ORIGINAL PAPER
Polish Journal of Microbiology
2024, Vol. 73, No 1, 69–87
https://doi.org/10.33073/pjm-2024-008

Isolation, Identification, Antimicrobial Resistance, Genotyping, and Whole-Genome Sequencing Analysis of *Salmonella* Enteritidis Isolated from a Food-Poisoning Incident

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Submitted 17 September 2023, accepted 21 January 2024, published online 8 March 2024

Abstract

Salmonella enterica is a common pathogen in humans and animals that causes food poisoning and infection, threatening public health safety. We aimed to investigate the genome structure, drug resistance, virulence characteristics, and genetic relationship of a *Salmonella* strain isolated from patients with food poisoning. The pathogen strain 21A was collected from the feces of patients with food poisoning, and its minimum inhibitory concentration against commonly used antibiotics was determined using the strip test and Kirby-Bauer disk methods. Subsequently, WGS analysis was used to reveal the genome structural characteristics and the carrying status of resistance genes and virulence genes of strain 21A. In addition, an MLST-based minimum spanning tree and an SNP-based systematic spanning tree were constructed to investigate its genetic evolutionary characteristics. The strain 21A was identified by mass spectrometry as *S. enterica*, which was found to show resistance to ampicillin, piperacillin, sulbactam, levofloxacin, and ciprofloxacin. The WGS and bioinformatics analyses revealed this strain as *Salmonella* Enteritidis belonging to ST11, which is common in China, containing various resistance genes and significant virulence characteristics. Strain 21A was closely related to the SJTUF strains, a series strains from animal, food and clinical sources, as well as from Shanghai, China, which were located in the same evolutionary clade. According to the genetic makeup of strain 21A, the change G > A was found to be the most common variation. We have comprehensively analyzed the genomic characteristics, drug resistance phenotype, virulence phenotype, and genetic evolution relationship of *S.* Enteritidis strain 21A, which will contribute towards an in-depth understanding of the pathogenic mechanism of *S.* Enteritidis and the effective prevention and control of foodborne diseases.

K e y w o r d s: *Salmonella*, whole-genome sequencing, resistance genes, virulence genes, evolution

Introduction

Foodborne diseases are a major international public health problem. According to the latest study, of the order of 420,000 people die of illness due to eating contaminated food every year. *Salmonella* is the most common pathogen, especially in developing countries (Soubeiga et al. 2022). *Salmonella* Enteritidis is a harmful zoonotic pathogen with a wide range of hosts and can colonize humans, animals, plants, and even the environment. After invading the body, *S.* Enteritidis invades the intestinal epithelium of the ileum and colon through intestinal cells, entering microfold cells (M cells) or being taken up by mucosal dendritic cells, which either causes neutrophilic gastroenteritis or spreads throughout the body to cause sepsis (Knodler and Elfenbein 2019). In addition, *Salmonella* has developed multi-resistance to certain clinically available antibiotics. Multidrug-resistant *Salmonella* infects animals or contaminates food, and subsequently enters human bodies through the food chain, accumulating antibiotic-resistant genes in humans, which in turn affects the efficacy of antibiotic use (Founou et al. 2016). According to one research, the most common phenotypic resistances of *Salmonella* was

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resistance to ampicillin (76.0%), tetracycline (60.4%), and ciprofloxacin (56.0%), and there is a high correlation (96.8%) between drug resistance phenotype and the presence of drug resistance genes (Woh et al. 2021). Therefore, understanding the resistance of multidrug-resistant *Salmonella* is important for treating human *Salmonella* infection. In recent years, genotyping methods have been rapidly developed, including pulsed-field gel electrophoresis, multilocus sequence typing (MLST), core genome MLST (cgMLST), whole-genome MLST (wgMLST), and whole-gene single-nucleotide polymorphism (wgSNP) analysis. These methods have high resolution and accuracy and can be used to track bacterial evolution and the epidemic survey of foodborne diseases (Radomski et al. 2019). To date, wgSNP and cgMLST have been widely used to study and trace bacterial evolution (Wang et al. 2020b; Hyeon et al. 2021).

The present study has systematically characterized and determined the genome structure, drug resistance, virulence characteristics, and genetic relationship of *S.* Enteritidis isolated from patients after food poisoning. The findings of this study have comprehensively revealed the resistance and virulence mechanisms associated with *S.* Enteritidis, which is important for preventing and controlling its infection; moreover, the present study provides a reference for containing food safety problems caused by *S.* Enteritidis and for formulating effective public health policies.

Experimental

Materials and Methods

Sample collection, strain isolation and identification. In May 2021, one patient with food poisoning was admitted to a rank A tertiary hospital in Shanxi, China, and we collected the patient's clinical data using the electronic medical record system. The patient's feces sample was collected, inoculated into SBG enrichment solution (Qingdao Haibo Biotechnology Co., Ltd., China), and subsequently incubated at 35°C in an incubator containing 5% CO_2 for 24 h. The enrichment solution was then inoculated into *Salmonella-Shigella* culture (SS culture) medium (Hangzhou Binhe Microbial Reagent Co., Ltd., China), and incubated at 35°C in an incubator containing 5% CO_2 for 24 h. The suspicious colonies on the SS culture medium were selected for identification. The isolate was identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry as *S.* Enteritidis.

Antimicrobial susceptibility testing. According to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Humphries et al. 2021), the resistance to 15 antibiotics was detected using a VITEK®2-compact automatic drug sensitivity analyzer (bioMérieux, France) and the disc diffusion method (or Kirby-Bauer method). The antibiotics were as follows: ampicillin, piperacillin, ampicillin/sulbactam, piperacillin/sulbactam, ceftriaxone, ceftazidime, cefepime, aztreonam, imipenem, meropenem, compound sulfamethoxazole, furadantin, levofloxacin, ciprofloxacin, and cefoperazone. *Escherichia coli* ATCC® 25922™ and *Staphylococcus aureus* ATCC® 25923™ were used for quality control.

Biofilm formation assay. The experiment on biofilm formation ability was conducted based on previous research (Zhou et al. 2019). The TSBG medium was used as the negative control, and the strain 21A was used as the experimental group. Among them, $p < 0.05$ was considered to indicate statistical significance.

DNA extraction. 200 mg of the sample was transferred to a grinding tube. CTAB lysis buffer was added straightly and mixed thoroughly. The mixture was incubated at 65°C for 60 min with 400–1,400 rpm, then cooled to room temperature and centrifuged at 12,000 × *g* for 5 min. The supernatant was transferred to a new 2.0 ml centrifuge tube, and an equal volume of supernatant of phenol : chloroform : isoamyl alcohol (25:24:1) was added and vortexed to mix thoroughly. Then, the samples were centrifuged at 12,000 × *g* for 10 minutes at room temperature. The supernatant was transferred to a new 1.5 ml tube carefully add ⅔ volume of supernatant of isopropyl alcohol and ⅒ volume of 3M sodium acetate was added, gently mixed by inverting, and precipitated at −20°C for 2 hours. Then, the samples were centrifuged at 18,213 × *g* for 15 minutes at room temperature, and the supernatant was removed. 1 ml 75% ethanol was added, precipitated, and then centrifuged at 18,213 × *g* for 3 minutes at room temperature. The supernatant was removed. The content was air dried for 3–5 min. 20–200 μl of TE buffer was finally added.

Genome sequencing and assembly. The 21A genome was sequenced using a PacBio Sequel II (Pacific Biosciences of California, Inc., USA) and DNBSEQ platform at the Beijing Genomics Institute (BGI, China). Four SMRT Cell Zero-Mode Waveguide sequencing arrays were used by the PacBio platform to generate the subreads set. PacBio subreads (length < 1 kb) were removed. The software Canu (https://github.com/marbl/canu) was used for self-correction. Draft genomic unitigs, which are uncontested groups of fragments, were assembled using Canu and a high-quality-corrected circular consensus sequence subreads set. To improve the accuracy of the genome sequences, the Genomic Analysis Toolkit (GATK) (https://www.broadinstitute.org/gatk) was used to make single-base corrections.

