Antibiotic resistance of *Riemerella anatipestifer* and comparative analysis of antibiotic-resistance gene detection methods

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ABSTRACT *Riemerella anatipestifer* is an important pathogen in waterfowl, and is generally multidrug resistant. This study assessed the current status of *Riemerella anatipestifer* antibiotic resistance and antibiotic-resistance genes (**ARG**s), compared the results of different detection methods, and evaluated a new method of studying the association between antibiotic resistance and ARGs in *Riemerella anatipestifer*. In this study, 51 strains of *Riemerella anatipestifer* were isolated from ducks on several farms, their resistance to 28 antibiotics was assessed, and the isolates were subjected to whole-genome sequencing. The number of ARGs carried by *Riemerella anatipestifer* was predicted, compared, and analyzed, and the consistency between ARGs and antibiotic-resistance phenotypes was assessed. The potential for loss of resistance genes during the sequencing and assembly of genome-wide framework map was assessed, and a new ARG detection method was pilot tested. The 51 strains of *Riemerella anatipestifer* were multidrug resistant (**MDR**) and had high level of resistance to aminoglycosides, trimethoprim, lincosamides, polypeptides, and macrolides. Based on the genome-wide framework map of the 51 strains, 3 local databases of ABRicate software and 1 online database of CARD website were used to detect ARGs, and a mean of 4 to 5 ARGs were identified per isolate. Although the detection results differed according to the database used, the general performance was consistent. The online website detected more types of ARGs than the ABRicate software. The association between ARGs and antibiotic-resistance phenotypes was assessed, and the *ermF* gene was identified as a possible key ARGs regulating macrolide resistance of *Riemerella anatipestifer*. The method used to investigate and detect *Riemerella anatipestifer* ARGs was convenient and rapid, and had strong accuracy and pertinence. The ARGs detection method reported here combined the advantages of PCR and genome detection, and could greatly reduce workload and detect ARGs more precisely.

Key words: antibiotic-resistance genes, antibiotic resistance, bioinformatics, Riemerella anatipestifer, genome

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INTRODUCTION

Riemerella anatipestifer infection is a bacterial disease caused by *Riemerella anatipestifer*, which is harmful to poultry, especially ducks (Yu et al., 2008). *Riemerella anatipestifer* is currently highly resistant to antibiotics (Zhong et al., 2009; Shousha et al., 2021). Horizontal gene transfer of ARGs across bacteria has gradually become the main mode of spread of antibiotic resistance (Zhu et al., 2020). There are many types of ARGs and detection methods, and the results of different detection methods are different; the risk of ARG transmission is high (Hall, 1997; Marano et al., 2019) and the impact is huge (Jian et al., 2021), hence increasing the difficulty of clinical prevention and treatment of *Riemerella anatipestifer*. Therefore, antibiotic resistance of *Riemerella anatipestifer* needs to be understood well, the distribution and prevalence of ARGs of *Riemerella anatipestifer* need to be investigated, the differences across various ARGs detection methods need to be evaluated, and a simple and rapid ARGs detection method needs to be established. This study aimed to compare and analyze the differences in detection results of various detection methods and databases, evaluate the accuracy of bacterial genome framework for predicting bacterial antibiotic resistance. We used a new detection method using an online database of ARGs. The prevalence of ARGs of Riemerella anatipestifer were investigated first, then found out the ARGs with high detection rate in the epidemic survey, and take this ARGs as the epidemic antibiotic resistance gene, and then PCR was used to detect the strains to be tested for epidemic resistance genes. This took advantage of the high throughput and high representativeness of genomics and genome databases,

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and combined the rapidity and specificity of PCR. We report the results of the study and describe the new detection methods to contribute to the clinical treatment of *Riemerella anatipestifer* and to inform further ARGs research.

MATERIALS AND METHODS

Isolation and Identification of Riemerella Anatipestifer

The strains used in this study were isolated and purified from the brain tissue of dead diseased ducks collected from farms in 7 provinces, including Shandong, Henan, Hebei, Guangdong and other three Provinces. The collected tissue samples were transported to the laboratory within 24 h in ice packs for bacterial isolation and culture.

The head of a dead duck was first wiped and disinfected with alcohol cotton balls, the brain tissue was aseptically extracted, streaked, and inoculated on a Tryptic Soy Agar (**TSA**) (Guangdong Huankai Microbiology Co., Ltd) plate (containing 10% newborn calf serum), followed by incubation at 37°C for 24 h in a CO_2 incubator (Shanghai Yiheng Scientific Instrument Co., Ltd.). A single colony was picked and streaked again on a TSA plate (containing 10% newborn calf serum); after 2 to 3 repeats, a single colony was picked from the last streak plate, inoculated in Tryptic Soy Broth (**TSB**) (Guangdong Huankai Microbiology Co., Ltd.) medium (containing 5% newborn calf serum), and incubated at 37°C for 24 h on a shaker (Shanghai Yiheng Scientific Instrument Co., Ltd.) to obtain the purified bacterial sample. The purified bacterial solution was frozen in a −80℃ refrigerator (Qingdao Haier Biomedical Co., Ltd) in a 20% glycerol-physiological saline.

