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Characterization of *Escherichia coli* pathogenicity and drug resistance in yolk peritonitis

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ABSTRACT Yolk Peritonitis can lead to a rapid decline in egg production, which seriously affects the health of laying hens and the profitability of chicken farms. *Escherichia coli* (*E. coli*) is the most common cause of yolk peritonitis in laying hens. In this study, bacterial samples were collected from the ovaries and fallopian tubes of laying hens with suspected yolk peritonitis from a laying farm in Jiangsu Province, and their pathogenicity and drug resistance were investigated. Initially, morphological and biochemical detection methods were employed to isolate and identify the pathogenic bacteria. The results showed that a total of 16 strains of *E. coli* were isolated from laying hens with yolk peritonitis. Subsequently, the drug resistance and pathogenicity of a randomly selected *E. coli* strain were analyzed and predicted by genome sequencing technology, and the drug resistance of E. coli was verified by drug sensitivity test and PCR. Finally, the virulence was verified by infection experiment in mice. The study revealed that the egg-yolk peritonitis in laying hens was caused by *E. coli* infection, and the genome sequencing analysis revealed that the bacteria had multidrug resistance and high virulence. The drug susceptibility testing indicates that *E. coli* exhibited resistance to aminoglycosides, β-lactam, macrolides, fluoroquinolones, and sulfonamides. In this study, resistance genes including KdpE, aadA5, APH(3 ")-ID, APH(6)-ID, and TEM-1 were identified, and their expression levels varied across different stages of bacterial growth. The results of virulence analysis indicated a mortality rate of 50% in mice infected with *E. coli* at a concentration of 2.985×10^7 CFU/mL. *E. coli* infection resulted in damage to various tissues and organs in mice, with the intestinal tissue structure being the most severely affected. This study provides a reference for the study of drug resistance mechanisms in *E. coli* and provides valuable insights into the selection of drugs for the treatment of vitelline peritonitis.

Key words: *Escherichia coli*, yolk peritonitis, genome sequencing, multidrug resistance, pathogenicity

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INTRODUCTION

Yolk peritonitis is a systemic disease with a high incidence in egg farming. The main symptoms of the affected chickens are a cheese-like yellowish-white exudate in the abdominal cavity and varying degrees of inflammatory reaction in the peritoneum (Pors et al., 2014; Olsen et al., 2016). In the laying hen industry of China, small-scale breeding still constitutes a significant proportion. These farmers encounter various challenges related to diseases, among which yolk peritonitis stands out as one of the most prevalent and impactful ailments. Yolk peritonitis directly impacts the economic value of laying hens by reducing their egg-laying rate and significantly increasing the occurrence of egg deformities and soft shells (Srinivasan et al., 2013; Landman and Van Eck, 2015). Clinical causes of yolk peritonitis in chickens are complex and are usually due to improper feeding management and infestation by various pathogenic microorganisms in the environment, such as *Escherichia coli* (***E. coli***) (Landman et al., 2021). Previous studies have shown that 15.39% of reproductive tract abnormalities in commercial laying hens from 21 to 80 wk of age were associated with *E. coli* yolk peritonitis. Yolk peritonitis caused by avian pathogenic *E. coli* can lead to a 3 to 4% mortality and a 2 to 3% reduction in egg production in hens (Malik et al., 2022; Joseph et al., 2023). In fact, the effective prevention, control, and treatment of

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yolk peritonitis caused by avian pathogenic *E. coli* has been a long-standing problem in egg farming (Telli, 2022).

E. coli is widely distributed in the environment and is an important zoonotic conditional pathogen (Zhu et al., 2021). Antibiotics are the main method of treating the *E. coli* disease, but with the continuous development of intensive and large-scale farming mode, antibiotic abuse is widespread worldwide, leading to the enhancement of bacterial resistance and the increasing number of superbugs (Afridi et al., 2020). Back in 2014, economist Jim O'Neill already predicted that 10 million people worldwide will die each year from superbug infections by 2050, costing the world up to $100 trillion (O'Neill, 2014). The report of the United Nations Environment Programme also points out that the abuse of antibiotics has become a serious problem, especially in animal husbandry, where antibiotics are widely used as growth promoters, resulting in serious environmental pollution (Murray et al., 2022). At the same time, people and livestock are infected by exposure to more drug-resistant bacteria and drug-resistant genes through air, food and water, increasing the risk of health and medical failure of antibiotic treatment (O'Flaherty et al., 2018, 2019; Schmiege et al., 2021). More seriously, these drug-resistant genes may be transferred horizontally from the environmental host bacteria to the pathogens, or from the pathogens to the primary host bacteria in the environment, making the spread more rapid and widespread (Wang et al., 2019). *E. coli* is a typical multi-drug-resistant bacteria, and in the course of broiler farming, studies have found that *E. coli* with broad spectrum cephalosporinase from imported broiler parent flocks can be clonally and horizontally transmitted to the meat of broilers even in countries where cephalosporins are not used (Agersø et al., 2014; Apostolakos et al., 2021). Some studies have even claimed that pathogenic *E. coli* strains with virulence genes may act as zoonotic pathogens and virulence reservoirs and may jump to other species and cause human infections (Poulsen et al., 2017; Arimizu et al., 2019; Brisola et al., 2019; Kim et al., 2020).

Currently, the homeostasis of biological populations changes with environmental changes, especially the study of strain mutation and drug resistance generation in the environment has become a current hotspot, while the discovery of *E. coli* mutation and novel drug-resistance genes in the farming industry is still lacking. Therefore, the aim of this study was to sequence and analyze the whole genome sequencing of *E. coli* causing yolk peritonitis in laying hens, and to screen the key virulence factors and drug-resistance genes of this strain in combination with drug sensitivity tests, so as to provide a strong basis for the prevention and treatment of yolk peritonitis in laying hens, and also to provide a reference for drug screening of novel drug-resistance genes for yolk peritonitis in laying hens infected with *E. coli*.

MATERIALS AND METHODS

Sample Source and Ethics Statement

The experimental samples were obtained from ovaries and fallopian tubes of laying hens with suspected yolk peritonitis in a chicken farm in Changzhou, Jiangsu Province. There are a total of 12,000 grass hens in this laying farm. Every 3 chickens are kept in a cage measuring 40 cm in long, 40 cm in wide and 38 cm in high. For this study, we collected 96 affected chickens and randomly selected 10 individuals for bacterial isolation from their ovaries and oviducts. The isolated bacterial cultures were sent to Jiangxi Provincial Key Laboratory for Animal Health at Jiangxi Agricultural University in Nanchang, Jiangxi, China for pathogenicity and drug resistance testing.

The mice were purchased from the animal experimental base of Nanchang University (China). All the animals were acclimated under standard laboratory conditions (ventilated room, 25 ± 1°C, 60 ± 5% humidity, 12 h light / dark cycle) and had free access to standard water and food. All procedures were conducted following the "Guiding Principles in the Care and Use of Animals" (China) and were approved by the Laboratory Animal Ethics Committee of Jiangxi Agriculture University.

Isolation and Identification of Pathogenic Bacteria

Ten diseased laying hens were randomly selected for anatomical observation, and fresh diseased materials were smeared with sterile cotton swabs in a 37℃ incubator on a common nutritional agar medium for 16 h and suspected *E. coli* was observed by Gram staining. After 3 generations of purification and culture, a single colony was selected and inoculated on an eosin methylene blue (**EMB**) culture medium and cultured in an incubator at 37℃ for 16 h.

