×10^8 CFU caused 40 to 60% death of *G. mellonella* after 24-h infection (Figures 5B and C), while at the same dose, Ra37 resulted in 80 to 100% survival (Figure 5D). The LD_{50} of S63 in *G. mellonella* was 1.99×10^{10} CFU.

We further explored the effect of different doses of S63 (1×10^5 CFU, 1×10^6 CFU, and 1×10^7 CFU) on chicken embryos. The findings indicated that administration of 1×10^7 CFU of S63 on d 21 p.i. resulted in 100% death, while treatment with 1×10^5 CFU and 1×10^6 CFU of S63 on d 21 p.i. resulted in 50% and 20% survival, respectively (Figure 6A). Surprisingly, all dead embryos displayed a jelly-like, rather than chick-shaped, appearance (Figure 6B). there were no other pathogenic bacteria, such as *E. coli* and *Salmonella* spp., detected in the dead embryos. We raised seven chicks, and they were all dead after 21 d. S63 DNA was detectable in the



C

Antimicrobial agent	MIC(μg/mL)
Florfenicol	1
Gentamicin	16
Amoxicillin-clavulanate	1
Ampicillin	1
Ceftiofur	0.125
Polymyxin	256
Neomycin	16
Enrofloxacin	8
Meropenem	0.0039
Doxycycline	2
Apramycin	256

Figure 2. Growth characteristics and drug sensitivity of S63. (A) Scanning electron microscopy of S63. (B) Growth curve of S63. *R. anatipestifer* was grown on agar containing 20% sheep blood at 37°C for 48 h with 5% CO_2. This experiment was performed in triplicate and data are expressed as means ± SD. (C) Determination of minimal inhibitory concentrations (MICs) of several antibiotics according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) 2018.

brain, liver, and reproductive organs of all chicks (Figure 6C). Additionally, S63 was isolated from all other tissues (data not shown). Changes to the morphology of chicken embryos caused by different pathogenic bacteria are presented in Figure S1. As shown in the above results, *R. anatipestifer* S63 resulted in jelly-like dead embryos and reduced virulence in *G. mellonella* and chicken embryos.

R. anatipestifer *S63 Infected Chicks Without Causing Death and Infected Ducklings But Were Completely Cleared From the Body*

It is well known that *R. anatipestifer* can infect chicks and ducklings. Therefore, we assessed the effect of S63 on chicks and ducklings. After postchallenge, none of the S63-infected chicks displayed any overt clinical symptoms (Figure S2A). No deaths were recorded in the treatment and PBS groups (Figure S2B). Based on S63 processing, the Ra37-infected chicks had respective death rates of 33.3% and 100% with 1×10^6 CFU and 1×10^7 CFU (Figures S2C and D; Figure S3).

We also evaluated the effect of S63 on ducklings by inoculating them with 100 μL (1×10^7 CFU) of S63. The challenged ducks were monitored for 42 d (Figure S4A). None of the ducklings had obvious clinical symptoms or signs of illness on the first d after challenge (Figures S4B and C). From d 3 p.i. to d 14 p.i., S63 DNA was detectable in the brain, liver, and reproductive organs; however, no S63 DNA was detectable by qPCR after d 42 p.i. (Fig. S4D). The above results showed that