Colonies on the TSA plate were collected to make bacterial smears, and Gram-staining (Hunan Bikeman Biotechnology Co., Ltd.) was performed to observe the bacterial staining characteristics and morphology. Referring to the 16S rRNA gene of *Riemerella anatipestifer* (NR-074429.1), *Riemerella anatipestifer*-specific 16S rRNA primers (Table 1) were designed and synthesized by Shanghai Sangon Bioengineering Co., Ltd. Bacterial DNA was extracted by the boiling method. The PCR reaction system (total volume, 25 μL) included the following: 12.5 μL of 2× Taq Master Mix (Nanjing Vazyme Biotech Co., Ltd.), 1 μL of upstream and downstream primers, 2 μL of bacterial DNA, and 6.5 μL of diethylpyrocarbonate-treated water (Shanghai Sangon Bioengineering Co., Ltd.). The amplification program was as follows: predenaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 45 s, a final extension at 72°C for 10 min, and then storage at 4°C. Five microliters of the PCR product were used for electrophoresis, conducted on 1% agarose gel (Shanghai Sangon Bioengineering Co., Ltd.). The electrophoresis was observed with a gel imaging system, photographs were taken, and the PCR product was then sent to Shanghai Sangon Bioengineering Co. Ltd. for sequencing. The primers were verified by BLAST alignment.

Bacterial Antibiotic Susceptibility Test

Referring to the Clinical and Laboratory Standards Institute (**CLSI**) antibiotic susceptibility test standards (CLSI VET01S ED5: 2020, CLSI M100 ED32: 2022) and the current antibiotic-use practices among local waterfowl, a total of 28 antibiotics, namely piperacillin, ampicillin, amoxicillin, cefepime, cefotaxime, cefixime, aztreonam, imipenem, meropenem, gentamicin, kanamycin, streptomycin, neomycin, amikacin, tobramycin, spectinomycin, enrofloxacin, ofloxacin, norfloxacin, ciprofloxacin, levofloxacin, chloramphenicol, florfenicol, trimethoprim, lincomycin, polymyxin B, azithromycin, and tetracycline, were selected for testing bacterial antibiotic susceptibility. The resistance profile of the isolated strains was determined using the Kirby-Bauer method, as recommended by CLSI. Colonies on the TSA plate were inoculated into TSB medium (containing 5% newborn calf serum) and incubated at 37℃ on a shaker for 12 h. One milliliter of the bacterial sample was centrifuged to discard the culture medium, and the purified bacterial sample was adjusted to 0.5 McFarland units with normal saline. A sterile cotton swab was dipped into the diluted bacterial solution, and spread evenly on the Mueller-Hinton (**MH**) agar medium; and the culture dish lid was opened and blow dried for 3 min. An antibiotic-sensitive tablet (Hangzhou Binhe Microbial Reagent Co., Ltd.) was attached to MH medium surface, and subsequently placed in a 5% CO_2 incubator and cultivated at 37℃ for 24 h, provided that the size of the inhibition zone of the quality control strain ATCC 25922 (Guangdong Huankai Biotechnology Co., Ltd.) met the quality control requirements. The diameter of the inhibition zone of each strain was measured, and antibiotic resistance was determined according to the breakpoint specified in the CLSI antibiotic sensitivity test standard. Strains that were resistant to 3 or more classes of antibiotics were defined as MDR strains (Magiorakos et al., 2012).

Riemerella anatipestifer Genome Sequencing and Assembly

A single colony of *Riemerella anatipestifer* was selected, inoculated into TSB medium (containing 5% newborn bovine serum), and cultured by shaking the

Table 1. Primer for the identification of *Riemerella anatipestifer* 16S RNA gene.

Gene ID	Forward (5′-3′)	Reverse (5′-3′)	Product size/bp
16S	GTATTGAAAGCTCTGGCGG	TCGCTTAGTCTCTGAACCC	644

solution at 180 r/min at 37℃. After 24 h, 1 mL of this seed liquid was inoculated into 35 mL of TSB medium (containing 10% newborn bovine serum), and incubated at 180 r/min at 37°C for 24 h. The culture was then centrifuged at 8500 rpm for 10 min, the supernatant was discarded, and the cells were harvested and sent to Beijing Nuohezhiyuan Technology Co., Ltd. for DNA extraction and whole-genome sequencing. Illumina PE150 was used as the sequencing platform, using a NovaSeq6000 System (Illumina). Fast QC software was used for sequencing data quality control, and Unicycler (Wick et al., 2017) software was used for sequencing raw data assembly. Fragments shorter than 200 bp were filtered out, and the sequencing data was completed for subsequent statistical analysis of genetic prediction.

Antibiotic-Resistance Gene Detection

We detected ARGs using ABRicate software. ABRicate software contains several ARGs databases, including NCBI AMRFinderPlus, CARD, and ResFinder, and each database contains data on thousands of ARGs. Based on the database selected by the user, the ABRicate software compares all ARGs in the database with genes in the input genome to derive genetic similarities. Genes with similarity to ARGs in the database are predicted as possible ARGs, and the higher the similarity the more likely that the input genome will develop antibiotic resistance.

ARGs were detected in the draft genome of the 51 strains of *Riemerella anatipestifer* by matching gene sequences to the AMRFinderPlus database (Feldgarden et al., 2019), CARD database (Alcock et al., 2020), and ResFinder database (Zankari et al., 2012) of ABRicate (https://github.com/tseemann/abricate); and the CARD online database (https://card.mcmaster.ca/home); and TB tools (Chen et al., 2020) was used to draw a heat map of the distribution of ARGs. Because ARGs with a low detection rate were not statistically representative or statistically significant in the consistency test, we calculated the degree of agreement between the genotype and phenotype of ARGs that were detected in more than 50% of strains; and then compared the ARGs and phenotypes detected by the different methods. We used both the traditional coincidence rate and the kappa value to determine level of agreement between ARGs and antibiotic-resistance phenotypes. Traditional coincidence rate = (true resistance + true sensitive)/total number of strains, kappa value was calculated by IBM SPSS statistics 25.0 software.