From the isolated bacteria, a random strain was selected for PCR identification. The genomic DNA was extracted in accordance with the instructions provided by the bacterial genomic DNA extraction kit (TIANGEN Biotech, Beijing, China). Subsequently, PCR amplification was conducted using 16s rRNA universal primers(27F: 5′-CTACTTCTTTTGCAACCCACTCCCA-3′, 1492R: 5′-CATGCAGTCGAACGGTAACAGGAA-3′) and the extracted genomic DNA as a template. The PCR products were recovered by a DNA gel extraction kit (Beyotime Biotech, Shanghai, China) and sent to Sangon Biotech (Shanghai, China) for sequencing, and sequence homology was analyzed using GenBank BLAST tools (BLAST: Basic Local Alignment Search Tool (nih.gov)). This Whole Genome Shotgun project has been deposited at GenBank under the accession JAXCVA000000000.

The Strain Genome Sequencing and Analysis

Genomic DNA of the strain was extracted with the SDS method. The harvested DNA was detected by the agarose gel electrophoresis and quantified by Qubit. Whole-genome sequencing was performed on the Illumina HiSeq PE150 platform with a target sequencing depth of 100x. A-tailed, ligated to paired-end adaptors and PCR amplified with a 350 bp insert was used for the library construction at the Beijing Novogene Bioinformatics Technology Co., Lt. (Beijing, China) The original data obtained from sequencing were filtered to obtain valid data, and the analysis method based on K-mer statistics was used to estimate the genome size. SOAPdenovo (http://soap.genomics.org.cn/soapdenovo.html), SPAdes, and ABySS assembly software were used to recombine the data and integrate them through CISA to obtain the final sequence. The GC content and reads coverage depth of the assembled sequence were calculated to summarize the GC bias of the genome. We used 5 databases to predict gene functions. They were respective the Non-Redundant Protein Database (**NR**), Cluster of Orthologous Groups of proteins (**COG**), Kyoto Encyclopedia of Genes and Genomes (**KEGG**), Gene Ontology (**GO**), Pathogen Host Interactions Database (**PHI**). For pathogenic bacteria, we added the drug resistance and pathogenicity analyses. We used the Comprehensive Antibiotic Research Database (**CARD**), Antibiotic Resistance Genes Database (**ARDB**), and Virulence Factors of Pathogenic Bacteria (VFDB) to perform the above analyses. The sequences of the 7 housekeeping genes (adk, fumC, gyrB, icd, mdh, purA, and recA) of *E. coli* were extracted from the genomic sequencing results and uploaded to the Enterobase database (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search) to determine the corresponding sequence numbers for multilocus sequence typing (**MLST**) analysis.

Analysis of Drug Resistance of the E. coli Strain: Drug Sensitivity Test and Detection of Drug Resistance Gene

The strain, identified as *E. coli*, was subjected to a drug sensitivity test using the paper disc method (Kirby-Bauer method) to determine the susceptibility of 17 antimicrobial drugs in 6 classes: aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin), betalactams (cefoperazone, ceftazidime, cefazolin, penicillin); macrolides (erythromycin, azithromycin), sulfonamides (cotrimoxazole); fluoroquinolones (ciprofloxacin, ofloxacin, norfloxacin), tetracyclines (doxycycline, tetracycline, minocycline). The results were interpreted as sensitive, intermediate, or resistant, according to the standards of the National Committee for Clinical Laboratory Standards.

Validation analysis of *E. coli* drug-resistance genes by PCR based on the predicted results of the whole gene sequencing and drug sensitivity test results. The PCR amplification primers were designed according to the gene sequences published in GenBank (Table 1) and synthesized by Sangon Biotech (Shanghai, China). The PCR reaction conditions were as follows: 35 cycles of 95° C for 5.5 min, 54°C for 60 s, and 72°C for 60 s. The PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide and visualized under UV illumination.

Table 1. PCR amplification primers.

Gene name	Primer sequences(5′ - 3′)	Product length
aadA5-F	CTCCGCCACGACATCCTTT	206bp
aadA5-R	CCCTTCCAATCCGACTCTGC	
kdpE-F	CCAGATTTGATTATTCTCGAT	510bp
kdpE-R	AGTAATGAAATGGCGTGGG	
APH(3′′)-Ib-F	GCAGGAGGAACAGGAGGGT	464bp
APH(3′′)-Ib-R	CTTCGGCGTTAGCAATCA	
APH(6)-Id-F	GGGACTCCTGCAATCGTC	498bp
APH(6)-Id-R	GCCAAAGCCCACTTCACC	
TEM-1-F	GCGGTATTATCCCGTGTTG	295bp
TEM-1-R	CGTCGTTTGGTATGGCTTC	
mphA-F	TGCTGGCAATGCTCAAGAATC	555bp
mphA-R	CCGCTTCATACGTGAGGAGGA	
Mrx-F	AGGACAGTGAGCTGCCCAAAC	447bp
Mrx-R	CAATGCCAAGGAGACCACCAG	
leuO-F	GGTTCAGTTCGTCAGGCAT	439bp
leuO-R	GCTTGCTTATCTACCGTGTC	
sul1-F	TATTGCGCCGCTCTTAGACG	496bp
sul1-R	CGCTGGACCCAGATCCTTTA	
sul2-F	CGGTCCGGCATCCAGCAAT	426bp
sul2-R	AGCGAGGTTTCGGGAGCAG	
parC-F	GAATCTGACCACGAGAACCC	424bp
parC-R	CCAGTTTGGCAAGATGACG	
gyrA-F	TGCGGTCGGTGAAGTTGT	433bp
gyrA-R	TTGCTGTTCGGTCAGGTAG	
mfd-F	TGCCAACGACGCCAAACTGTA	503bp
mfd-R	CGATACGTACCGGCCAGTTGG	

Growth Curve Determination of the E. coli Strain

Draw the bacterial growth curve to determine the time point of gene expression. In short, the known concentration of bacterial liquid was inoculated into a Mueller-hinton broth medium so that the final concentration was 5×10^5 CFU/mL and cultured in a 37℃ constant temperature oscillating incubator. A tube of bacterial liquid was taken out every 2 h and its absorbance was detected. Finally, the growth curve was drawn by the Graphpad software. The measuring points are when the logarithm of bacteria increases, when the number of bacteria reaches the maximum and when the number of bacteria is stable.

Test for Antibiotic Susceptibility

One antibiotic from each of the 5 drug classes was selected as a representative and its minimum inhibitory concentration was measured to determine the drug concentration required for the experiment. The antibiotics selected for this study were: aminoglycosides (streptomycin), β-lactams (penicillin), macrolides (erythromycin), sulfonamides (cotrimoxazole), fluoroquinolones

(norfloxacin), and tetracyclines, all of which were purchased from G-CLONE Biotech (Beijing, China). The drug powder was dissolved in accordance with the requirements of the Clinical and Laboratory Standards Institute and then diluted in a multiplicative gradient so that the final concentrations were 1024, 512, 256, 128, 64, 32, 16, 8, 4, and 2 μg/mL when mixed with known concentrations of bacterial broth. The mixture of the drug and the bacterial solution was incubated in a constant temperature shaker at 37℃ for 12 h, and then the turbidity of the bacterial solution was observed.