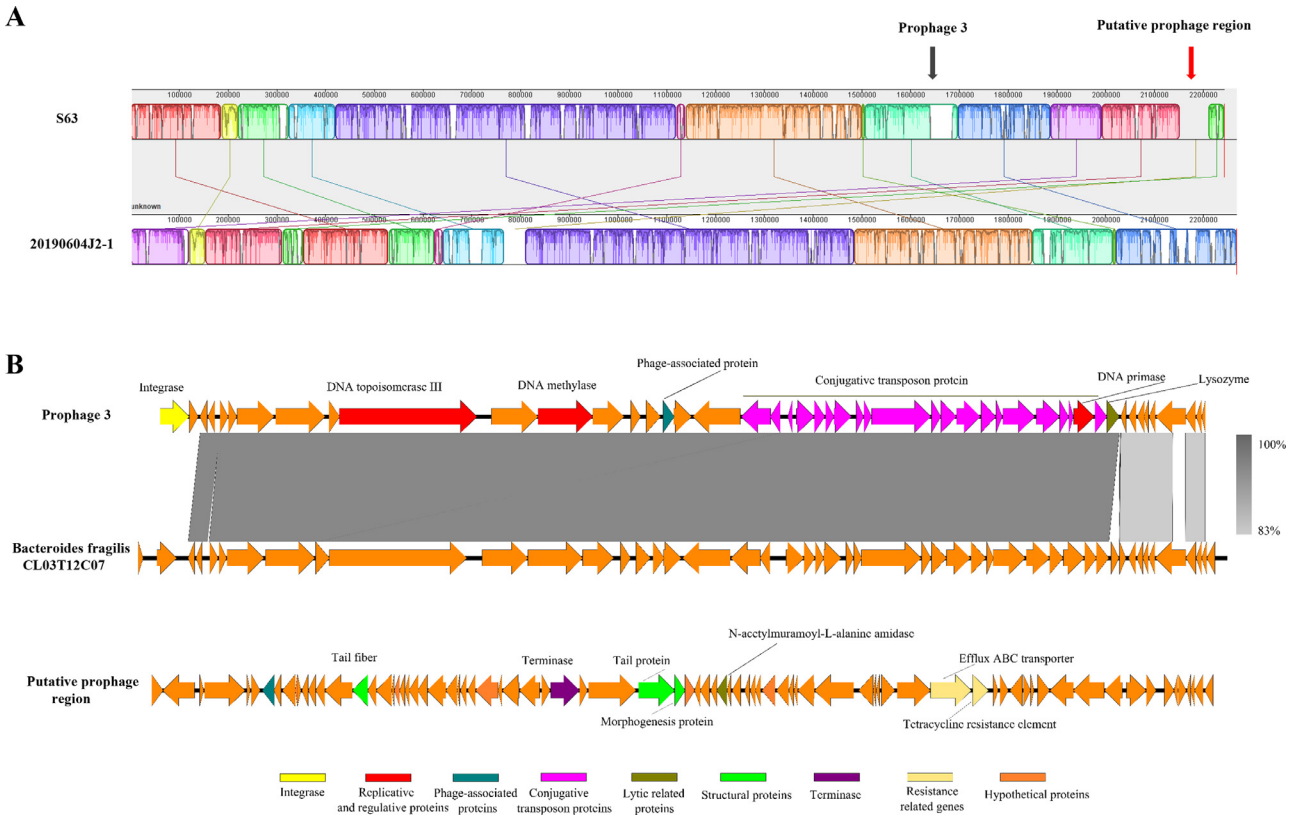


Figure 3. Organization of S63 genome and its prophage. (A) Comparative genomic analyses of chicken-origin S63 and 20190904J2-1 using progressive Mauve alignment. (B) Comparative genomic analysis of prophage in S63 genome using Easyfig software.

R. anatipestifer S63 infected the chicks and ducklings without causing acute toxicity or death, and it was completely cleared from the body of S63-infected ducklings.

Chickens Carry R. anatipestifer *S63 for Their Entire Lives, Infected Through Semen and Vertical Transmission*

S63 can cause jelly-like dead embryos, and hatched chicks carry S63, but no dead chicks have been observed. However, adult chickens on large-scale farms were shown to have different degrees of neurological symptoms as well as decreased egg production and hatchability.

As shown in Figure 7, we conducted an experiment to investigate the aforementioned clinical symptoms. A total of 72 chicks, including 24 roosters and 48 hens, were randomly divided into 2 groups. Each chick was inoculated i.v. with 100 μL (1×10^7 CFU) of S63 and monitored for 267 d. On d 201 p.i., some hens began to exhibit neurological symptoms (Figure S5), but gradually returned to normal by d 245 p.i. without drug intervention. The roosters occasionally exhibited neurological symptom, but they recover quickly. On d