Genome prediction and annotation. The assembled contigs were used for predicting the genome components, including tRNAs (Lowe and Eddy 1997), rRNAs (Lagesen et al. 2007), small RNAs (sRNAs)

(Gardner et al. 2009), tandem repeats, minisatellite DNA, microsatellite DNA and CRISPR identification. Subsequently, the best matches were abstracted using the BLAST alignment tool for function annotation, including the Virulence Factor Database (VFDB) (Chen et al. 2016), Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al. 2020), Clusters of Orthologous Genes (COG) (Galperin et al. 2015), Gene Ontology (GO) (Ashburner et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Jones et al. 2014) databases. In addition, the entire gene sequences were submitted to the website (http://genomicepidemiology.org/services) for the multi-locus sequence type, serotype, and mobile genetic elements, and the plasmid sequence was submitted to the website (https://cge.food.dtu.dk/services/PlasmidFinder) for the plasmid replicon. Subsequently, *Salmonella* from Asia was selected from the PubMLST database (https://pubmlst.org) (Jolley et al. 2018), and PHYLOViZ (http://www.phyloviz.net) was used as the minimum spanning tree based on MLST.

Comparative genomics and phylogenetic analysis. The synteny of strain 21A and reference strains was performed using MUMmer (a bioinformatics software system for sequence alignment), and BLAST core/pan genes of these strains were clustered using the CD-HIT rapid clustering of similar proteins software (https://nmdc.cn/analyze/details?id=60067b3f0b38496ee0c90951) with a threshold of 50% pairwise identity and 0.7 length difference cutoff in amino acids. The SNP-based tree was also generated based on the total genomic sequence results through using the NJ method. The phylogenetic tree was constructed using TreeBeST software using the NJ method, and subsequently iTOL was used for visual analysis (Nandi et al. 2010). The clean data from each sample were then aligned to the reference genome using the alignment software, BAM (Li and Durbin 2009; 2010), to obtain an initial alignment result file in the BAM format. SNP and insertions and deletions (InDel) analyses were subsequently performed according to the optimal variation detection and analysis procedures recommended by the GATK official website (McKenna et al. 2010; DePristo et al. 2011).

Results

Identification of the strain and clinical information of the patients. The patient, 21 years old, was hospitalized for fatigue, dizziness, nausea, and fever for three days and had experienced aggravated diarrhea for two days. According to the laboratory tests, the level of C-reactive protein was increased. And combined with the initial complaint, a final diagnosis of infectious diarrhea was made. Feces samples were taken from the patient for isolation and cultivation (Fig. S1). The colonies were identified as *S. enterica* according to mass spectrometric analysis (Fig. 1 and Table I). During the period of hospitalization, the patient was treated with a combination of levofloxacin and cefotaxime and received symptomatic and supportive treatment through fluid replacement therapy. On day five after hospitalization, the patient had no symptoms of fever, abdominal pain or diarrhea, and the patient's general condition was satisfactory. No *Salmonella* was found in the fecal culture after re-examination, indicating

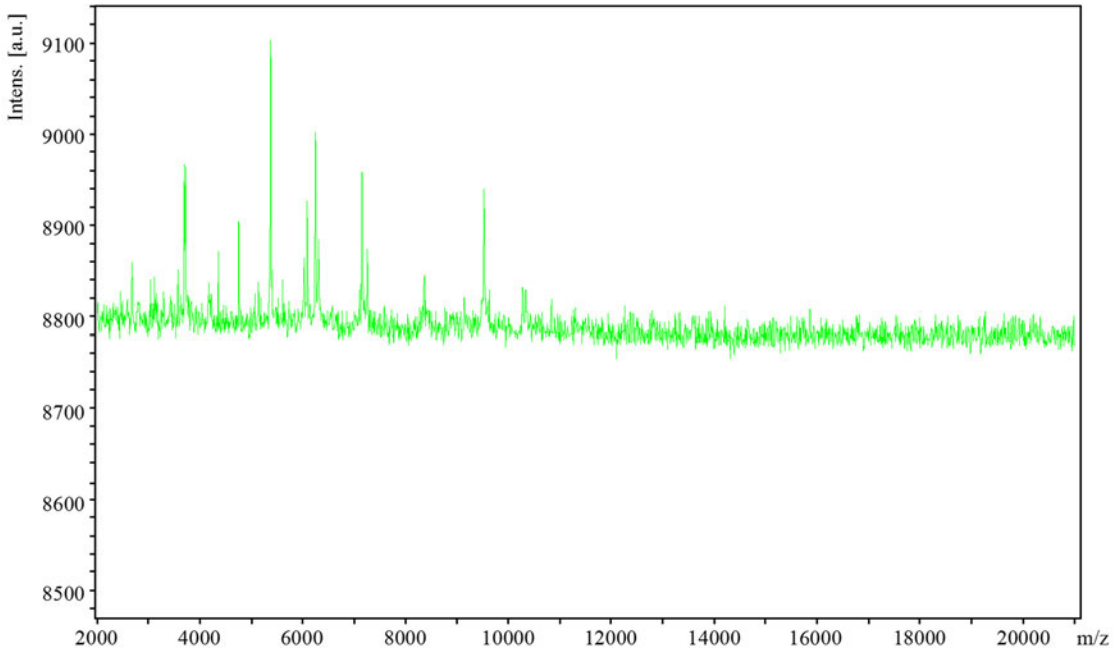


Fig. 1. Clinical symptoms and treatment of patient 21A.

Table I
Matching scores of mass spectrometry identification results.

Grade (quality)	Matching mode	Score	NCBI identifier
1 (++)	*Salmonella* sp. (enterica st Hadar)Sa05 506 VAB	2.030	149385
2 (++)	*Salmonella* sp. (enterica st Anatum)11 LAL	2.023	58712
3 (+)	*Salmonella* sp. (choleraesuis)08 LAL	1.998	591
4 (+)	*Salmonella* sp. (enterica st Dublin)Sa05 188 VAB	1.972	98360
5 (+)	*Salmonella* sp. (enterica st Enterica)DSM 17058T HAM	1.915	59201
6 (+)	*Salmonella* sp. (typhimurium)12 LAL	1.894	602
7 (+)	*Salmonella* sp. (enteritidis)25089078 (PX)MLD	1.885	592
8 (+)	*Citrobacter koseri* 9553-1 CHB	1.783	545
9 (+)	*Citrobacter koseri* Mu15167-1 CHB	1.753	545
10 (+)	*Salmonella* sp. (enterica st Stanley)15 LAL	1.752	192953

Table II
Basic genomic component information of strain 21A.

Type		Number	Total length (bp)	GC content (%)
Genome		4663	4,118,712	53.34
		Number	Total length (bp)	In genome (%)
ncRNA	tRNA	21	1,647	0.0347
	5S rRNA	8	920	0.0193
	16S rRNA	7	10,702	0.2253
	23S rRNA	7	20,453	0.4306
	sRNA	68	7,926	0.1669
Tandem Repeat	TRF	73	9,851	0.2074
	Minisatellite DNA	50	3,611	0.076
	Microsatellite DNA	5	190	0.004
CRISPR		2	1,155	0.0243
Prophage		9	217,435	4.5787

clinical recovery had been made from *Salmonella* infection. The patient was discharged (Fig. 2). After the investigation, patient 21A and other diarrhea patients admitted either on the same day or the day before had consumed sandwiches that may have been contaminated with *Salmonella*-infected eggs and meat.