Detection of the Expression of Drug-Resistant Genes in Different Growth Stages of the E. coli *Strain*

The highest antibiotic concentration that cannot affect bacterial growth was determined as the drug concentration for the experiment based on the results of the drug sensitivity test and growth curve measurement. The sampling time of the experiment was when the number of bacteria was logarithmically increasing, when the number of bacteria reached the maximum and when the number of bacteria was stable, and the mixture of different concentrations of different drugs and bacteria was incubated in a biochemical incubator at 37℃.

DNA samples of the bacterial broth were extracted using ONE-4-ALL Genomic DNA Mini-Preps Kit (Sangon Biotech, Shanghai, China) and then detected by RT-QPCR using a StepOnePlu real-time fluorescence quantitative PCR (Thermo Fisher Scientific Inc, Grand Island, NY, U.S.A) instrument, the data were analyzed with one-way ANOVA (Tukey's test, $p < 0.05$) by using SPSS. RT-QPCR data were log-transformed before analysis.

Virulence Testing and Measure of LD50 Values

The 50% lethal dose (**LD50**) values of mice were estimated according to improved Spearman-Kärber method (Spearman, 1908; Kärber, 1931). Kunming mice weighing 18 to 22 g were used in this study. Forty mice were randomly divided into 4 groups (A, B, C, and D) of 10 mice. The mice in groups A, B, and C were intraperitoneally injected with 2 mL of the diluted solution of *E. coli* with concentrations 10^7, 10^8, and 10^9 CFU/mL, respectively. In the control group, mice in group D were injected with 2 mL of normal saline. The mice were observed continuously and after 24 h, the number of dead mice was counted in each group. The percentage of mice that had died in each group was transformed to LD50 values determined by the improved Kärber formula: $\mathrm{LD50} = \lg^{-1}\left[\mathrm{X_n} - \mathrm{i}\left(\frac{2\sum \mathrm{m+h}}{2\mathrm{n}} - 0.75\right)\right]$. The dead mice were autopsied, various organs and intestines of the mice were preserved with paraformaldehyde, and then the pathological sections were made. Sections were counterstained with thionin solution (Nissl stain) and hematoxylin and eosin (HE stain), and histopathological changes were observed under an optical microscope.

RESULTS

The Bacteria Were Isolated and Identified as E. coli *Strain*

The selected 10 sick chickens were all diagnosed with yolk peritonitis by anatomy. In the autopsy of the sick and dead chickens, it was found that there were adhesion of the peritoneum, necrosis, and degeneration of follicles in the abdominal cavity, hyperemia of the theca, obvious dilatation of the fallopian tube and thinning of the tube wall (Figure 1-A2). There were turbid yellowish-brown liquid and ruptured yolk in the abdominal cavity, and give off a foul smell, with yellow cheese-like substances attached to the surface of the abdominal organs (Figure 1-A3). After the diseased materials were cultured in the ordinary medium for 16 h, round, smooth, moist, and translucent grey-white colonies could be seen (Figure 1-A4). Cultured in EMB selective medium, there is round colonies with dark purple-black, smooth, moist, and metallic lustre (Figure 11-A5). Gram staining showed pink rod-shaped bacteria, and it was preliminarily determined that the disease was caused by *E. coli* infection (Figure 1-A6).

The results of biochemical identification of the strain showed that the amino acids in the control tube did not change, indicating that the experimental process was not contaminated. Hydrogen sulfide, peptone, phenylalanine, urea, and citric acid were negative. Lysine and ornithine changed from brown to purple, glucose, lactose and dulcitol all changed from purple to yellow, showing positive, in which glucose fermentation tube produced acid and gas. These are in line with the characteristics of *E. coli* (Figure 1B). Sixteen strains exhibiting these characteristics were isolated from 10 diseased chickens, and the positive rate of infection was 100%.

From the isolated bacteria, a random strain was selected for PCR identification to further confirm that the isolated bacteria was *E. coli*. The target band of the PCR product was detected by polyacrylamide gel electrophoresis at the expected position of about 1400bp, which was consistent with the size of the target band (Figure 2A). The 16S rRNA PCR products of the isolated strains were sequenced and compared with the gene sequences of the reference strain *E. coli* in the GenBank database (Figure 2B). The results showed that the homology of the 16S rRNA gene sequence between the isolated strain and the *E. coli* strain was as high as 99.79% (Figure 2C). The strain was named *E. coli*. L350_DMS27638.

Functional Annotation and Classification of the E. coli *Genomic*

The sequencing data were filtered to remove the sequence containing Adapter and low-quality data, and

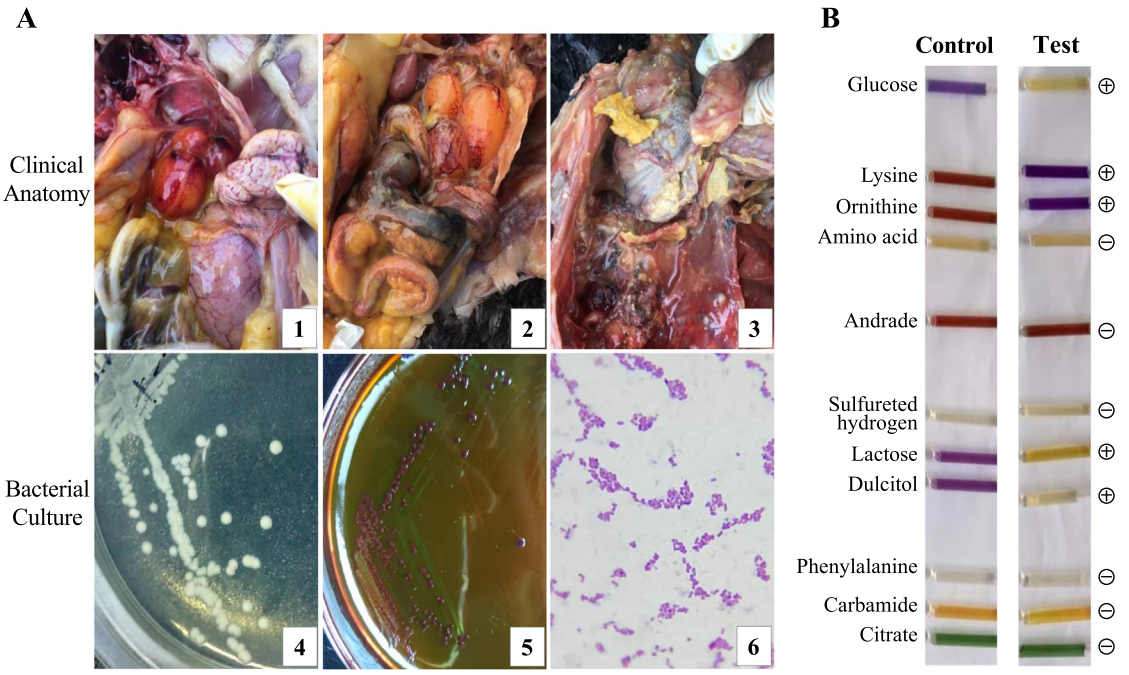

Figure 1. The autopsy of diseased chickens and preliminary identification of pathogens. (A) The autopsy and bacterial culture identification of diseased hens. 1: Normal chicken; 2-3: Severe yolk peritonitis in diseased hens; 4: Colony morphology on ordinary Agar medium; 5: Colony morphology on EMB medium; 6: Gram staining of bacterial fluid. (B) Biochemical identification of bacteria. Row 1: Name of the biochemical experiments; Row 2: Control group; Row 3: Test group; Row 4: Results of biochemical experiments.

the obtained Clean Data was used for follow-up analysis. The result of this experiment was that Q20(Percentage of Phred quality score > 20) was 97.95% and Q30(Percentage of Phred quality score > 30) is 94.09%. The detection of GC content distribution was 50.85%. The sequencing results showed that the genome length of the *E. coli* was 4,977,738 bp and the number of genes was 4,754, of which the coding gene was 4,350,870 bp, accounting for 87.41% (Figure 3). The E. coli genome contig N50/N90 and scaffold N50/N90 were 193,452 bp/50,282 bp and 193,452 bp/52,460 bp, respectively.