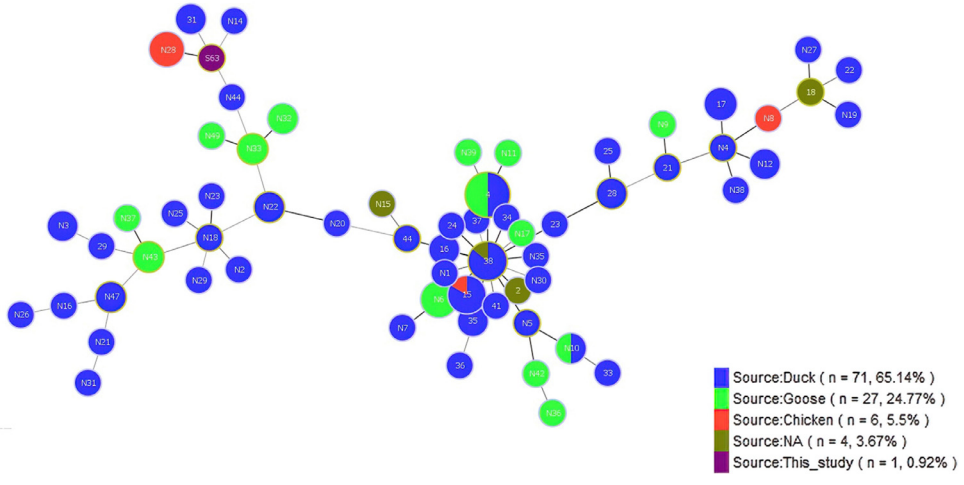


Figure 4. Minimum spanning tree based on the MLST data. Numbers indicate ST of each node.