Genomic characteristics of 21A. Upon sequencing of the 21A strain, 8,764,210 original reads were obtained, and 8,474,991 valid reads were retained after quality control with an effective rate of 96.70%. The genome size of 21A was found to be 4,748,863 bp (Table II), including a circular chromosome and two

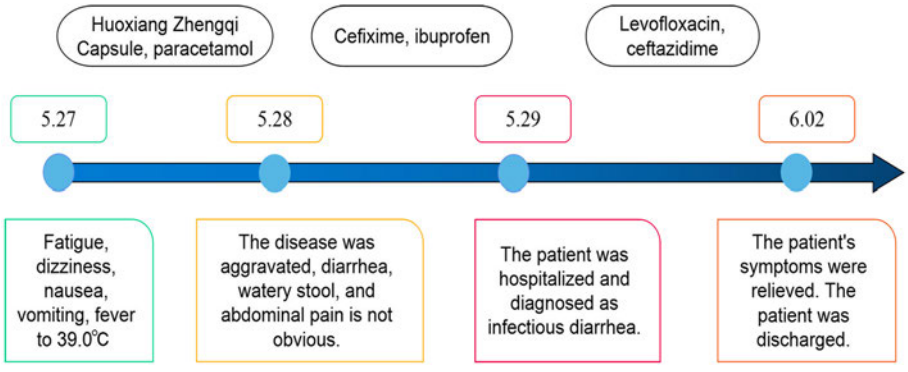


Fig. 2. The clinical symptoms and treatment of patient 21A.

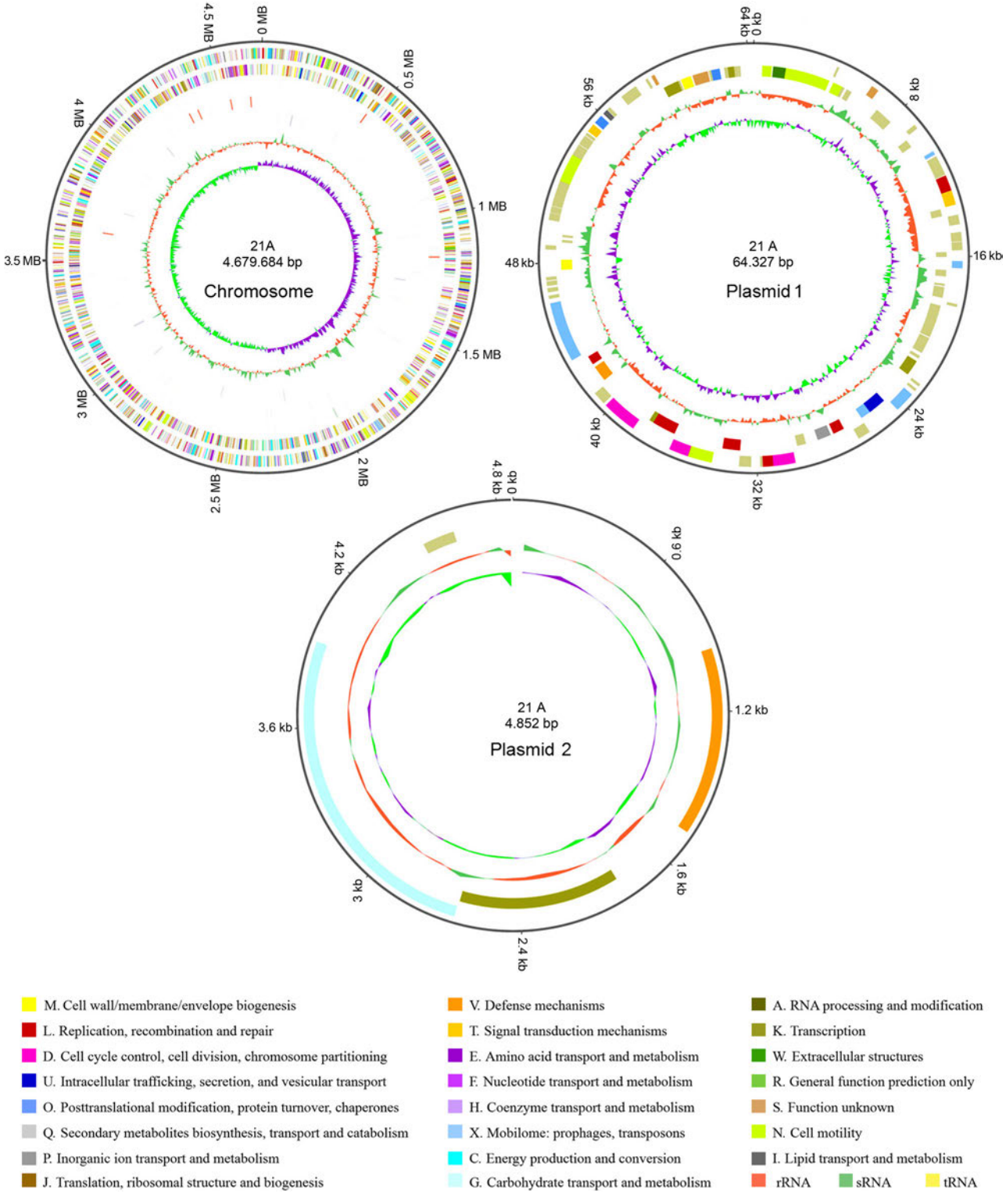


Fig. 3. Circular representations of the genome and plasmid.

plasmids, encoding 4,663 genes with a gene length of 4,118,712 bp and an average length of 883.38 bp. The length of the gene accounted for 86.73% of the genome length. The chromosome length was 4,679,684 bp, and the GC content was 52.17%. The lengths of the two circular plasmids were 64,327 and 4,852 bp, and the GC content was found to be 51.76 and 59.70%, respectively (Fig. 3). Non-coding RNA was found mainly to include tRNAs, rRNAs, and sRNAs, accounting for 0.08768% of the genome. Tandem repeats included tandem repeats finder (TRF), minisatellite DNA, and microsatellite DNA, accounting for 0.2874% of the genome. All genes were functionally annotated. Totals of 45, 554, 3,681, 3,086, and 3,258 genes were

Table III
Resistant antimicrobial phenotype for strain 21A.

Type of antibiotics	Drug	MIC	Resistance
β-lactam	Ampicillin	≥ 32 μg/ml	R
	Piperacillin	≥ 128 μg/ml	R
	Ampicillin/sulbactam	≥ 32 μg/ml	R
	Piperacillin/sulbactam	≤ 4 μg/ml	S
	Cefoperazone	25 mm	S
	Cefatriaxone	≤ 1 μg/ml	S
	Ceftazidime	≤ 1 μg/ml	S
	Cefepime	≤ 1 μg/ml	S
	Aztreonam	≤ 1 μg/ml	S
Carbopenem	Imipenem	≤ 1 μg/ml	S
	Meropenem	≤ 1 μg/ml	S
Sulfonamide	Compound sulfamethoxazole	≤ 20 μg/ml	S
Nitrofuran	Furadantin	≥ 128 μg/ml	I
Quinolone	Levofloxacin	≥ 8 μg/ml	R
	Ciprofloxacin	≥ 4 μg/ml	R

S – susceptibility, I – intermediate susceptibility, R – resistance

annotated in the CARD, VFDB, COG, GO, and KEGG databases, respectively.

Subsequently, the genome sequencing results were submitted to the Center for Genomic Epidemiology to identify strain 21A as ST11 *S.* Enteritidis (Table SI), serotype 9 : g, m: – (Table SII). In addition, the detection of mobile genetic elements in the 21A strain showed four insertion sequences, including ISKpn2, ISSty2, ISEcl10, and ISSen7. Among them, ISSen7 is located on plasmid 1, while the rest of the insertion sequences are located on the chromosome. Plasmid 1 contains two plasmid replicons, IncFIB(S) and IncFII(S) (Table SIII).

The resistance phenotype and genotype of 21A. The 21A strain showed resistance to ampicillin, piperacillin, sulbactam, levofloxacin, ciprofloxacin, and intermediate susceptibility to furantoin (Table III). Based on the CARD database annotation, 45 resistant genes were annotated, among which only 28 genes were associated with *Salmonella*. Fig. 4 shows the resistance mechanism of different resistance genes and the associated antibiotics. A total of 67.8% of the resistance genes were found to exert drug resistance via efflux pump. Among the resistant genes associated with the efflux pump, the *kdpE* gene was only associated with one antibiotic, and the others were associated with four or more antibiotics that could mediate resistance to multiple antibiotics through either different or the same efflux pump transporters. Further analysis of Fig. 4 and Table III revealed that the antibiotic resistance phenotype of the 21A isolate was highly consistent with the resistance genotype. Further drug resistance testing was conducted on strain 21A, and the results of biofilm formation ability testing showed that the biofilm formation ability of strain 21A was weak (Fig. S2).