Using Diamond software, the amino acid sequences of the target species were compared with the NR database, and the genes of the target species were combined with their corresponding functional annotation information. the results showed that the number of genes annotated with *E. coli* was 4734 (Figure 4A). All the annotated genes in the strain were compared with the COG database, and a total of 3,793 sequences were assigned to COG classification. Among the 24 COG categories, carbohydrate transport and metabolism (421, 11.1%) is the largest group, followed by amino acid transport and metabolism (371, 9.8%), transcription (336, 8.9%) and general function prediction (304, 8.0%) (Figure 4B). By comparing the amino acid sequence of the *E. coli* strain with the KEGG database, it was found that a total of 4,651 genes were annotated in 207 pathways of KEGG. Among them, 335 genes are related to the carbohydrate metabolic pathway. Secondly, 272 genes were annotated in the membrane transport pathway (Figure 4C). The genes of the *E. coli* strains were analyzed by GO using IPRscan software to classify their biological functions. A total of 3,198 genes have been successfully assigned to the GO vocabulary with 7,540 functional terms. Among them, genes 3,443 (45.67%), 2,940 (39.0%) and 1,157 (15.3%) are assigned to the following categories: biological processes, cellular components, and molecular functions (Figure 4D).

Resistance Genes and Pathogenic Genes of the E. coli *Genomic*

The resistance gene identifier software provided by the CARD database was used to compare the amino acid sequences of the target species with the CARD database to annotate the resistance gene information in the database. A total of 88 genes were annotated in the result database, among which 4 genes were associated with aminoglycoside resistance: KdpE, aadA5, APH (3″)-Ib, APH(6)-Id, 2 were associated with β-lactam resistance: CYM-62, TEM-1, 2 genes were associated with macrolide resistance: Mrx, mphA, 3 genes related to quinolone resistance: MFD, ParC, gyrA, and 3 genes related to sulfonamide resistance: LeuO, sul1, sul2. In addition, there were some factors related to other antibiotic resistance, such as polymyxin resistance, aminocoumarin resistance, diaminopyrimidine resistance and so on. It was worth noting that many genes related to antibiotic resistance exist in efflux pumps. The results revealed that 33 resistance genes were obtained by matching the annotated genes with the ARDB database, and clustering of antibiotic resistance genes revealed

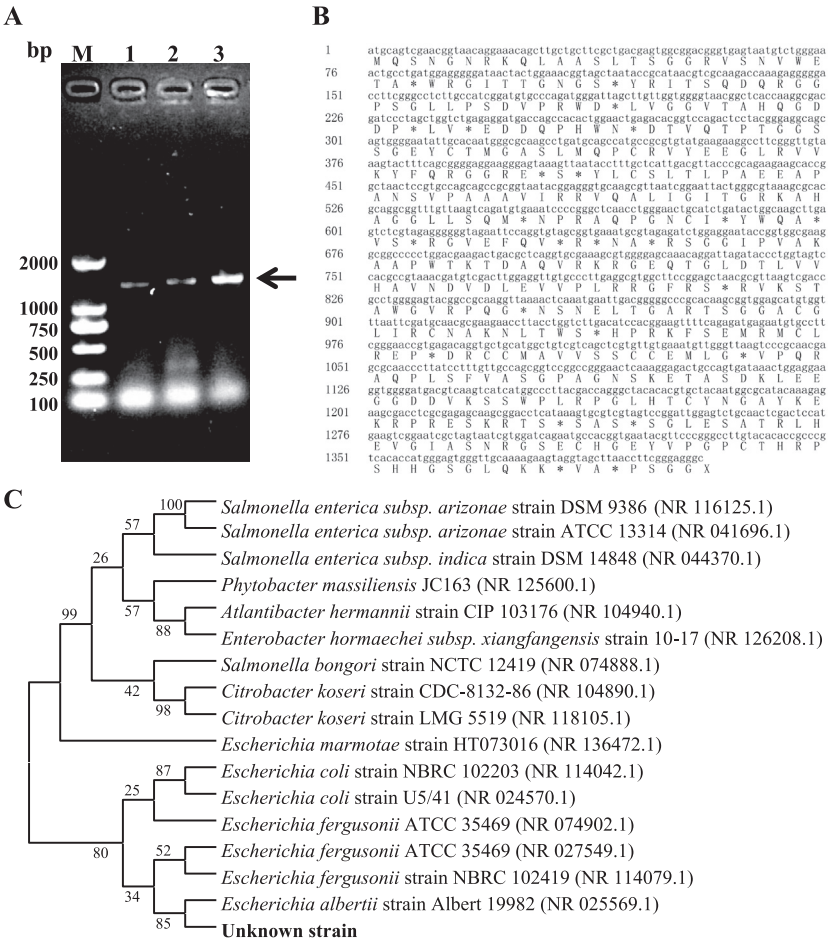

Figure 2. PCR identification and homologous evolutionary tree of the unknown strain.
(A) Identification of bacterial liquid by PCR. M: Mark; 1-3: Bacterial liquid amplification result is about 1400 bp. (B) The result of the unknown strain sequencing. (C) The phylogenetic tree of the 16S rRNA gene sequence of the unknown strain. The numbers in parentheses represent the accession numbers of each strain in GenBank; the numbers on each branch represent the bootstrap value analysis for 1000 replicates.

that these genes were mainly bacterial Multidrug resistance efflux pump genes.

Regarding the analysis of bacterial virulence and pathogenicity, 454 genes were annotated with the PHI database, of which 273 genes were associated with diminished bacterial virulence. A comparison with the VFDB database, combining the genes of the target species and their corresponding annotated information on the function of virulence factors, yielded 485 virulence-related genes. The MLST analysis revealed that the *E. coli* strain used for genomic sequencing in this study was classified as sequence type 155(ST155).

Drug Resistance of the E. coli *Strain*

As shown in Table 2, the *E. coli* strain was sensitive to β-lactams (cefoperazone, ceftazidime, cefazolin), and aminoglycosides (amikacin). It is intermediate to aminoglycosides (gentamicin, kanamycin), tetracyclines (minocycline), resisting to aminoglycosides (streptomycin), β-lactams (penicillin), macrolides (erythromycin, azithromycin), sulfonamides (cotrimoxazole), fluoroquinolones (ciprofloxacin, ofloxacin, norfloxacin), tetracyclines (doxycycline, tetracycline).