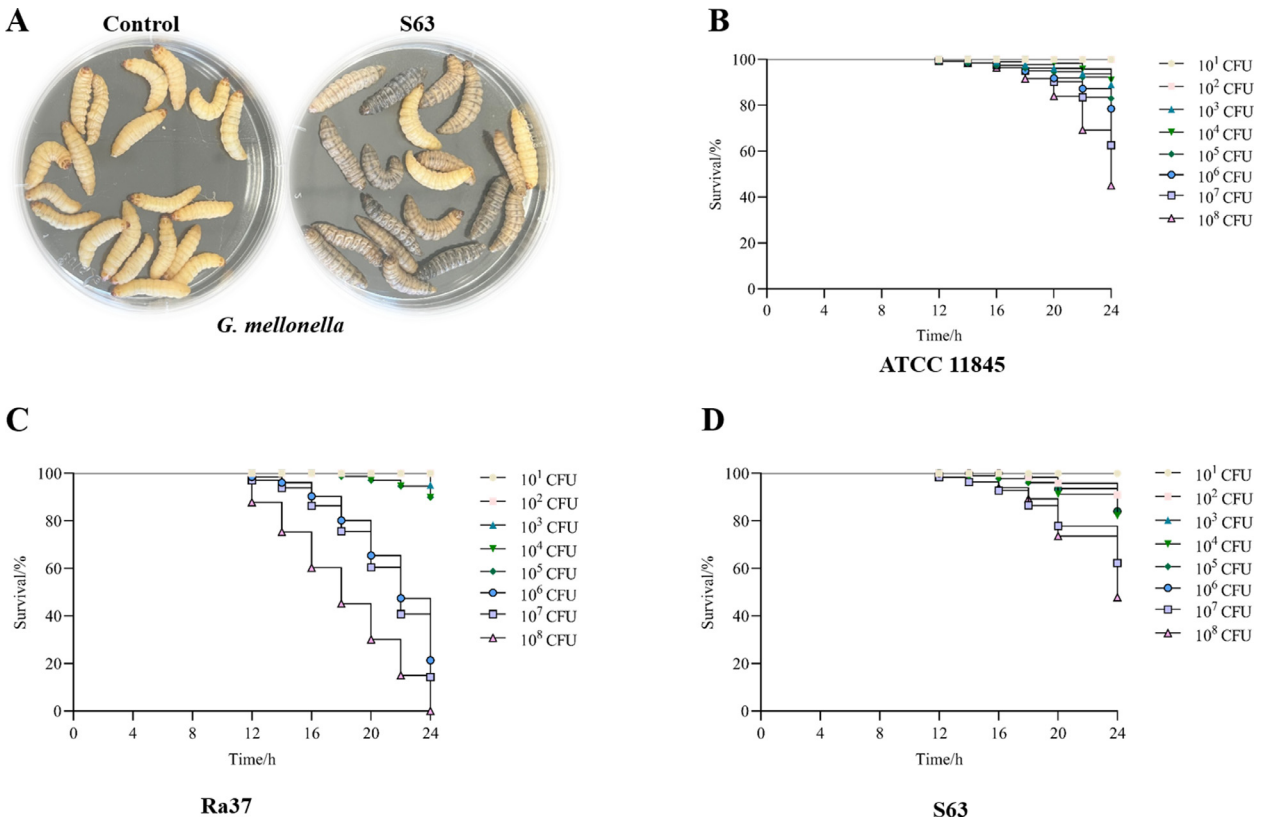


Figure 5. Experimental infection of *G. mellonella* larvae. (A) Morphological characteristics of dead (black) and surviving (beige) *G. mellonella* larvae. *R. anatipestifer* ATCC 11845 (B), Ra37 (C), and S63 (D) of different doses (from 10^1 to 10^8 CFU) were injected into the worm.

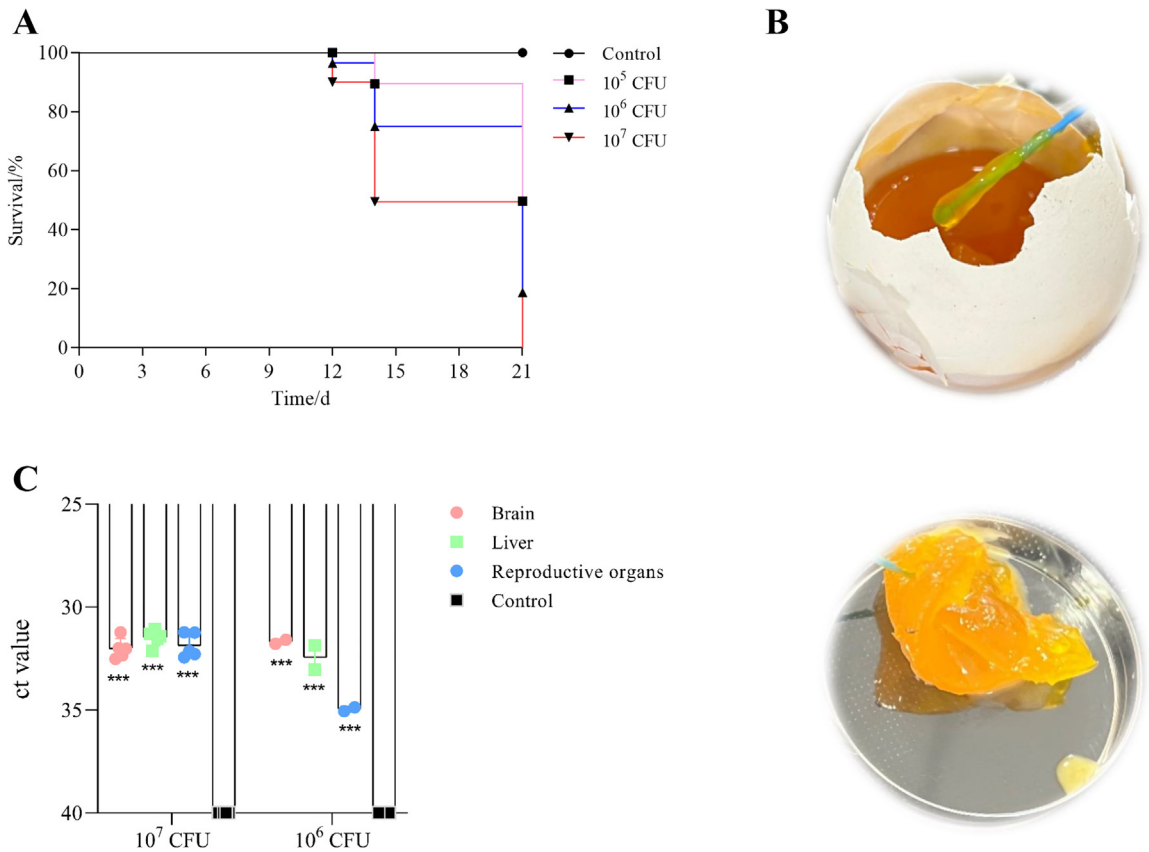


Figure 6. Experimental infection in chicken embryos. (A) The rate of S63 infection of chicken embryos. (B) Morphology of "jelly-like" lifeless embryos. (C) Quantitation of S63 DNA by qPCR analysis. The significance level was set at $P < 0.001$ (***).