Detection of Antibiotic-Resistance Genes Using PCR, and Missed Detection in the Draft Genome

All 33 complete genomes of *Riemerella anatipestifer*, included in the NCBI Whole Genome Database (https://www.ncbi.nlm.nih.gov/genome), were downloaded, and the CARD online website, which is updated frequently and comprehensively, was used to detect ARGs; the detection rate of each ARG was counted. ARGs with a detection rate higher than 50% were considered to be the dominant ARGs in the strain. Primers were designed for the dominant ARGs using Primer 5.0 software. The reference sequences of each ARG were downloaded from the CARD ARGs database. The primers were tested for sensitivity and specificity using Primer 5.0 and NCBI Primer BLAST. The primer sequences with high scores and good specificity were used for ARGs detection. The dominant ARGs of *Riemerella anatipestifer* were detected by PCR, the frequency of detection of each ARG was recorded, and the PCR products were sent to Beijing Qingke Bio Co., Ltd. for sequencing and BLAST verification using the NCBI database.

In order to evaluate the degree of loss of ARGs from the draft genome, we compared and analyzed the PCR detection results and the draft sequence detection results of the ARGs detected by ABRicate software, and calculated the missed detection rate of each gene using the formula: Missed detection rate of a certain gene = (the number of strains in which the gene is missed in the draft sequence) / (total number of strains) × 100%.

RESULTS

Isolation and Identification of Strains

A total of 51 *Riemerella anatipestifer* strains were isolated, including 34 from Shandong, 4 from Hebei, 4 from Jiangsu, 3 from Guangxi, 3 from Fujian, 2 from Henan, and 1 from Guangdong (Table 2). Ten of the 51 *Riemerella anatipestifer* strains, with reference numbers starting NN, were donated by Professor Zhang Wei from Nanjing Agricultural University. The isolated strains were cultured on TSA plates (containing 5% newborn calf serum), and morphologically, each colony was small, white, and translucent. Gram staining revealed that all the isolated strain was Gram-negative. Agarose gel electrophoresis result of PCR products showed that all the isolate could amplify the target band of 644 bp. After PCR product sequencing, the BLAST result showed that the similarity with *Riemerella anatipestifer* 16s rRNA was 99.83%.

Strain Antibiotic Sensitivity Results

The antibiotic susceptibility classification of the 51 strains of *Riemerella anatipestifer* is shown in Figure 1, detailed antibiotic susceptibility data are shown in Supplementary Table 1; Forty-five of the 51 strains (88%) were resistant to 6 aminoglycoside antibiotics; and all strains were resistant to gentamicin, kanamycin, streptomycin, and neomycin. The 51 strains were resistant to 4 of the 5 quinolones, of which the highest prevalence of resistance was toward enrofloxacin and norfloxacin (45% and 55%, respectively), whereas the prevalence of ciprofloxacin and levofloxacin resistance was only 9.8%. The prevalence of resistance to methicillin, lincomycin, polymyxin B, and azithromycin was 88%, 94%, 98%, and 78%, respectively. The prevalence of resistance to 9 types of β-lactam antibiotics and 2 types of amido

Table 2. Strains used in the experiment.

Strain name	Separating regions	Separation time	Accession number
R-1	Shandong	2021-01	GCA_024124845.1
R-2	Hebei	2021-01	GCA_024124915.1
R-3	Shandong	2021-01	GCA_023914185.1
R-4	Shandong	2021-01	GCA_024124985.1
R-5	Shandong	2021-01	GCA_024124945.1
R-6	Henan	2021-04	GCA_024124315.1
R-7	Shandong	2021-11	GCA_024436155.1
R-8	Shandong	2021-01	GCA_024436195.1
R-9	Shandong	2021-03	GCA_024436185.1
R-10	Shandong	2021-01	GCA_024437765.1
R-11	Hebei	2021-01	GCA_024437815.1
R-12	Shandong	2021-01	GCA_024437825.1
R-13	Hebei	2021-01	GCA_024437955.1
R-14	Hebei	2021-01	GCA_025246525.1
R-15	Shandong	2021-01	GCA_025246685.1
R-16	Shandong	2021-03	GCA_025246665.1
R-17	Shandong	2021-05	GCA_024124875.1
R-18	Shandong	2021-01	GCA_025246705.1
R-19	Shandong	2021-01	GCA_025246725.1
R-20	Shandong	2021-11	GCA_025246815.1
R-21	Guangdong	2021-08	GCA_024124965.1
R-22	Shandong	2021-01	GCA_025246865.1
R-23	Shandong	2021-01	GCA_025246805.1
R-25	Shandong	2021-01	GCA_025246775.1
R-26	Shandong	2021-07	GCA_025246765.1
R-27	Henan	2021-04	GCA_025246845.1
R-28	Shandong	2021-01	GCA_025246905.1
R-29	Shandong	2021-11	GCA_025246895.1
R-30	Shandong	2020-11	GCA_025629305.1
R-31	Shandong	2021-01	GCA_025629325.1
R-32	Shandong	2021-01	GCA_025629335.1
R-33	Shandong	2021-03	GCA_025629395.1
R-34	Shandong	2021-01	GCA_025629365.1
R-35	Shandong	2021-01	GCA_025629385.1
R-36	Shandong	2021-03	GCA_025629445.1
R-37	Shandong	2021-01	GCA_025629415.1
R-38	Shandong	2021-01	GCA_025629465.1
R-39	Shandong	2020-11	GCA_025629505.1
R-40	Shandong	2021-11	GCA_025629545.1
R-41	Shandong	2022-03	GCA_025629485.1
R-42	Shandong	2022-04	GCA_025629515.1
NN71	Guangxi	2020-01	GCA_025629525.1
NN74	Guangxi	2020-01	GCA_025629585.1
NN118	Fujian	2020-05	GCA_025629665.1
NN133	Jiangsu	2020-07	GCA_025629635.1
NN137	Fujian	2020-09	GCA_025629605.1
NN151	Jiangsu	2020-10	GCA_025629615.1
NN159	Jiangsu	2021-01	GCA_025629705.1
NN162	Jiangsu	2021-01	GCA_025629725.1
NN187	Fujian	2021-04	GCA_025629685.1
NN210	Guangxi	2021-09	GCA_025629715.1