The resistance gene of the *E. coli* was amplified by PCR, sulfonamide resistance genes leuO, sul1, and sul2, fluoroquinolone resistance genes parC, gyrA and mfd, aminoglycoside resistance genes aadA5, kdpE, APH (3′′)-Ib, and APH (6)-Id, β-lactam resistance genes TEM, macrolide resistance genes mphA and Mrx were detected. The size of each amplified gene was consistent with the expected fragment size of the target gene (Figure 5A).

The Expression of Drug-Resistance Genes in Different Growth Periods of the E. coli *Strain*

By measuring the growth curve of *E. coli*, we found that the bacteria were in the retarded phase from 0 to 2 h, in the logarithmic growth phase from 2 to 12 h, and in the plateau phase after 12 h (Figures 5B). With this as a reference, we selected the 6, 12, and 18th h of bacterial growth as the measurement points of gene expression.

The minimum inhibitory concentrations of 5 classes of drugs against *E. coli* were obtained by dilution method, and the results were 1,024 μg/mL for penicillin and streptomycin, 256 μg/mL for erythromycin, 16 μg/mL for norfloxacin, and 512 μg/mL for cotrimoxazole. The

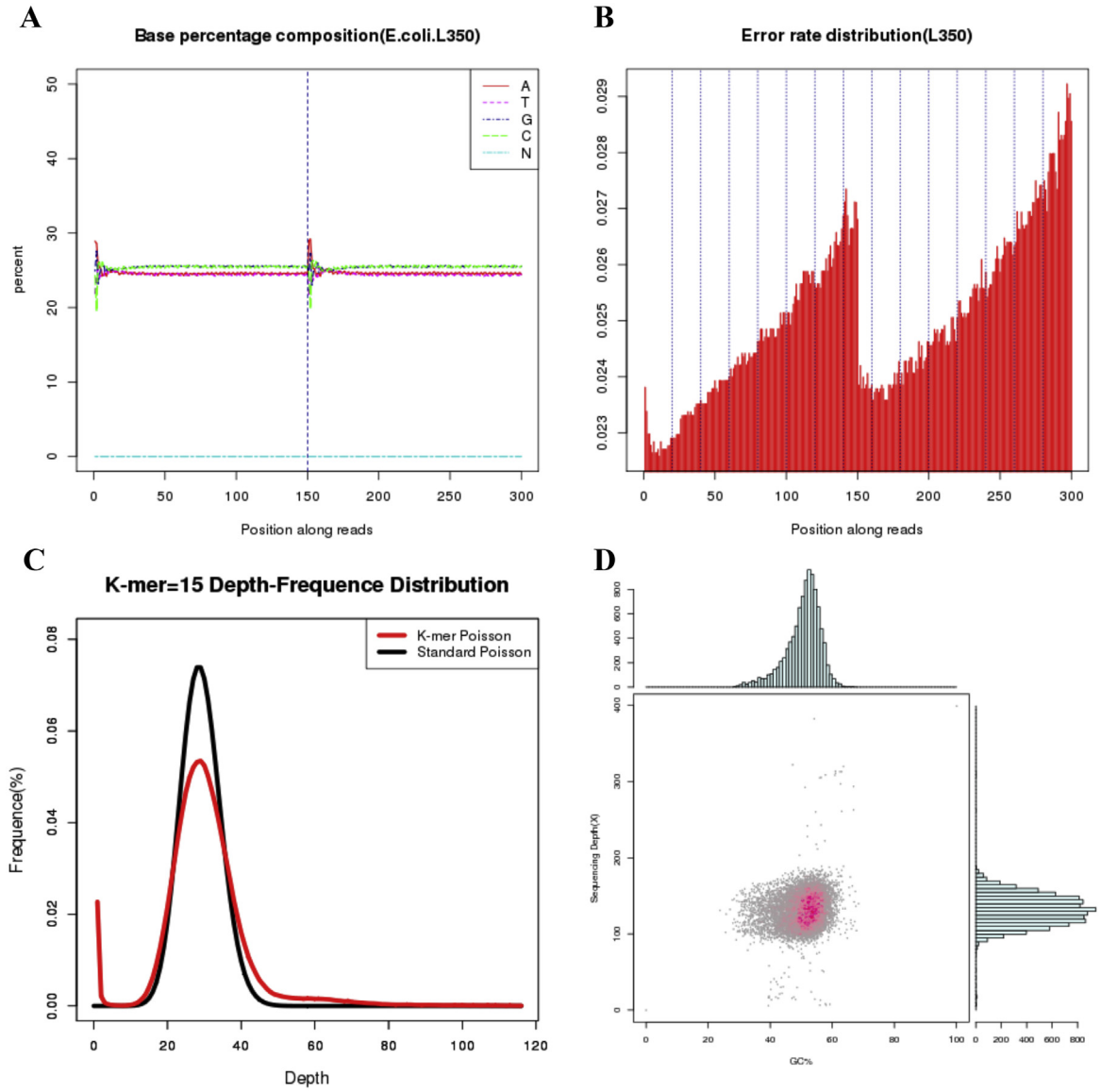

Figure 3. Gene sequence quality analysis of the *E. coli* strains. (A) The distribution of base content in the sample. The abscissa is the position on Reads, the ordinate is the content of a certain base at that position, and the 5 colors represent the content ratio of the 4 ATGC bases and the undetected N respectively.

(B) Sample library mass distribution map. The abscissa is the position on the Reads, and the ordinate is the average error rate percentage of the Reads at that position. (C) K-mer (k-mer =15) statistical chart. The abscissa denotes k-mer depth, ordinate represents the ratio of frequency to the total frequency at each depth. The red curve in the figure is the 15-meter depth distribution curve of sequencing data, and the black curve is the closest standard Poisson distribution curve. (D) GC content associated with depth of *E. coli* genomic sequences. The abscissa represents the GC content, and the ordinate represents the sequencing depth.

maximum concentration of drugs that can make bacteria grow was used as the experimental concentration, that is, the experimental concentration of each drug was determined as follows: penicillin and streptomycin were 512 μg/mL, erythromycin was 128 μg/mL, norfloxacin was 8 μg/mL, and cotrimoxazole was 256 μg/mL.

The analysis of qPCR results showed that the genes with increased expression at 12 h compared with 6 h were TEM-1, sul1, mfd, gyrA, aadA5, APH(6)-Id, and decreased genes were kbpE and APH(3″)-Ib. The genes with increased expression at 18 h compared with 12 h were TEM-1, mphA, Mrx and leuO, decreasing genes were sul1, sul2, parC, mfd, APH(3′′)-Ib, and APH(6)-Id. 18 h compared to 6 h, increasing genes were: TEM-1, mphA, Mrx, leuO, aadA5, and the genes with decreased expression were sul2 and APH(3′′)-Ib (Fig. 5-C).