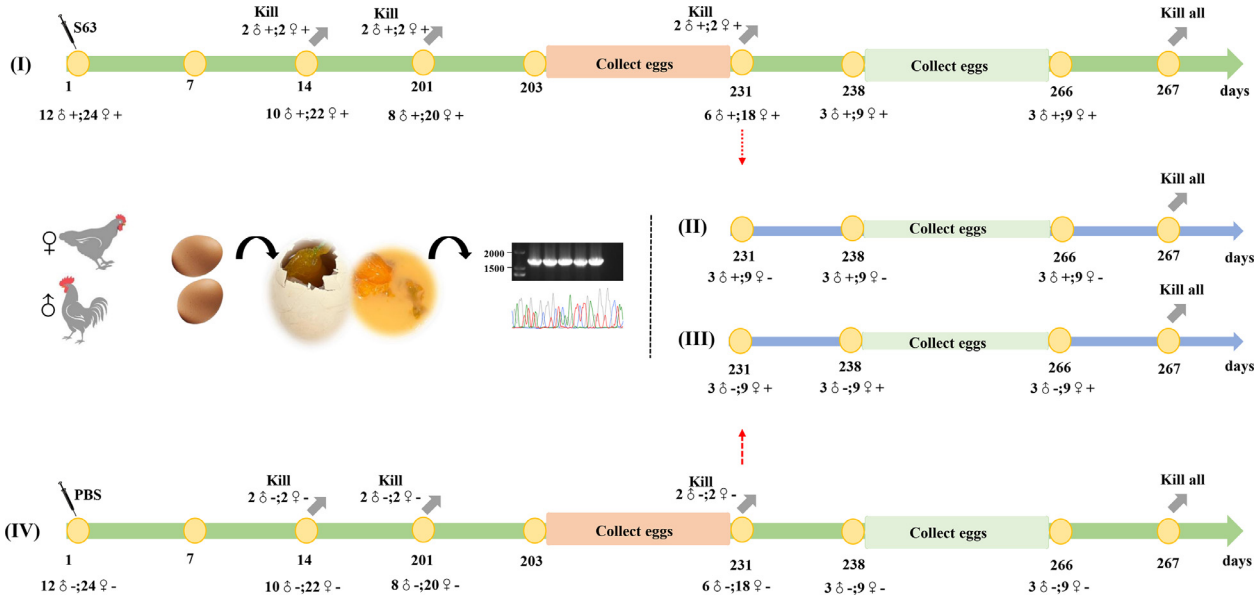


Figure 7. Experimental scheme for the evaluation of S63 threat to chickens.

14 p.i., 201 p.i., and 232 p.i., we executed 2 hens and 2 rooster in groups I and IV. The autopsy results revealed that the chickens had enlarged spleens (Figure S6A), tracheal hemorrhage (Figure S6B), and diffuse hemorrhaging in the duodenum and jejunum (Figures S6C and D). Pathological changes and histopathology of the reproductive organs, brain, livers, and spleen were observed (Figure 8). S63 DNA was detectable in the brain, liver, and reproductive organs on d 7 p.i., 201 p.i., and 231 p.i. via qPCR (Figure S7A).

On d 231 p.i., 6 roosters and 18 hens were randomly divided into two groups. Group II consisted of 3 positive roosters and 9 negative hens, while group III consisted of 3 negative roosters and 9 positive hens (Figure 7). On d 267 p.i., all chickens were executed. The qPCR showed that bacterial DNA was detectable in the brain, liver, and reproductive organs (including rooster semen) of Group I and Group II, (Figure S7B). No S63 DNA was detected in the tissues of roosters in Group III (Figure S7B).