alcohols was below 10%. The 51 strains of *Riemerella anatipestifer* showed high resistance to aminoglycosides, trimethoprim, lincosamides, polypeptides, and macrolides, and high sensitivity to β-lactams and amido alcohols. Forty-five of the 51 strains (88%) were resistant to 5 or more types of antibiotics: The strain with the highest level of resistance was resistant to 7 types of antibiotics, 24 strains were resistant to 6 types of antibiotics, and 16 strains were resistant to 5 types of antibiotics.

Results of Antibiotic-Resistance Genes Comparisons

Comparisons of ARGs Using the ABRicate Software Database The genome framework of 51 *Riemerella anatipestifer* strains were searched for ARGs using ABRicate (Supplementary Table 2), and TBtools were used to draw a heat map of the distribution of ARGs (Figure 2). The searches using the NCBI database detected a total of 9 ARGs in 51 genomes, of which *OXA-209, erm(F), floR*, and *tet(X)* were the most frequently detected, with a prevalence of 90%, 76%, 67%, and 73%,respectively. A minority of strains harbored *aadS* and *ere(D)*, with a prevalence of 27% and 16%, respectively. Only one of the 51 strains carried *lnu (AN2), mef(En2)*, and *tet(Q)* genes at the same time. The CARD database also detected 9 ARGs, of which the detection rate of 7 was consistent with the information in NCBI database; the *tet(X4)* gene was also detected in 20 strains of bacteria, although the *lnu (AN2)* gene was not. Compared to the results of above 2 databases, fewer ARGs were found in the ResFinder database: a total of 6 ARGs were found, all of which were also found in the above 2 databases. The prevalence of *erm(F), floR, tet(Q), OXA-209*, and *ere(D)* detected by the ResFinder database was consistent with that of the NCBI and CARD databases. However, *aadS, lnu(AN2)*, and *mef(En2)* genes were not detected using the ResFinder database, whereas 47 of 51 strains had *tet (X)* genes detected by the ResFinder database, higher than that of the NCBI and CARD databases. Overall, detection of the ARGs of the 51 strains of *Riemerella anatipestifer* the 3 databases was generally consistent: 5 ARGs, *OXA-209, erm(F), floR, ere(D)*, and *tet(Q)*, were detected identically in all 3 databases, whereas the detection rate of *tet(X)* gene in the ResFinder database was higher than that in the other 2 databases.

CARD Online Database ARG Comparison Results The 51 strains of *Riemerella anatipestifer* were submitted to the CARD website for online detection of ARGs. More ARGs were detected in the CARD online database than in the 3 ABRicate databases. Eleven types of ARGs were detected, of which *RanB, VanT, RanA, erm (F)*, and *tet(X4)* were the most frequently detected (100%, 98%, 73%, 73%, and 71%, respectively). The prevalence of *aadS, tet(X), EreD*, and *tet(X6)* genes, was 22%, 33%, 16%, and 6%, respectively; *tet(Q)* and *Mef(En2)* genes were only detected in one strain. Compared to the above 3 local databases, the online database detected more types of ARG. This may have been related to the more frequent updating of the online database and more comprehensive data on ARGs.

PCR Results for ARG The downloaded 33 complete genomes of *Riemerella anatipestifer* were tested for ARGs using the CARD online website (Table 3). The types of ARGs in the *Riemerella anatipestifer* genome were relatively concentrated, The 10 most common ARGs detected were *RanA, RanB, aadS, ErmF, tet (X4), tet(X), tetQ, Mef(En2), EreD*, and *tet(X6)*. Among them, *RanA, RanB, aadS, ErmF*, and *tet(X4)* had the highest detection rates (100%, 100%, 67%, 52%, and 85%, respectively). We designed primers (Table 4) for the 5 ARGs with a detection rate of more than 50%. PCR amplification was conducted on the 51 isolated strains of *Riemerella anatipestifer*, and results of PCR product sequencing sequence BLAST showed similarity

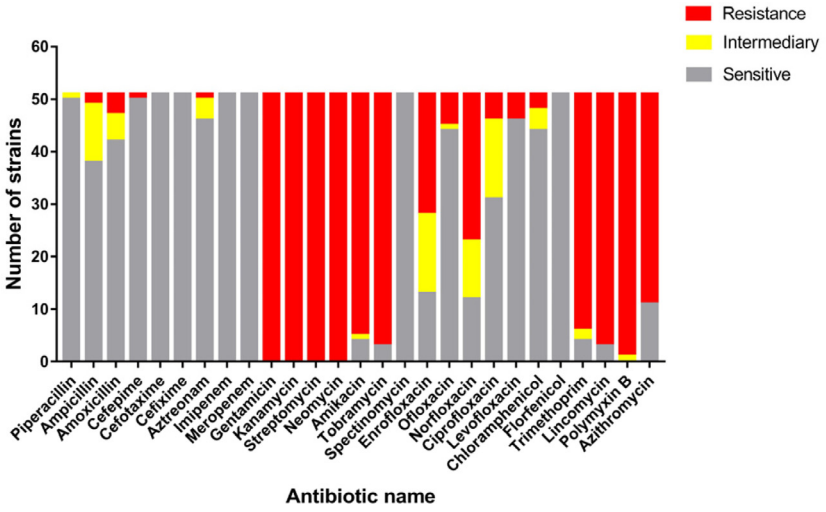

Figure 1. Statistical of antibiotics resistance of *Riemerella anatipestifer*.