Pathogenicity Analysis of the E. coli *Strain*

The half-lethal dose of the *E. coli* was calculated to be 2.985×10^7 CFU/mL by the Spearman-Kärber method. The mice infected with the *E. coli* were dissected and their organs were performed HE and Nissl staining to examine their pathological changes. It was observed that compared with the control group, the infected mice had swelling and oozing blood in multiple organs, and the intestines were severely damaged (Figure 6). With the help of the HE staining technique, we observed that the intestinal tissue structure of the infected group of mice was heavily abnormal, with exposed lamina propria, extensive necrosis of the mucosal layer (arrow 1), no normal villous crypt structure (arrow 2), and fragmented or absent nuclei (arrow 3). Many mucosal epithelial cells in the cecum were eroded

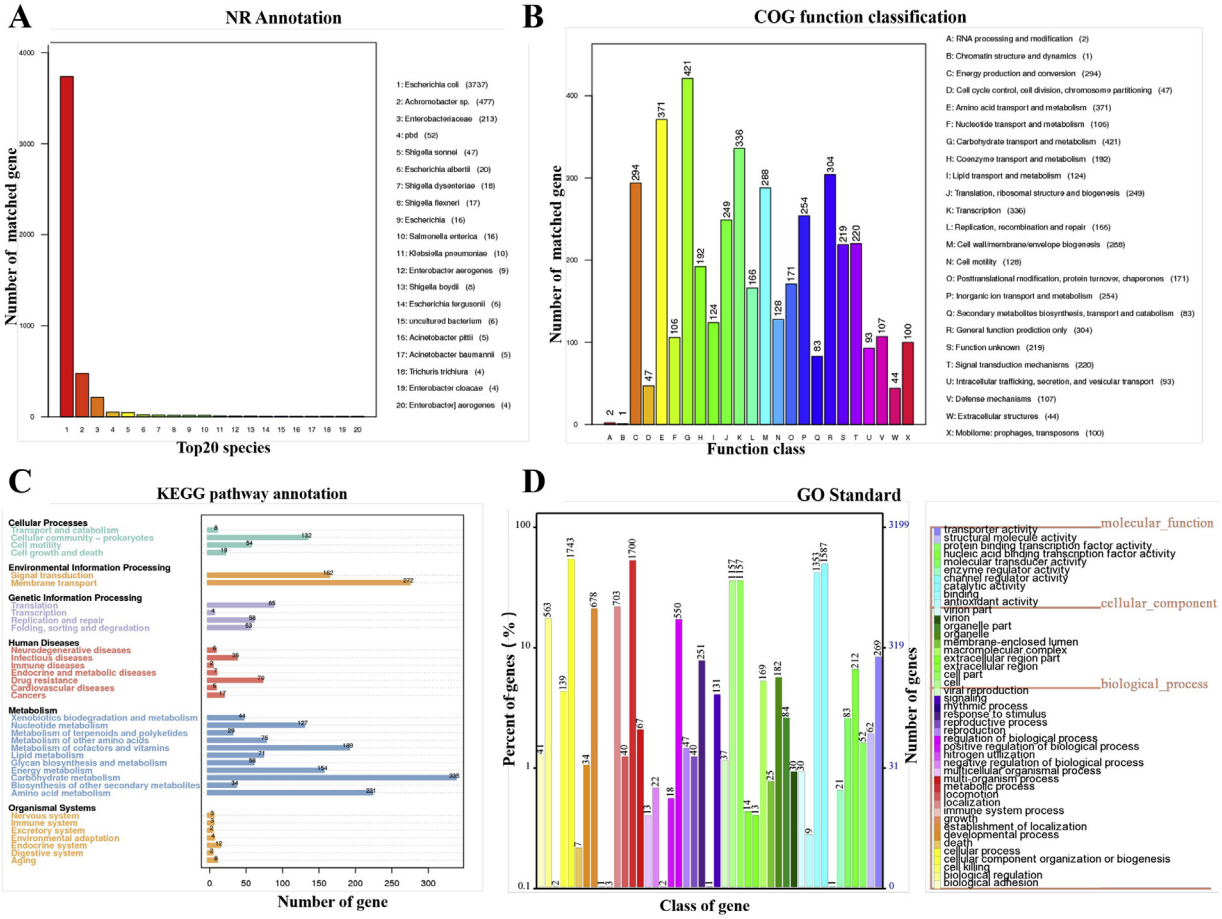

Figure 4. Function annotation of the *E. coli* strain genes. (A) Statistical Chart of species Annotation in NR Database. The abscissa represents the species ID, and the ordinate indicates the number of genes on the annotation. (B) COG function classification. The abscissa represents the functional type of COG, and the ordinate indicates the number of genes on the annotation. (C) KEGG metabolic pathway classification. The number on the bar chart represents the number of genes on the annotation; the other axis is the code of each functional class of level1 in the database, which is explained in the corresponding legend. (D) GO function classification. The Abscissa represents the GO functional classification on the sample annotation, the right ordinate indicates the number of genes on the annotation, and the left ordinate represents the percentage of the number of genes in the annotation as a percentage of all encoded genes.

and detached, and inflammatory cell infiltration was seen (arrow 4). The kidney tissue was structurally abnormal, with massive hemorrhage and a small amount of neutrophil infiltration visible in the interstitium (arrow 5). The alveolar structure was not clear in some areas of the lungs, and the alveolar epithelial cells were proliferating, resulting in the thickening of the alveolar wall (arrow 6). Nissl staining showed degeneration of some neurons in parts of the brain with concentrated deep staining of the cytosol (arrow 7). Some areas of the heart had broken myocardial fibers (arrow 8). The liver tissue structure was mildly abnormal, and the hepatic sinusoids were not significantly dilated, but a small amount of inflammatory cell infiltration was seen around them (arrow 9). In contrast to the control group, the spleen, thymus, testes, and ovaries of the infected mice showed lesions such as swelling and congestion, but the lesions were not obvious when observed after staining. These results showed that *E. coli* caused pathological changes in several organs of the mice, especially their intestinal tissue was severely damaged.

Table 2. Analysis of drug sensitivity tests of the *E. coli* strain.

Antibiotics	Antimicrobial zone diameter	Results
Cefoperazone	26	S
Ceftazidime	27	S
Cefazolin	21	S
Amikacin	17	S
Gentamicin	17	M
Kanamycin	17	M
Minocycline	17	M
Ciprofloxacin	11	R
Ofloxacin	10	R
Doxycycline	10	R
Tetracycline	8	R
Penicillin	9	R
Norfloxacin	0	R
Streptomycin	0	R
Erythromycin	0	R
Azithromycin	0	R
Cotrimoxazole	0	R

The results were interpreted as sensitive (S), intermediate (M) or resistant (R), according to the standards of the National Committee for Clinical Laboratory Standards.

DISCUSSION

E. coli is an important zoonotic pathogen. Pathogenic *E. coli* causes most of the yolk peritonitis in the laying hens, resulting in a rapid decline in egg production and causing huge economic losses (Kim et al., 2020;