Analysis of virulence factors by the VFDB database. A total of 554 virulence genes were identified through the VFDB database annotation of 21A isolates. According to their locations and functions, 327 of these genes were grouped into seven categories: 'Type III secretion system (TTSS)', 'flagella and chemotaxis', 'fimbriae', 'lipopolysaccharide', 'adhesins', 'capsule', 'efflux pump', and 'others' (Table IV). TTSS was found to be the most important of the virulence factors, mainly composed of SPI-1 and SPI-2. The *sopABDEE2*, *sipA*, *avrA*, and *sptP* genes were annotated as effectors of SPI-1, whereas the *sseFGJLKI*, *sifAB*, *pipB2*, *sspH*, and *sopD2* genes were annotated as effectors of SPI-2. Most genes were located on the chromosome, with 15 genes on plasmids 1 and 2 free of virulence factors. Virulence genes located on plasmid 1 included the *spvA*, *spvB*, *spvC*, *spvD*, *spvR*, *pefB*, *pefA*, *pefC*, *pefD*, *rck*, *cofT*, *virB*, *pilW*, *mig-5*, and *mlr6326* genes. The *rck* gene was annotated as 'resistance to complement killing'. *spvB* and *spvC* were TTSS effectors, annotated as 'ADP-ribosylation activity' and 'phosphothreonine lyase', respectively. Finally, *pefA* was annotated as a plasmid-encoded fimbriae major subunit, whereas *pefB* was annotated as a plasmid-encoded fimbriae regulatory protein.

Functional annotation of resistance genes and virulence genes. Functional annotation analysis of 45 resistance genes in strain 21A was subsequently performed. The COG database predicted 40 genes into four categories: 'cellular', 'information', 'metabolism', and 'poorly'. These genes were mainly found to

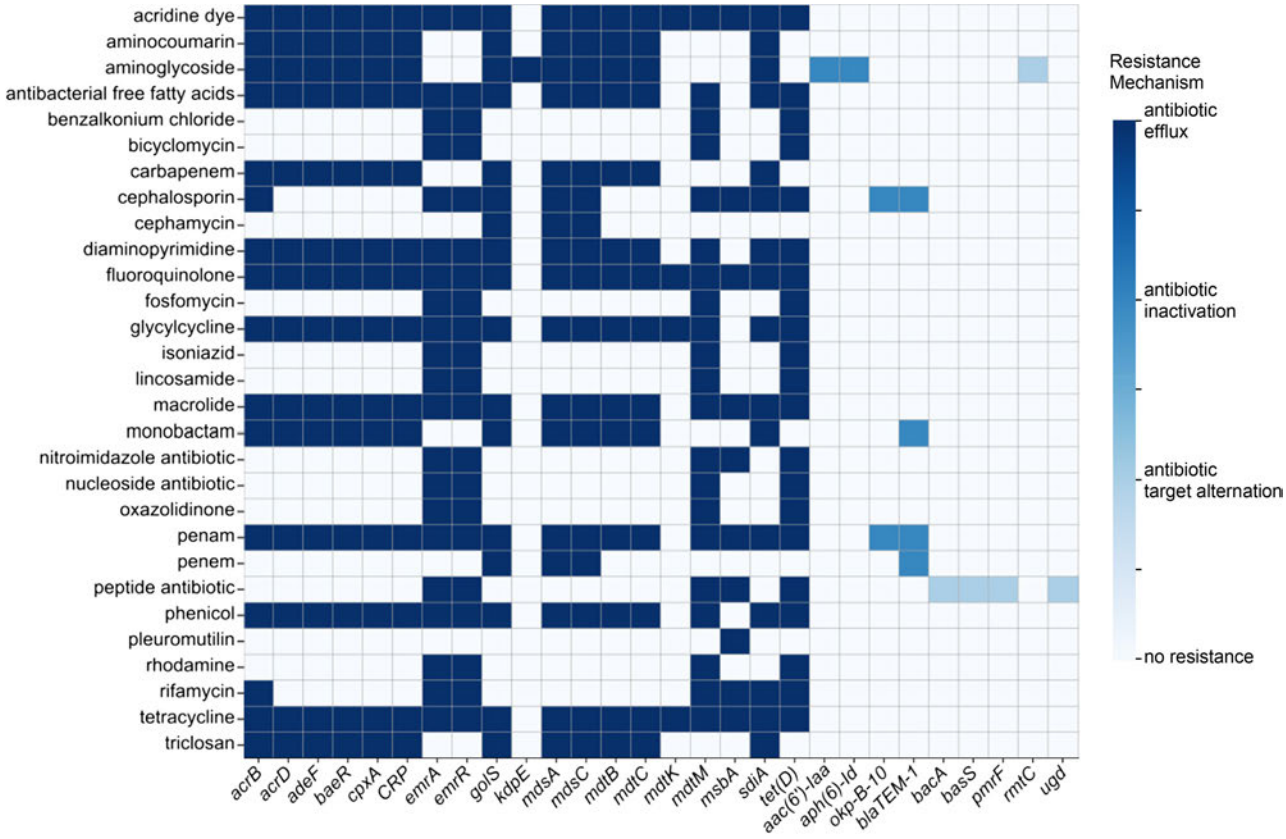


Fig. 4. Resistance genes annotated with 21A based on CARD database.

Table IV
VFDB database predicted the virulence factors of strain 21A.

Type		Genes
TTSS	SPI-1	*avrA*, *hilCD*, *iacP*, *iagB*, *invABCEFGHIJ*, *orgABC*, *prgHIJK*, *sicAP*, *sipABCD*, *sitABCD*, *sopABDEE2*, *spaOPQRS*, *sprB*, *sptP*, *steA*
	SPI-2	*pipBB2*, *sifAB*, *sopD2*, *ssaCDEGHIJKLMNOPQRTUV*, *sscAB*, *sseBCDEFGIJKL*, *sspH2*, *ssrAB*, *steC*
	SPI-3	*mgtBC*, *misL*
	SPI-4	*siiE*
	T3SS	*ABB77417*, *hopAN1*, *hrpH*, *mlr6326*, *RSp0731*, *spvABCDR*
Flagella and chemotaxis	Flagella	*fleQ*, *fleR*
	Polar flagella	*fleR/flrC*, *nueA*, *flrA*, *flmH*
	Lateral flagella	*lafK*
	Peritrichous flagella	*cheABDRWYZ*, *flgABCDEFGHIJKLMN*, *flhABCDE*, *fliABDEFGHIJKLMNOPQRSTYZ*, *fljB*, *flk*, *motAB*, *tar/cheM*
Fimbriae	Fimbrial subunit	*bcfADEF*, *lpfE*, *pefA*, *pegA*, *safD*, *stbA*, *steADEF*, *stfAEFG*, *sthDE*, *stiA*
	Fimbrial chaperone	*bcfBC*, *fimC*, *lpfB*, *safB*, *sefB*, *stbBE*, *stdC*, *steC*, *stiB*
	Type IV pili	*vfr*, *rpoN*, *pilRTW*
	Other related genes	*algU*, *bcfC*, *fimADFHIWYZ*, *lpfAD*, *pefB*, *pegC*, *safC*, *sefCR*, *stbCD*, *stdAB*, *steB*, *stfC*, *sthA*, *stiCH*
Lipopolysaccharide (LPS)	LOS	*galU*, *gmhA*, *htrB*, *kdsA*, *lpxABCDHK*, *msbA*, *opsX*, *orfM*, *rfaDEF*, *wbaP*, *wecA*
	O-antigen	*ddhACD*, *prt*, *wbcC*
	Other related genes	*acpXL*, *bplF*, *fabZ*, *gtrAB*, *hisH2*, *kdtB*, *orfH*, *pagP*, *waaGP*
Adhesins		*bcfH*, *csgABCDEFG*, *lpfC*, *pagN*, *pefCD*, *stdD*, *stfD*, *sthBC*, *upaGH*
Capsule		*cap8J*, *cpsF*, *galF*, *gmd*, *gnd*, *kpsF*, *manB*, *oppF*, *rmlAB*, *uppS*, *wbfBD*, *wcaHI*, *wcbN*, *wzb*
Efflux pump		*adeFG*, *ccmB*, *fbpC*, *fepC*, *hitC*, *mtrE*, *phuU*, *sugC*
Others		*hlyA*, *entABCDEFS*, *rck*, *sodB*, *sodCI*, *acrB*, *allABCDRS*, *bfmR*, *btpA*, *cesT*, *clpEP*, *fes*, *icl*, *mip*, *pvcC*, *ratB*, *ricA*, *sinH*, *cofT*, *virB*, *mig-5*