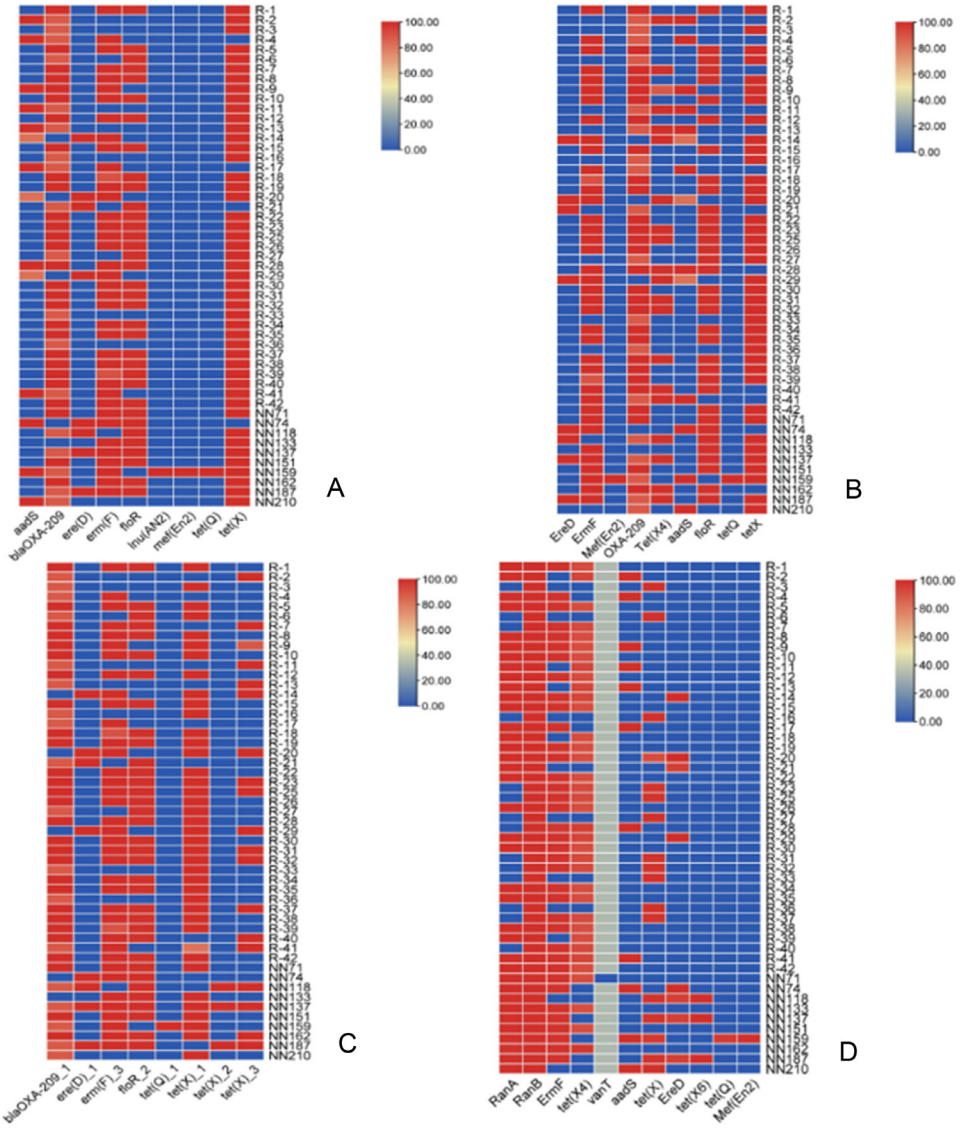

Figure 2. Statistical of the AGRs detection results of *Riemerella anatipestifer*. (A) NCBI database; (B) CARD database; (C) ResFinder database; (D) CARD online database. The depth of color represents the completeness of drug resistance gene detection. Darker the red, more complete is the detection of the drug resistance gene. Dark blue represents that the drug resistance gene is not detected.

Table 3. Comparison results of antibiotics resistance genes in genomes which downloaded in NCBI database.