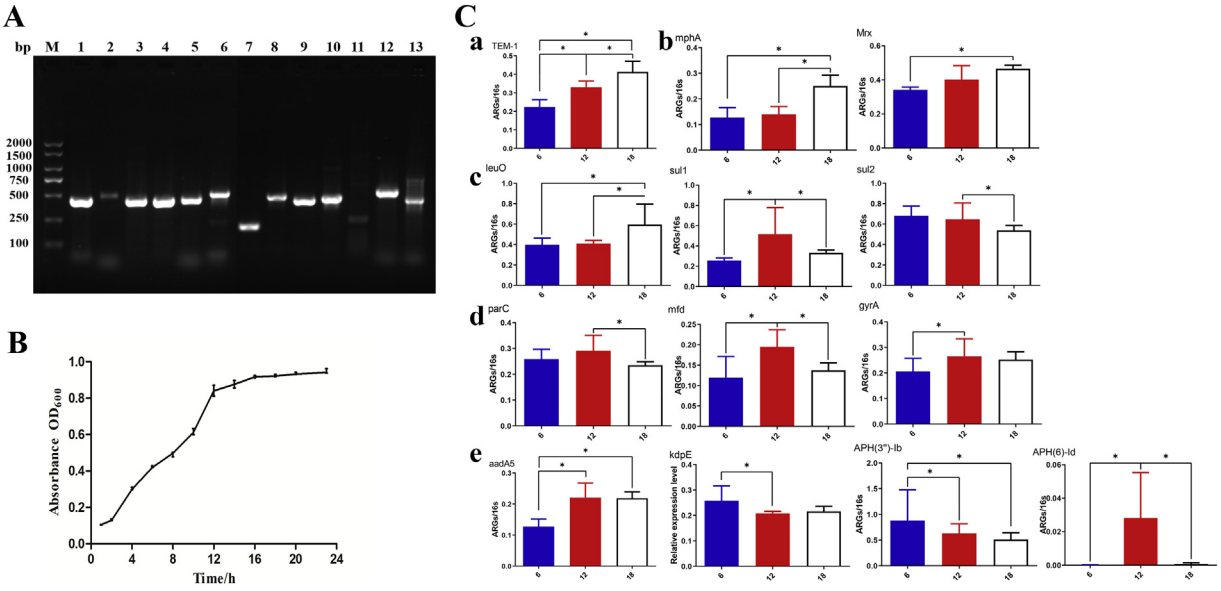

Figure 5. Analysis of drug-resistance of the *E. coli* and determination of its growth curve. (A) Detection of *E. coli* resistance gene. M: DL 2000 DNA marker. 1-13: In order, they are drug-resistance genes: leuO, sul1, sul2, parC, gyrA, mfd, aadA5, kdpE, APH(3'')-Ib, APH(6)-Id, TEM-1, mphA and Mrx. (B) Growth curve of the *E. coli* strain. (C) Expression of drug-resistance genes of different drugs in different time periods. a: β-lactam resistance genes: TEM-1. b: macrolide resistance genes: mphA and Mrx. c: sulfonamide resistance genes: leuO, sul1 and sul2. d: fluoroquinolone resistance genes: parC, gyrA and mfd. e: aminoglycoside resistance genes: aadA5, kdpE, APH(3'')-Ib, and APH(6)-Id.

Apostolakos et al., 2021). Avian pathogenic *E. coli* is widely present in the breeding environment of laying hens. With each egg laying, *E. coli* could infect the reproductive system of laying hens from the cloaca upstream and even infect the entire abdominal cavity of laying hens (Poulsen et al., 2017). *E. coli* can attack the reproductive system by releasing virulence factors, causing redness and congestion in the oviducts and uterus, accompanied by a series of inflammatory diseases (Olsen et al., 2016). In severe cases, the follicles may become necrotic and rupture, contaminating the abdominal cavity and causing serious abdominal infections secondary to multiple organ infections (Srinivasan et al., 2013). The results of this experiment showed that a severe case of yolk peritonitis occurred in a farm in Jiangsu Province, China. In addition to the above typical symptoms, yellow cheese-like material left behind by the absorption of the ruptured yolk was observed adhering to the surface of the abdominal organs, so we suspected that this was yolk peritonitis caused by *E. coli*. To confirm this suspicion, this study combined the isolation and multiple identification of strains from the abdominal cavity of diseased chickens, and after whole genome sequencing, the results showed that the strains infecting these diseased chickens were 99.79% homologous to *E. coli*.

In this study, the whole genome of the isolated strain was sequenced and analyzed, and from the results, the genome length of the strain was 4,977,738 bp and the number of genes was 4,754, of which 4,350,870 bp, or 87.41%, were coding genes. The annotation results of the NR database, a non-redundant protein database created and maintained by NCBI, showed that the number of genes annotated to *E. coli* was 4,734. In other words, this again proves that it was *E. coli* that infected the laying hens in this study. The results showed that the *E. coli* genome was annotated with the COG database to obtain 3,793 genes, which are involved in biological processes such as carbohydrate transport metabolism, amino acid transport and metabolism and transcription, respectively, to varying degrees, and maintain the basic life activities of *E. coli*. For the prediction of biological functions, besides COG databases, GO and KEGG databases are widely used, which are databases of gene related functions stored based on different classification ideas (Kanehisa, 2006). In this study, the annotation results of GO and KEGG databases showed that most of the annotated genes in *E. coli* were related to catalytic activity, linkage and transport activity, and many genes were significantly enriched in membrane transport, sugar metabolism amino acid metabolism and other pathways. It has been shown that these important enrichment functions or pathways are importantly linked to intracellular transport in *E. coli*, regulation of *E. coli* gene expression, establishment of a favorable intracellular environment, and virulence of *E. coli* (Afridi et al., 2020; Chen et al., 2021).

In recent years, with the widespread use of antibiotics and various chemically synthesized drugs in animal husbandry, large amounts of antibiotics and disinfectants have continued to contaminate soil and rivers, causing a serious ecological burden (Alves et al., 2018; Varga et al., 2018). This has also led to an expanding resistance spectrum and increasing levels of drug resistance in *E. coli*. The analysis revealed that the *E. coli* strain in this study was classified ST155. The ST155 genotype is a prevalent strain of avian-derived *E. coli*, significantly impacting poultry production across various nations (Maluta et al., 2014). Notably, many reports point out that avian-origin *E. coli* of the ST155 type demonstrates pronounced multidrug resistance (Kluytmans et al., 2013; Pires-Dos-Santos et al., 2013).