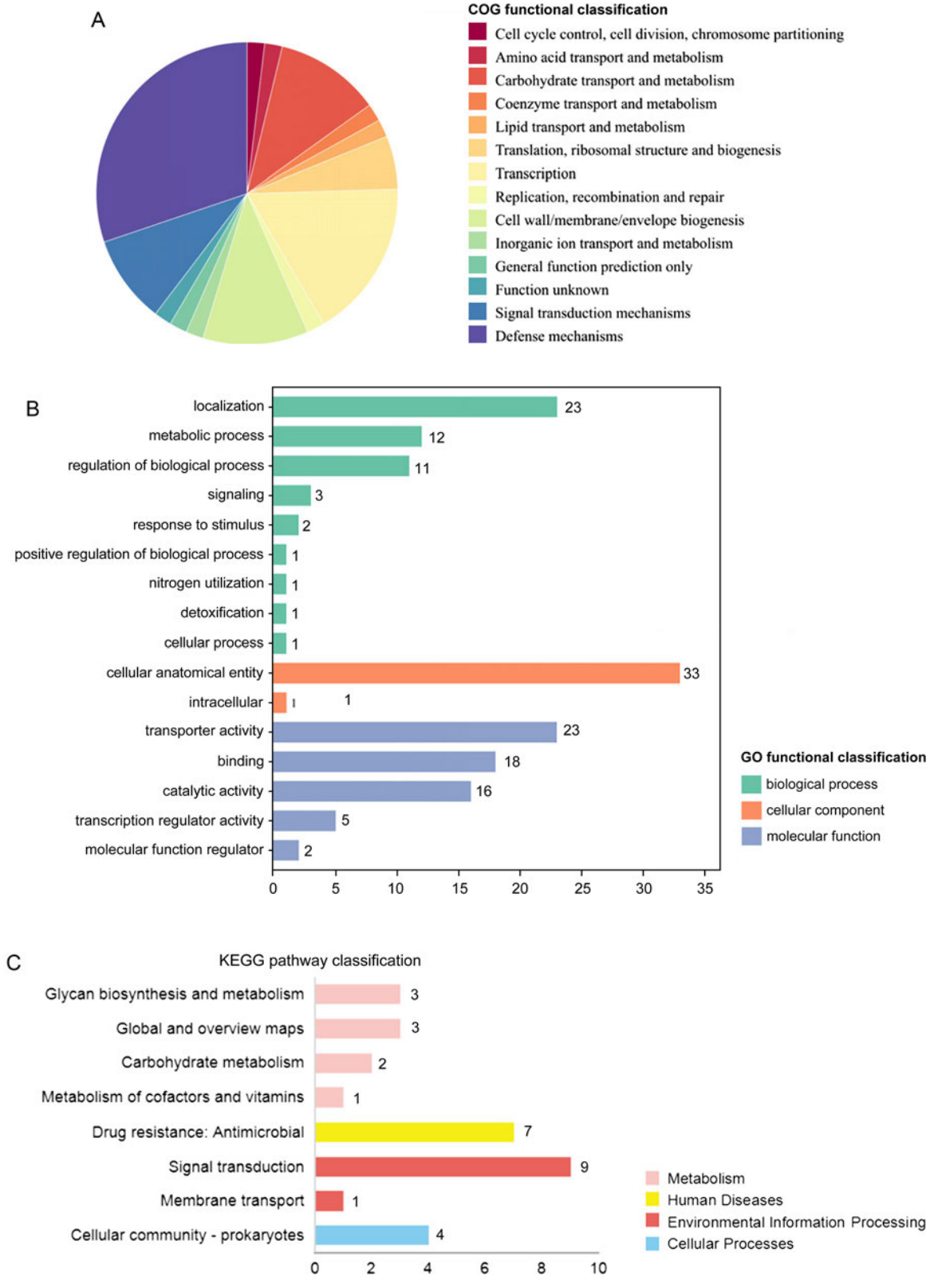


Fig. 5. A, B, C. Functional annotations of 21A resistance genes and virulence genes based on database searches.

be involved in defense mechanisms (Fig. 5A). The GO annotation indicated that 40 genes were involved in the categories 'biological processes', 'cellular component', and 'molecular function', and 82.5% of the genes were involved in 'cellular anatomical entity', 57.5% were associated with 'localization', and 57.5% were involved in 'transporter activity' (Fig. 5B). In the KEGG database, only 20 resistance genes were annotated, and these were involved in metabolism, environmental information processes, cellular processes, and human