Accession number	Release Date	RanA	RanB	aadS	ErmF	Tet(X4)	Tet(X)	tetQ	Mef(En2)	EreD	Tet(X6)
CP072186.1	2021/8/11	+	+	−	+	+	−	−	−	−	−
LT906475.1	2017/8/15	+	+	−	−	−	−	−	−	−	−
CP003388.1	2012/3/15	+	+	−	−	−	−	−	−	−	−
CP002346.1	2010/11/23	+	+	−	−	−	−	−	−	−	−
CP081925.1	2021/8/30	+	+	+	+	+	−	−	−	−	−
CP081934.1	2021/8/30	+	+	+	+	+	−	−	−	−	−
CP029760.1	2018/6/1	+	+	+	−	−	+	−	−	−	−
CP072188.1	2021/8/11	+	+	+	+	+	+	+	+	−	−
CP072190.1	2021/8/11	+	+	+	+	−	+	+	+	−	−
CP011859.1	2017/3/13	+	+	+	+	+	−	−	−	−	−
CP076675.1	2021/6/21	+	+	+	+	+	−	−	−	−	−
CP081923.1	2021/8/30	+	+	+	+	+	−	−	−	+	−
CP072196.1	2021/8/11	+	+	+	+	+	−	−	−	−	−
CP041029.1	2019/6/22	+	+	−	−	+	−	−	−	−	−
CP088073.1	2021/11/30	+	+	+	−	+	−	−	−	+	−
CP081928.1	2021/8/30	+	+	+	+	+	−	−	−	−	+
CP072185.1	2021/8/11	+	+	−	+	+	+	−	−	−	−
CP081924.1	2021/8/30	+	+	+	+	+	−	−	−	−	−
CP045564.1	2019/11/3	+	+	+	−	+	−	−	−	−	−
CP031845.1	2020/11/9	+	+	+	−	+	−	−	−	+	−
CP081927.1	2021/8/30	+	+	−	−	+	+	−	−	−	−
CP081931.1	2021/8/30	+	+	+	+	+	−	−	−	−	+
CP081929.1	2021/8/30	+	+	+	+	+	−	−	−	−	+
CP081937.1	2021/8/30	+	+	−	+	+	−	−	−	−	−
CP079205.1	2021/7/21	+	+	−	−	+	−	−	−	−	−
CP003787.1	2012/9/7	+	+	+	+	+	−	−	−	−	−
CP006649.1	2014/7/28	+	+	+	−	+	−	−	−	−	−
CP007204.1	2015/7/14	+	+	−	−	+	−	−	−	−	−
CP004020.1	2013/1/14	+	+	+	−	+	−	−	−	+	−
NZ_JAGFUR010000001.1	2021/5/24	+	+	−	−	+	−	−	−	−	−
CP007504.1	2015/7/9	+	+	+	−	+	−	−	−	+	−
CP007503.1	2015/7/9	+	+	+	+	+	−	−	−	+	−
CP002562.1	2011/3/7	+	+	+	−	+	−	−	−	−	−
Percentage(%)		100	100	66.67	51.52	84.85	15.15	6.06	6.06	18.18	9.09

Table 4. Primer of high-frequency drug resistance genes detected by NCBI.

Gene ID	Forward (5′-3′)	Reverse (5′-3′)	Product size/bp
Tet(X4)	AAGAGACAACGACCGAGAGG	ACCAGGTTCAAGCATAACAAGT	468
aadS	TGGACGCTTAAATTCGGAAATC	CATCTCTCACAAGACACTTTGC	346
RanA	TCTGAGATAGGCACGGGTAT	GGTGTTGATAATCGGTCGTAG	354
RanB	TCTACCATTCCCATTCCACTGT	CCGTCACTCTCATCGTTCCT	148
ermF	GGAGGTTCCATTGTCCTTCAA	AGTTGGCGGTGGCAAGAA	147

between the amplified gene and the target gene to be more than 99%. *RanA, RanB, aadS, tet(X4)*, and *ErmF* genes were detected in 96%, 98%, 88%, 98%, and 76% of the 51 strains, respectively. The dominant ARG types obtained by the NCBI survey were similar to those identified in the 51 strains of *Riemerella anatipestifer*.

Statistics of Lost Antibiotic-Resistance Genes in the Bacterial Draft Genome

The missed detection of different ARGs varied (Table 5). The 4 database detection results of *ermF* and *RanB* genes were consistent with the PCR detection results. In draft genome, more than 30% of strains had undetected *aadS* and *tet(X4)* genes.

Table 5. Statistical of error rates of 4 database detection results.

Database	RanA	RanB	aadS	tet(X4)	ermF
CARD-online	31.37%	1.96%	66.67%	31.37%	3.92%
ResFinder	*	*	*	*	0.00%
CARD	*	*	60.78%	58.82%	0.00%
NCBI AMRFinderPlus	*	*	60.78%	*	0.00%

*indicates that the gene has not been detected in the related database and cannot be calculated.

Level of Agreement Between Antibiotic-Resistance Genes and Antibiotic-Resistance Phenotypes

The detection rate of 4 ARGs, *OXA-209, erm(F), floR*, and *tet(X)*, exceeded 50% in the NCBI database test results. The level of agreement between *erm(F)*-azithromycin was high, as reflected by a high kappa value, whereas the level of agreement between the remaining ARGs and their corresponding phenotypes was below 50%, and the kappa values were low or inconsistent. The level of agreement using searches of the other 3 databases showed similar results (Table 6). With the exception of *erm(F)*-azithromycin, which had a high level of agreement and good consistency; the level of agreement of other ARGs were poor or inconsistent. However, the

Table 6. results of level of agreement between ARGs and antibiotic-resistance phenotypes.