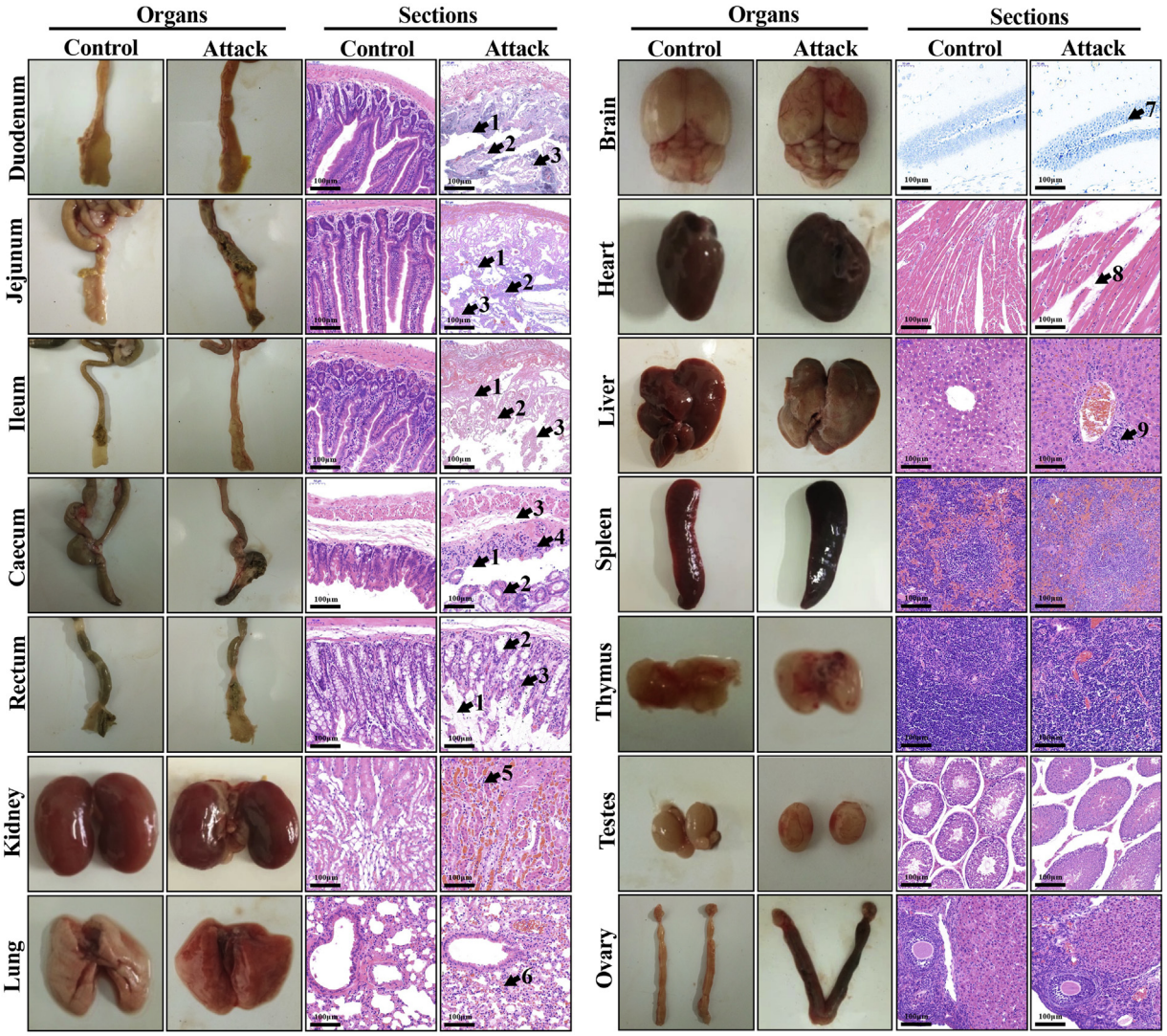

Figure. 6. The abdominal dissection and pathological specimen of mice. Organs: pathological changes in mouse organs. Sections: pathological sections observed under light microscope (200 ×, scale bar = 100 μm). Control: organs of normal control mice. Attack: organs of mice in the attack group of the *E. coli* strain. All arrows point to specific lesions.

In this study, a variety of resistance genes of *E. coli* were further screened and validated by drug sensitivity tests and PCR, which confirmed that *E. coli* isolated in this study caused yolk peritonitis in laying hens showed resistance to a variety of drugs such as aminoglycosides, β-lactams, quinolones, and sulfonamides, which means that the *E. coli* in this study showed multiple drug resistance. In this experiment, 3 genes encoding aminoglycoside modifying enzyme, aadA5, aph (3 ")-ID and aph (6)-ID, were detected and verified, which are all coding genes of aminoglycoside phosphotransferase. Studies have shown that these passivating enzymes can modify the antibiotic active molecules that enter cells, rendering them biologically inactive. In addition, KdpE was detected in this study, which is a response regulator combined with sensor kinase KdpD to form a KdpD/KdpE 2-component system, which is one of the regulators of high-affinity potassium ion pump and has been widely studied in potassium transport (Freeman et al., 2013). Potassium ion regulation is an important factor in bacterial virulence and survival, suggesting that KdpE also plays an important role in drug resistance of *E. coli*. The TEM-1 detected in this study is a plasmid-mediated β-lactamase. β-lactamases can catalyze the hydrolysis of 6-aminopenicillanic acid and 7-aminocephalosporanic acid and the inactivating enzymes of β-lactam cyclic amide bonds in their N-acyl derivatives, resulting in the inactivation of antibiotics (Drawz and Bonomo, 2010; Schmiege et al., 2021). The main reason for the resistance of *E. coli* to quinolones and sulfonamides is the mutation of the gene encoding the drug action site (Silver, 2011). The mfd, parC and gyrA genes detected in this study encode the helicase gene and topoisomerase, respectively. sul1 and sul2 are the mutated genes of dihydropteroate synthase (Sun et al., 2019).

From the above analysis, the *E. coli* antagonizes antibiotics in different ways, and these mechanisms of drug resistance depend on the expression and regulation of various drug-resistance genes. In this study, there were significant differences in the expression of different drug-resistance genes in different growth stages of bacteria. Indeed, the genetic background of bacteria and the external environment, such as genetic variation, transcriptional regulation, and antibiotic induction, can change the expression

level of drug resistance genes (Depardieu et al., 2007; López-Maury et al., 2008). The study on the expression and regulatory mechanism of drug-resistance genes is of great significance, which can predict the results of drug sensitivity test and guide clinical drug use.

Regarding the pathogenicity analysis of *E. coli*, this study was compared by the PHI database, *E. coli* in this study was annotated with 127 genes related to host action. Among these genes, 8 were found to enhance virulence, namely: hns, EF-P, PmrA, rcsC11, bcsA, rcsA, and QseB. Notably, hns, rcsC11, bcsA, and rcsA1 are biofilm formation-related genes. Biofilm is a complex microbial community formed in the environment and during the infection process. The ability to form biofilm allows *E. coli* to evade the attack of antibacterial drugs and the host immune system, resulting in high drug resistance. Moreover, biofilm dissociation can lead to new infections, making the infection chronic and difficult to control (Varga et al., 2018; Chen et al., 2021).

Additionally, a comparison with the VFDB database revealed 485 genes associated with virulence. This genomic information indicates that this *E. coli* strain is highly virulent. Our experimental analysis further supports this, as the LD50 of *E. coli* was determined to be 2.985×10^{7} CFU/mL. Furthermore, mice infected with *E. coli* developed severe diarrhea, and the dissection results and HE-stained histopathological pictures showed the most severe intestinal damage, including exposure of the lamina propria of the intestine and breakage and loss of intestinal villi. *E. coli* is a well-known intestinal pathogen that adheres to the intestinal surface. After invading the intestine, the bacteria primarily proliferate in the duodenum, jejunum, and upper ileum, releasing virulence factors such as endotoxins, pods, adhesins, and exotoxins. These factors cause intestinal dysfunction and lead to diarrhea (Olsen et al., 2016). In summary, mice infected with *E. coli* exhibited pathological changes like those seen in laying hens with vitelline peritonitis, further demonstrating the high virulence of the *E. coli* strain isolated in this study.

CONCLUSIONS

In summary, this study provides compelling evidence that *E. coli* is the etiological agent responsible for vitelline peritonitis in laying hens. Additionally, our results demonstrate the pronounced virulence of these *E. coli* strains, which induce severe intestinal damage in mice. Furthermore, the *E. coli* strains isolated in this study exhibited resistance to aminoglycosides, beta-lactam, quinolones, sulfonamides, and other antimicrobial drugs, indicating a worrisome prevalence of multi-drug resistance. Finally, we found that the expression of drug resistance genes in *E. coli* at different growth stages was different under different antibiotic environments. This study establishes a solid theoretical foundation for the investigation of *E. coli*'s multi-drug resistance mechanisms and offers valuable insights for the prevention and treatment of vitelline peritonitis caused by this pathogen.

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DISCLOSURES

The authors declare no conflicts of interest.

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