The experiments were conducted between d 203 p.i. and d 231 p.i., and the results showed that 193 eggs were laid in Group I compared with 313 eggs in Group IV (with hatching rates of 59% vs. 84%) (Table S4). In Group I, 94% of the 121 hatchlings tested positive (Table S4). In the period between d 238 p.i. and 266 p.i., the hatching rate of Group II (71%) was higher than that of Group I (58%) (Table S6). There was no

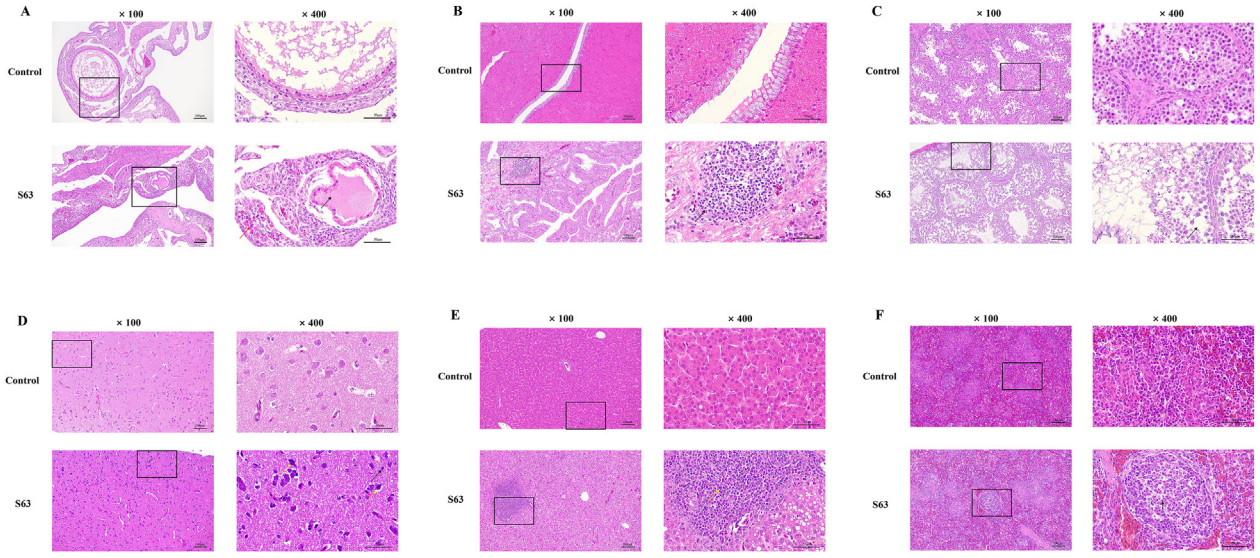


Figure 8. Histopathological analysis of organ tissues from chicken. (A) Ovarian: An irregular shape is evident in some follicles in the ovarian tissue, as indicated by black arrows; there are numerous eosinophils in the tissue, as indicated by red arrow; (B) Fallopian tube: as indicated by black arrows, fallopian tube tissue has been infiltrated with inflammatory cells; (C) Testis: according to the black arrow, the spermatogenic cells are loosely arranged and insignificantly numerous in the testis; (D) Brain: a large number of neurons have degenerated, the cell bodies are pyknotic and hyperchromatic, and the cells have irregular shapes, as shown by the yellow arrow; glial cells are significantly higher in the brain compared to the control group, as shown by the black arrow; (E) Liver: as indicated by yellow arrow, liver cells are infiltrated with inflammatory cells; (F) Spleen: the nucleus of the cell is broken, contracted and deeply stained, as shown by the black arrow.

significant difference between Group I and Group III in the total number of eggs laid or hatching rate (Table S5). Compared with the findings in the control group, there was no significant difference in body weight between roosters and hens in Group I (Table S6).

The above findings indicated that S63-infected chicks carry the *R. anatipestifer* for their entire lives. We found evidence S63 can cause neurological symptoms, reduced egg production (e.g., Group I, 93 eggs, vs. Group IV, 154 eggs, on d 238−266 p.i.), lower hatching rate (e.g., Group I 58% vs Group IV 82% on d 238−266 p.i.), and the production of jelly-like lifeless embryos. Roosters might transmit S63 to hens through their semen.

DISCUSSION

Ducks, geese, and turkeys, other poultry, and wild birds, are susceptible to infection by *R. anatipestifer*. The transmission route of *R. anatipestifer*, and particularly its effects on chickens, has received little attention from researchers in recent years—they have concentrated on duck infections of *R. anatipestifer* and its resistance to medication (Tang et al., 2018; Li et al., 2021; Zhang et al., 2022; Zhu et al., 2022). Studies have shown that *R. anatipestifer* spreads via air, tainted water, and feed; affects the digestive and respiratory systems; and causes skin infections after wounds (Pathanasophon et al., 2002). Several published papers have reported *R. anatipestifer* infections in chickens (Omaleki et al., 2021; Tzora et al., 2021), but the disease's effects on poultry are poorly understood.