Database	Pairing type	True Resistance	Error Resistance	Error Sensitive	Ture Sensitive	Number of Strains	Coincidence Rate	Kappa value	level of agreement
NCBI AMRFinderPlus	blaOXA-209-Ampicillin	13	33	0	5	51	35.29%	0.072	slight
	blaOXA-209-Amoxicillin	10	36	0	5	51	29.41%	0.052	slight
	blaOXA-209-Cefepime	1	45	0	5	51	11.76%	0.004	slight
	blaOXA-209-Aztreonam	4	42	1	4	51	15.69%	-0.024	No
	erm(F)-Azithromycin	38	1	2	10	51	94.12%	0.832	Perfect
	floR-Chloramphenicol	6	29	1	15	51	41.18%	0.074	slight
	tet(X)-Tetracycline	16	33	1	1	51	33.33%	-0.02	No
CARD	blaOXA-209-Ampicillin	13	33	0	5	51	35.29%	0.072	slight
	blaOXA-209-Amoxicillin	10	36	0	5	51	29.41%	0.052	slight
	blaOXA-209-Cefepime	1	45	0	5	51	11.76%	0.004	slight
	blaOXA-209-Aztreonam	4	42	1	4	51	15.69%	-0.024	No
	erm(F)-Azithromycin	38	1	2	10	51	94.12%	0.832	Perfect
	floR-Chloramphenicol	6	29	1	15	51	41.18%	0.074	slight
	tet(X)-Tetracycline	11	28	5	7	51	35.29%	-0.081	No
ResFinder	blaOXA-209-Ampicillin	13	33	0	5	51	35.29%	0.072	slight
	blaOXA-209-Amoxicillin	10	36	0	5	51	29.41%	0.052	slight
	blaOXA-209-Cefepime	1	45	0	5	51	11.76%	0.004	slight
	blaOXA-209-Aztreonam	4	42	1	4	51	15.69%	-0.024	No
	erm(F)-Azithromycin	38	1	2	10	51	94.12%	0.832	Perfect
	floR-Chloramphenicol	6	29	1	15	51	41.18%	0.074	slight
	tet(X)-Tetracycline	14	33	2	2	51	31.37%	-0.044	No
CARD-online	erm(F)-Azithromycin	36	1	4	10	51	90.20%	0.736	Substantial
	RanA-Gentamycin, Kanamycin, Neomycin, Streptomycin	37	0	14	0	51	72.55%	*	Uncountable
	RanA-Amikacin	33	4	14	0	51	64.71%	-0.139	No
	RanA-Tobramycin	34	3	14	0	51	66.67%	-0.107	No
	RanB-Gentamycin, Kanamycin, Neomycin, Streptomycin	51	0	0	0	51	100.00%	*	Uncountable
	RanB-Amikacin	47	4	0	0	51	92.16%	*	Uncountable
	RanB-Tobramycin	48	3	0	0	51	94.12%	*	Uncountable
	tet(X4)-Tetracycline	12	24	4	11	51	45.10%	0.048	slight
PCR	RanA-Gentamycin, Kanamycin, Neomycin, Streptomycin	49	0	2	0	51	96.08%	*	Uncountable
	RanA- Amikacin	45	4	2	0	51	88.24%	-0.055	No
	RanA-Tobramycin	46	3	2	0	51	90.20%	-0.049	No
	RanB-Gentamycin, Kanamycin, Neomycin, Streptomycin	50	0	1	0	51	98.04%	*	Uncountable
	RanB-Amikacin	46	4	1	0	51	90.20%	-0.032	No
	RanB-Tobramycin	47	3	1	0	51	92.16%	-303	No
	aadS-Gentamycin, Kanamycin, Neomycin, Streptomycin	45	6	0	0	51	88.24%	*	Uncountable
	aadS-Amikacin	41	4	5	1	51	82.35%	0.084	slight
	aadS-Tobramycin	42	3	6	0	51	82.35%	-0.085	No
	erm(F)-Azithromycin	38	1	2	10	51	94.12%	0.832	Perfect
	tet(X4)-Tetracycline	14	36	0	1	51	29.41%	0.051	slight

Note:True Resistance: having both AGRs and corresponding antibiotic-resistance phenotype.
Error Resistance: having AGRs but not a corresponding antibiotic-resistance phenotype.
Error Sensitive: no AGRs but has a corresponding antibiotic-resistance phenotype.
Ture Sensitive: neither a AGRs nor a corresponding antibiotic-resistance phenotype.
No agreement (Kappa value $\leq$ 0), None or slight (Kappa value = 0.00-0.20), Fair agreement (Kappa value = 0.21-0.40), Moderate agreement (Kappa value =0.41-0.60), Substantial agreement (Kappa value = 0.61-0.80), Almost perfect or Perfect agreement (Kappa value = 0.81-1).

level of agreement of the ARGs detected by PCR was above 82% except for *tet(X4)*-tetracycline, and the level of agreement was good.

DISCUSSION

Riemerella anatipestifer is currently prevalent in many regions, with high morbidity and mortality rates, causing great economic loss to the duck industry. At present, the resistance genes, virulence genes, and the serotyping and molecular typing system are not ideal, hence making prevention and treatment of *Riemerella anatipestifer* difficult. This study revealed that a few *Riemerella anatipestifer* ARGs were detected in different databases, which seems consistent with its severe MDR status, and therefore, may be related to a new antibiotic-resistance mechanism or *Riemerella anatipestifer* ARGs that we detected may not be involved in MDR. Antibiotic resistance is an important and urgent problem that needs to be understood and addressed (Davies and Davies, 2010). According to many other research, *Riemerella anatipestifer* has a high level of antibiotic resistance. The 51 *Riemerella anatipestifer* strains tested in this study showed a high level of resistance to aminoglycosides, trimethoprim, lincosamides, polypeptides, macrolides, and severe MDR. Zhong et al. (2009) isolated 224 strains of *Riemerella anatipestifer* between 1998 and 2005, and all isolates were more than 50% resistant to ampicillin, ceftazidime, cefazolin, cefepime, cefuroxime, benzathine, penicillin G, rifampicin, and methicillin/sulfamethoxazole. Resistance to amineptine, cefepime, benzathine, penicillin G, ceftazidime, and methotrexate/sulfamethoxazole ranged from 64% to 89% (Zhong et al., 2009). Egyptian researchers identified 7 strains of *Riemerella anatipestifer* in 60 freshly dead ducklings, and all the isolates were more than 70% resistant to penicillin, ampicillin, gentamicin, streptomycin, methicillin, cefoperazone, ceftazidime, and cefepime (Shousha et al., 2021). Hungarian researchers isolated 185 strains of *Riemerella anatipestifer* from local geese and ducks between 2000 and 2014, and the isolates were all more than 70% resistant to flumequine, tetracycline, erythromycin, and streptomycin (Gyuris et al., 2017).

Most of the ARG databases are based on gene sequences submitted by different researchers, existing ARG databases, and published papers. The databases are based on voluntary submission and data exchange, and databases such as the NCBI AMRFinderPlus database, which is a typical voluntary submission-based gene database, allow anyone to submit gene sequences to the database, and these sequences are shared in the website after review and collation. However, differences in algorithms, ARG coverage, and ARG classification rules among databases led to different ARG matching results. Therefore, when applying the whole-genome sequencing (**WGS**) method to predict ARGs, multiple databases should be used, and different prediction results should be combined to make judgments.