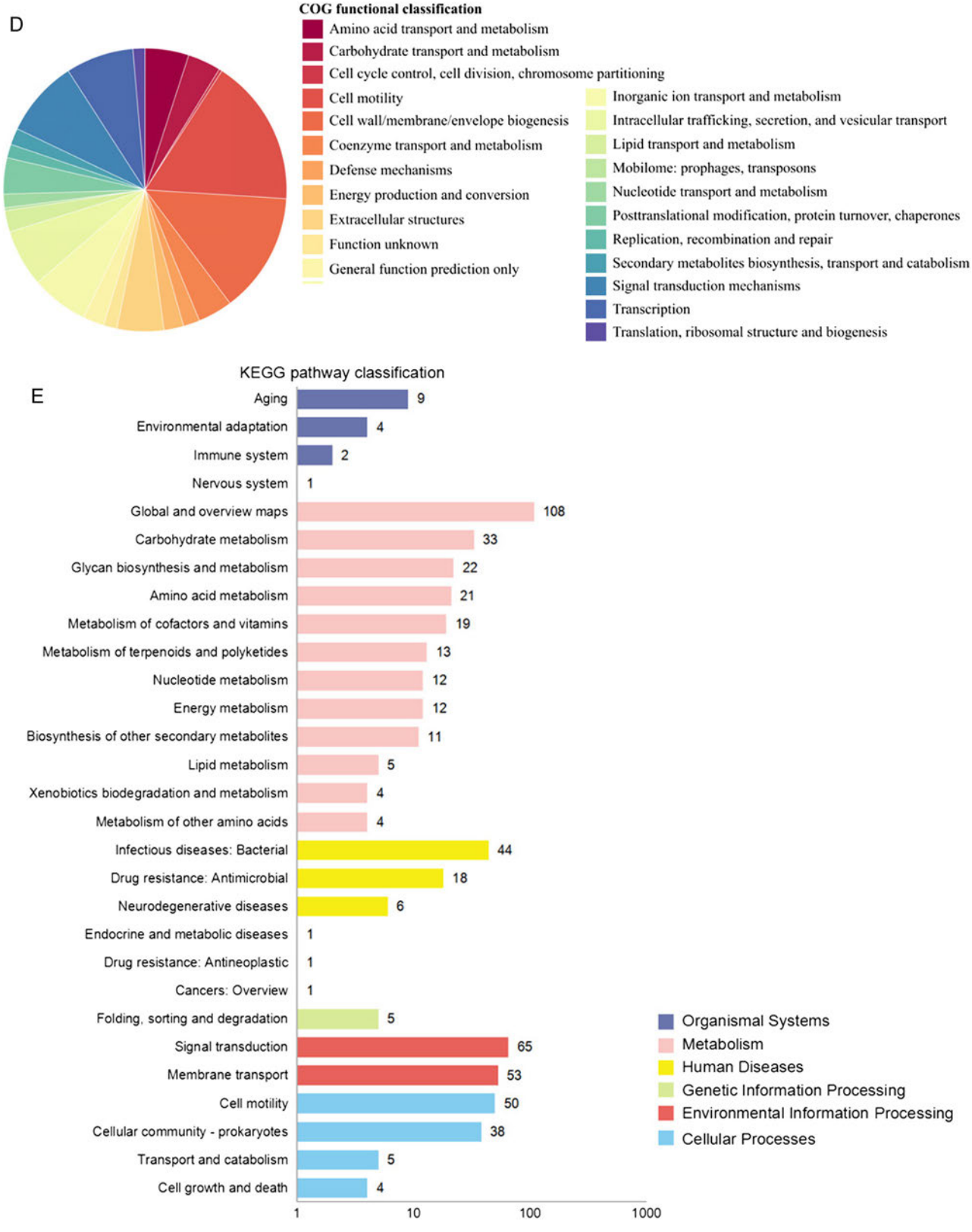


Fig. 5. D, E. Functional annotations of 21A resistance genes and virulence genes based on database searches.

diseases; 45% of these genes were involved in signal transduction (Fig. 5C).

The 554 virulence genes of 21A were also analyzed by functional annotation. The COG database predicted 477 genes, annotated into four categories of biological processes: cellular, information, metabolism, and poorly functioning. These genes mainly participated in intracellular processes and metabolic activities; 20.54% of them participated in cell motility, and 16.77% of the genes participated in cell wall/membrane/envelope

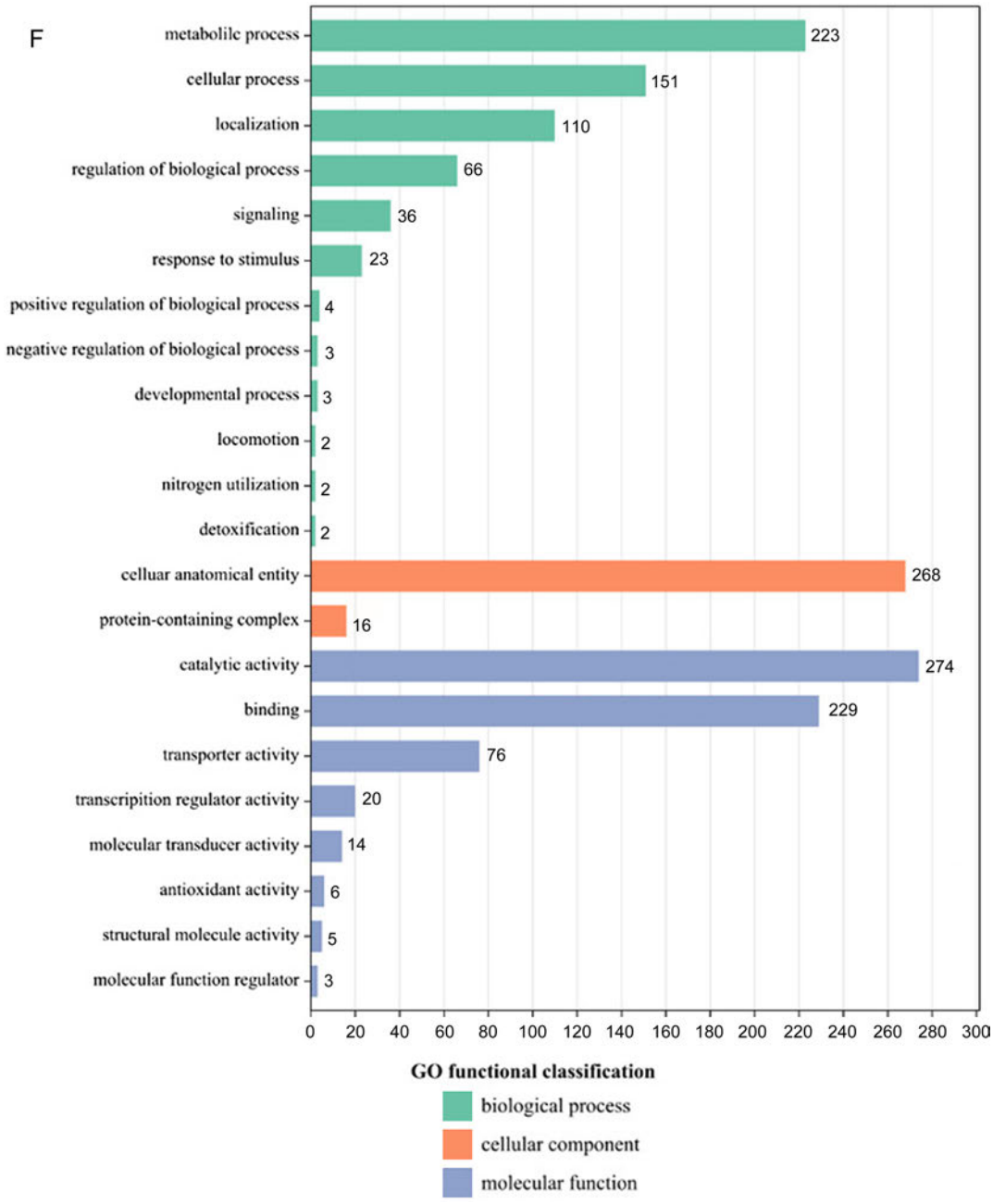


Fig. 5. F. Functional annotations of 21A resistance genes and virulence genes based on database searches.

biogenesis (Fig. 5D). According to the KEGG database, 321 genes were annotated in six functional categories, including 'metabolism', 'genetic information process', 'environmental information process', 'cellular processes', 'organismal systems' and 'human diseases'. Among these genes, 20.25% genes were involved in signal transduction, whereas 16.51% of them were involved in membrane transport (Fig. 5E). Based on the GO functional annotation, 456 genes were mainly involved in 'catalytic activity' (60.09%), 'cellular anatomical entity' (58.77%), 'binding' (50.22%) and 'metabolic process' (48.90%). Therefore, it was shown that the significant virulence gene process involved was 'catalytic activity' (Fig. 5F).

Phylogenetic analysis of strain 21A. A total of 959 *Salmonella* strains from Asia were found in the pubMLST database. After sorting out these strains' serovar and ST typing, 164 strains were screened out (Table SIV). Subsequently, the minimum spanning tree was generated based on the MLST strategy by combining the information of 164 strains and strain 21A (Fig. 6) to investigate the genetic associations and epidemiological relevance of strain 21A with other strains of *Salmonella*. As shown in Fig. 6, these strains were mainly divided into three clusters, and strains within the same clusters were further subdivided into several branches. ST11 formed the largest cluster, closely