In our experiment, the growth rates of ATCC 11845 and Ra37 were faster than that of S63 (Figure 2A), suggesting that S63 was cultivated under harsh conditions(Li et al., 2020; Li et al., 2023). There are a large number of *R. anatipestifer* bacterial genomes in the NCBI database, but very few of the *R. anatipestifer* strains are of chicken origin. Comparative genomic analysis revealed that the 20190604J2-1 strain of chicken origin lacks several distinct genes that are present in the S63 strain. Furthermore, a complete genetic sequence of prophage 3 was found in S63. Gene alignment analysis revealed that the prophage 3 found in the S63 genome may be related to the virulence of S63. For instance, a gene of prophage 3 encodes a functioning T4SS protein, which may mediate the dissemination of virulence and antibiotic resistance genes by conjugation. These results concur with the findings of previous investigations into *Pasteurella multocida* (Peng et al., 2017). The genome of S63 was distinct from those of other STs of *R. anatipestifer* strains that were previously identified. However, it was closely related to the ST N28 strain of chicken origin, suggesting that S63 might represent a new epidemic strain in China.

The hatching rate and egg production rate of S63-infected hens were reduced, either directly or indirectly. Our study of chicken embryos infected with S63 showed that, after more than 15 d of incubation, dead embryos displayed a jelly-like, rather than a chick-shaped, appearance (Figure 6B). In addition, we chose clinically epidemic strains, including *R. anatipestifer, E. coli, Salmonella, E. faecalis, S. aureus,* and *P. aeruginosa*, to contaminate the chicken embryos (Figure S1). Our work will serve as a guide for the poultry industry on how to identify dead embryos contaminated with *R. anatipestifer* in the future. This is because the findings demonstrated that each bacterial infection had a different appearance in chicken embryos (Figure S1).

In Group I, there were 193 eggs laid during the 203- to 231-d period, which was 120 eggs fewer than laid by the control group. In Group I, 63% of the eggs hatched, compared with 84% in the control group. In Group I, 94% of the 121 chicks that hatched did so with S63 infection. According to the findings, the S63-infected hens showed significantly reduced egg production and hatching rates. The potential threat of S63 infection to the poultry industry remained in the hatchling chicks.

Healthy hens were mixed with roosters with S63 infection in Group II. In Group II, 124 laying eggs were produced, fewer than in the control group (154 eggs). Group II had a hatching rate of 71%, which was lower than that of the control group (82%). Of the 88 chicks hatched in Group II, 47 tested positive for S63. However, no S63 DNA was detected in Group III. In subsequent experiments, we directly used S63-carrying semen to induce the same symptoms as observed before in hens (data not shown). According to the findings, S63 may be transmitted to hens by rooster semen or to offspring by S63-carrying hens. The limitation of the experiment is that there is no systematic confirmation of whether S63 are transmitted through air, contaminated water, and feeding, which will be further explored in future research.

In conclusion, this study was the first to give us an in-depth understanding of *R. anatipestifer*-infected chickens and revealed the infection can result in decreased egg production rates, decreased hatching rates, and jelly-like dead embryos, as well as the possibility that hens may be infected via rooster semen. We identified the potential hazards and risks to the poultry industry posed by *R. anatipestifer*-infected chickens.

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Author Contributions: Y.C drafted the main manuscript and performed the data analysis; X.L, Z.L, M.H, J.M, Y.L, Q.Z, L.L, X.Z, and M.Z planned and performed experiments; W.L, Y.C, and Y.L were responsible for experimental design. All authors reviewed and agreed the publication of this manuscript.

Ethics Statement: This study was approved by the Laboratory Animal Monitoring Committee of Shandong Academy of Agricultural Sciences and performed in strict accordance with the recommendations in the Guide for the Care and Use of Huahong Biology Co., Ltd., of Shandong Province, China.

Data Summary: The complete genome sequence of *R. anatipestifer* S63 with annotation has been deposited in GenBank under accession number CP110126.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103497.

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