In this study, the level of agreement between ARGs and phenotypes detected in *Riemerella anatipestifer* was generally low, except for *ermF*-aminoglycoside. Considering the low reliability of using the current ARGs of *Riemerella anatipestifer* to predict the resistance phenotype, and the results of previous studies, ARGs appear to not only determine antibiotic resistance, but also have far-reaching effects on the spread and transfer of ARGs in the livestock and poultry industries and to humans (O'Brien, 1997). Due to the transfer of ARGs between different hosts, genes that have not previously shown antibiotic resistance, may be expressed with a change in environment and host, resulting in the development of antibiotic resistance. Multiple studies have shown that although some ARGs do not manifest antibiotic resistance, they can serve as natural host reservoirs to provide a source of ARG transfer (Umar et al., 2021). Therefore, evaluating the role of ARGs should not be limited to assessing the relationship between ARGs and phenotypes, but should also consider their transmission potential. For the detection of ARGs, many researchers use PCR-based methods, whereas relatively few use genomics technology. If only some of the ARGs are detected, the accuracy and reliability of test results can be affected by other undetected ARGs. With the continuous development of open gene databases (such as NCBI) and online prediction sites (such as ResFinder and CARD), hundreds or thousands of bacterial genomes of a specific species from different regions and isolated at different times can be downloaded, using software or online websites that conduct rapid prediction of ARGs. These open gene databases can be used to determine the dominant ARGs of the bacteria at any time point, and then PCR can be used to identify the dominant ARGs of the strains to be tested, thereby reducing workload and improving work efficiency.

In this study, considering the sequencing cost of the complete genome and the operational difficulty, a bacterial draft genome was used to detect ARGs at low cost and with relative simplicity. The bacterial draft genome might lose some ARGs during the sequencing and splicing processes. If a gene is present in the bacterial genome, it can be detected by PCR, but if it is lost from the genome-frame map, it will not be detected by the database. Therefore, if a strain is PCR-positive for an ARG but the ARG is not detected by the database, this suggests that the ARG has been lost from the draft sequence. PCR results of the strains were compared with the genome draft results, and some ARGs were found to have been lost in the draft genome, resulting in decreased detection. Therefore, for large-scale bacterial epidemic ARG surveys, the use of bacterial draft genome can greatly reduce the workload and obtain more ARG types for analysis. For important pathogenic bacteria, PCR testing can be performed first, based on the results of dominant ARGs identified in surveys or clinical isolates. Alternatively, complete genome sequencing analysis can be performed directly in order to identify the prevalence of ARGs of the strain at a specific time point, conduct more in-depth research and analysis, and

predict and warn of the risk of ARG transmission. The ARG detection method established in this study aimed to detect ARGs in a targeted manner, improve work efficiency, and provide a new perspective for the investigation of the epidemiology of ARGs and the detection of ARGs of pathogens. Instead of using only one method, multiple methods should be used, comprehensively considering the antibiotic-resistance phenotype of the strain and the risk of ARG transmission, and the most appropriate detection method should be selected according to the research purpose or treatment needs. Various other assays involving antibiotic resistance may be considered, such as novel multiplex allele-specific PCR for antibiotic resistance in *Mycobacterium tuberculosis* (Evans and Segal, 2010), western blotting to detect fosfomycin resistance (García et al., 1994), and DNA microarray to detect genetic elements carrying glycopeptide resistance clusters in enterococci (Cassone et al., 2008).

Regarding the level of agreement between ARGs and antibiotic-resistance phenotypes, the level of agreement and consistency of the *ermF* gene was relatively high. Because other ARGs did not show good consistency, it can be inferred that the *ermF* gene of *Riemerella anatipestifer* might be the key ARG controlling macrolide resistance. Notably, the ARG level of agreement is usually used to measure the ability of the ARG to regulate the antibiotic-resistance phenotype, but there is a lack of corresponding statistical evaluation indicators. Some ARGs detected in this experiment, such as *RanA* and *RanB*, showed a level of agreement with antibiotic resistance higher than 90%, whereas the kappa value was lower than 0.1, showing poor agreement. Abdelaziz et al. (2021) used Pearson chi square to calculate the association between the phenotype of antibiotic resistance and the resistance genes, and found that the *blaSHV* and *MexA* genes were significantly associated with resistance to fluoroquinolones, amikacin, tobramycin, cotrimoxazole, and β-lactams other than aminoglutethimide. Kumburu et al. (2019) calculated the kappa values for cefazolin, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, and ampicillin, with corresponding resistance genes with kappa values ranging between 0.05 and 1, indicating slight to complete concordance. This indicated that the evaluation of regulatory status of ARGs on antibiotic-resistance phenotypes should not only rely on the simple calculation index of the level of agreement, but also combine several evaluation indices to determine the role of ARGs in antibiotic-resistance phenotypes, and consider other factors that control the expression of ARGs.

In this study, we performed resistance testing and genome sequencing on 51 strains of *Riemerella anatipestifer* and found differences in the results of different resistance gene detection methods; we had initially confirmed the possibility of missing resistance genes in the genomic-framework maps that are currently in use, and found that most of the ARGs that we detected do not appear to be the key genes controlling antibiotic resistance in *Riemerella anatipestifer*. With more researchers focusing on the antibiotic resistance and ARGs of *Riemerella anatipestifer*, the challenge of antibiotic resistance in *Riemerella anatipestifer* can eventually be solved.

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DISCLOSURES

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102405.

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