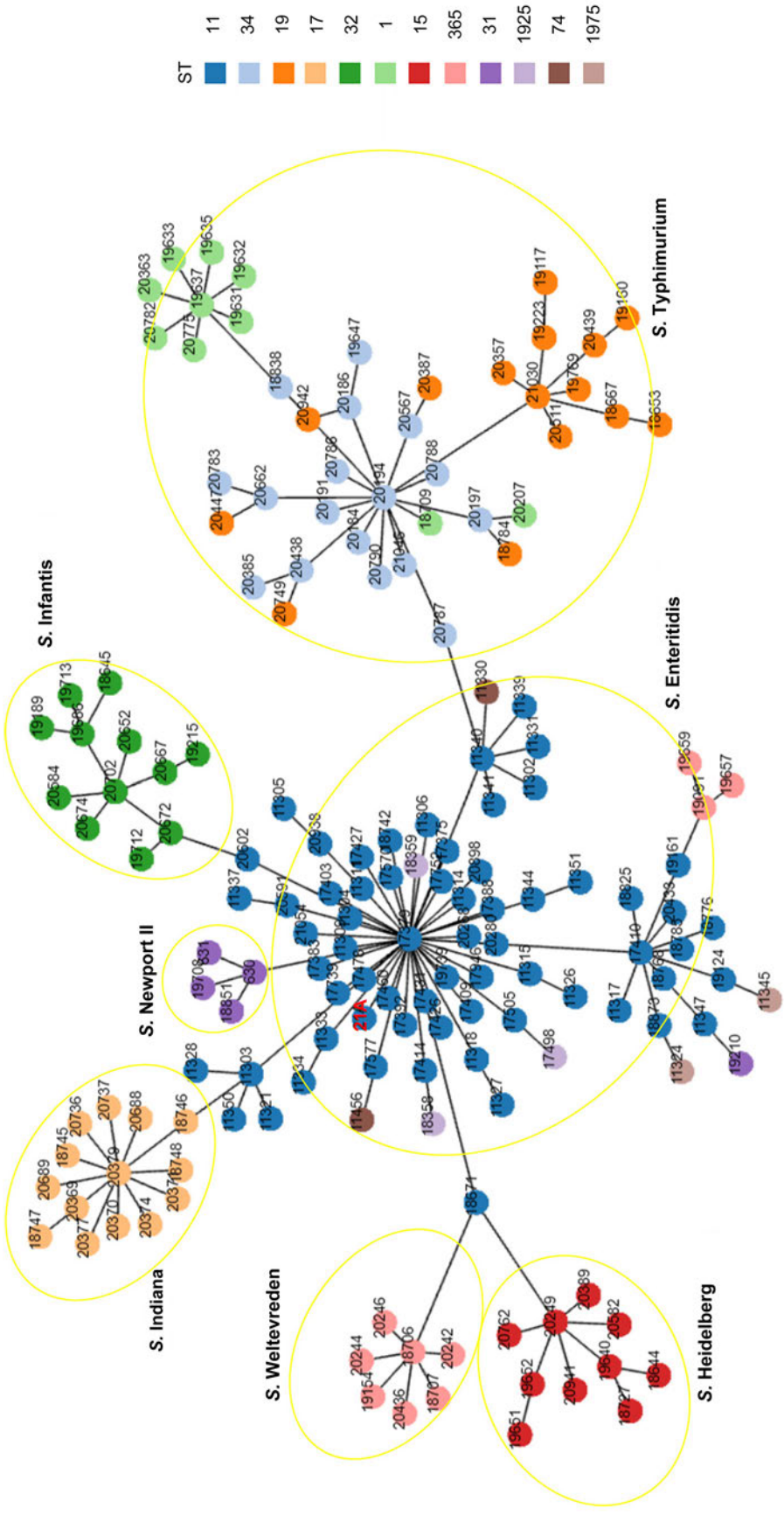


Fig. 6. Minimum spanning tree based on MLST.

associated with ST74 and ST1925, and had the closest kinship with them. ST34, ST19, and ST1 crossed each other to form a single cluster. Upon comparing the data shown in Fig. 6 and Table SII, it was revealed that the most common typing of *Salmonella* was ST11 in Asia, followed by ST34; in addition, the most common serovar was *S.* Enteritidis, followed by *Salmonella* Typhimurium. Different serovars had different typing. In Asia, *S.* Enteritidis was found to be mainly ST11 and ST1925, *S.* Typhimurium was mainly ST34 and ST19, *Salmonella* Indiana was ST17, *Salmonella* Infantis was ST32, *Salmonella* Heidelberg was ST15, *Salmonella* Weltevreden was ST365, and *Salmonella* Newport II was ST31.

A total of 61 ST11 *Salmonella* strains were selected from the NCBI database and combined with the 21A strain to analyze the SNP differences (Table SV); a phylogenetic tree featuring the distribution of resistance genes was constructed (Fig. 7). The phylogenetic relationship among the strains was very close, and the SNP strategy comprised dividing the 62 strains into three evolutionary clusters. The homologous relationship of strains within the same cluster was highly close, and the SNP differences were found to be the smallest. The phylogenetic tree showed that the genetic relationships among the strains were associated with the geographical location; moreover, the relationships among the strains from the same continent were found to be comparatively closer. The 21A strain in the present study was closely associated with the SJTUF series strains from Shanghai, China (Zhou et al. 2018). These strains [SJTUF11565 and SJTUF11653 (from animals), SJTUF11561 (from foods), and SJTUF11435 and SJTUF12519 (clinical)], being derived from animal, food, and clinical sources, suggested that the *Salmonella* strains that caused human illness were consistently associated with those strains of *Salmonella* linked with farms or food. Furthermore, regarding the distribution of resistance genes, the common resistance genes of *Salmonella* were identified as *aac(6')-Iaa*, *blaTEM-1*, *qnrB*, *floR*, *tetA*, *sul1*, and *sul2*. However, of these, only *aac(6')-Iaa* (aminoglycoside) and *blaTEM-1* (ampicillin) were found in strain 21A.

Core and pan-genome analyses. Twenty strains of *Salmonella* (Table SVI) closely related to strain 21A were screened from the phylogenetic tree, and all coding sequences (CDSs) from 21 *Salmonella* strains were used for core and pan-genome analyses. Core genes were considered the most conserved genes shared by all strains, whereas dispensable genes were shared by unique genes identified in only one of the genomes. The core genomes of these isolates comprised 3,555 genes, and dispensable genes were distributed in different strains in a range from 0–300 genes (Fig. 8A). The core and pan genomes of these 21 strains were subsequently analyzed according to the COG database (Fig. 8B). The core and pan genomes were found to be mainly associated with metabolism. Among the 3,555 core genes, 346 genes were assigned to category G (carbohydrate transport and metabolism), 322 to E (amino acid transport and metabolism), 244 to C (energy metabolism) and 210 to H (coenzyme transport and metabolism). Among the 330 pan genes, 89 were assigned to category X (mobilome: prophages, transposons), and 81 were assigned to G (carbohydrate transport and metabolism). Based on the distribution of the dispensable genes in different samples, heat-maps were drawn to show the clustering among the strains (Fig. 8C). The dispensable genes heat-map showed that the 21A strain was comparatively similar to *S. enterica* ASM950v1, and relatively different from *S. enterica* ASM276105v1 and *S. enterica* ASM694v2.

Genetic variation of strain 21A. Compared with *S.* Enteritidis ASM2413794v1, 38 single-nucleotide variants (SNVs) were identified in strain 21A, including three InDel mutations, 15 synonymous SNPs, and 20 non-synonymous SNPs. Of the three InDel mutations, two were insertion mutations, one of which had no significant effect on the open reading frame, and one was a deletion mutation, which was the deletion mutation located in the middle of the CDS. Among the 35 SNP sites, 34.3% of the variations were found to be the G > A variation, including the genes *trkA* (encoding the potassium transporter), *bamB* (encoding the outer membrane protein assembly factor), *yehD* (encoding fimbrial protein), *entF* (encoding enterobactin non-ribosomal peptide synthetase), *cdaR* (encoding DNA-binding transcription regulator), *moeA* (encoding molybdopterin molybdotransferase), *cpoB* (encoding the cell division protein), and specific genes encoding DNA-directed RNA polymerase subunit β and F0F1 ATP synthase subunit β, Among all the SNPs, only the gene *entF* was found to have two SNP sites, comprising the T > G and G > A variations (Fig. 9 and Table SVII).

Discussion

Salmonella is one of the most important pathogens causing foodborne illness (Xu et al. 2022). Its infection is closely associated with food contamination, and the leading cause of infectious diarrhea is the consumption of *Salmonella*-contaminated food (Qi et al. 2019). Therefore, understanding the drug resistance, pathogenic characteristics, and genetic evolution of *Salmonella* should provide a theoretical basis for the clinical treatment of *Salmonella* infection, which is of great significance for the prevention and control of *Salmonella*. The present study has collected a strain of *S.* Enteritidis 21A from a patient with food poisoning admitted to a rank A tertiary hospital in Shanxi, China. Via means

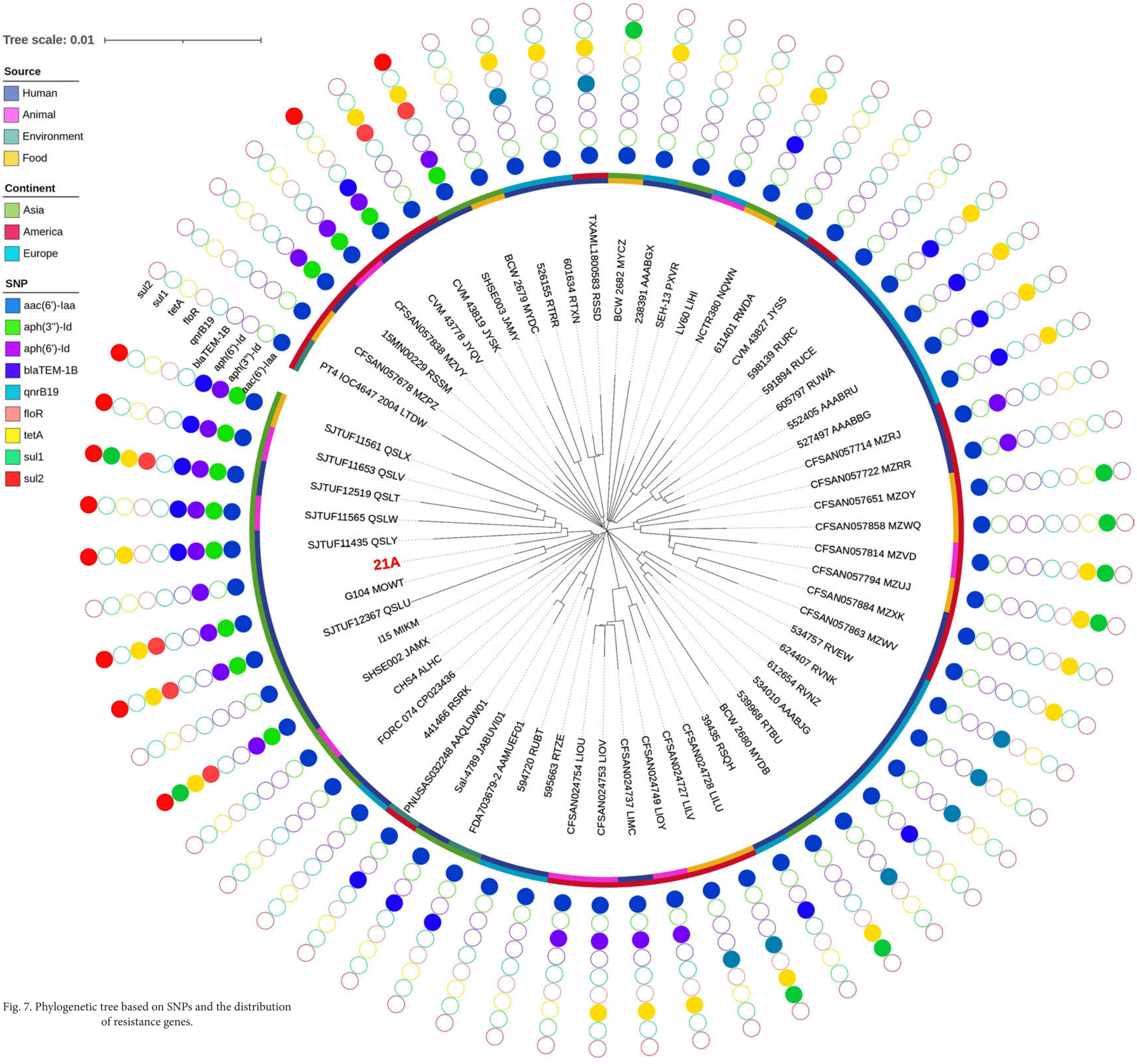


Fig. 7. Phylogenetic tree based on SNPs and the distribution of resistance genes.

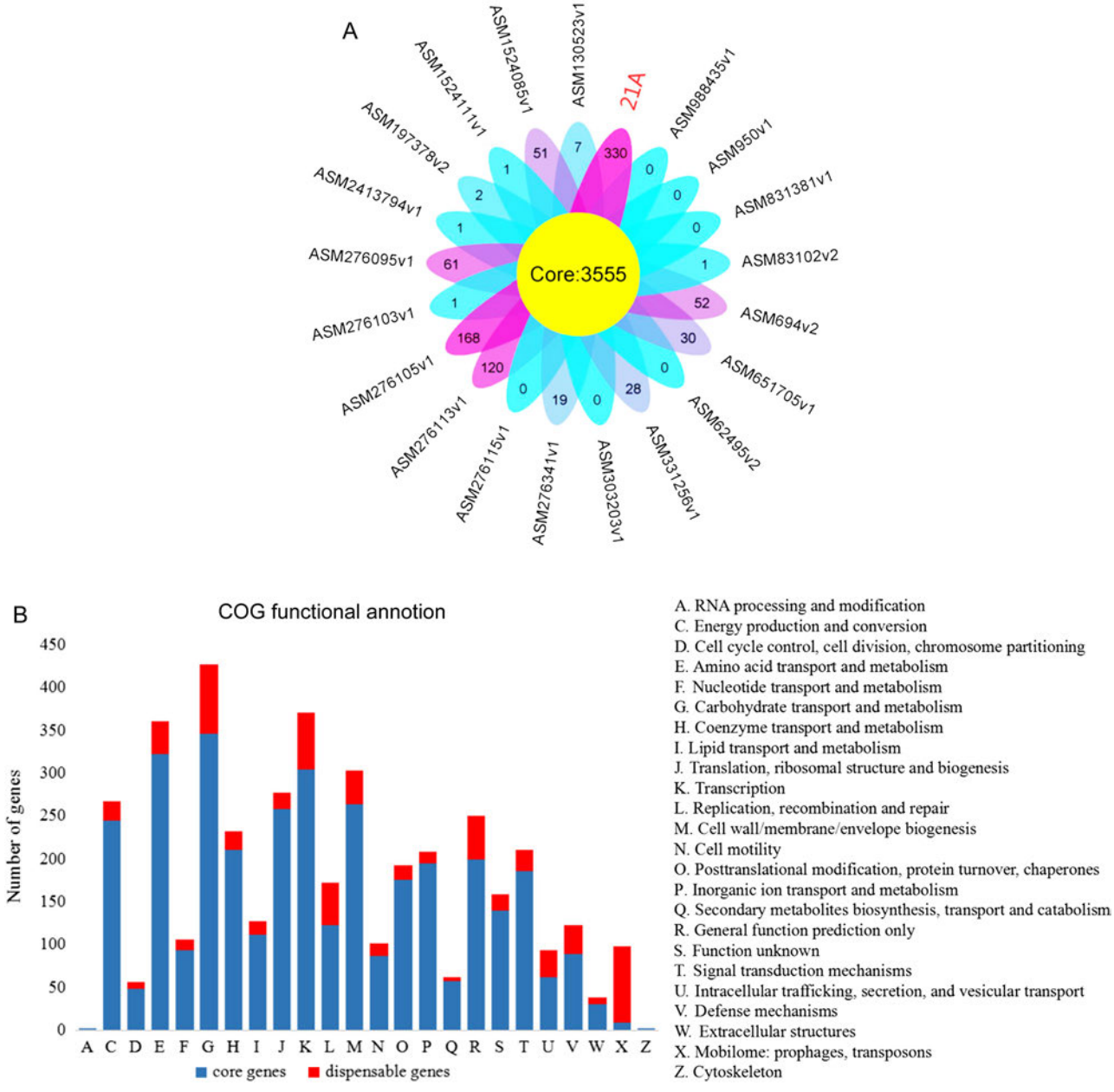


Fig. 8. A, B. Core and pan genome analysis.

of WGS, drug resistance determination, and genotyping, the underlying mechanisms of drug resistance and virulence, genome structural characteristics, and genetic evolution have been deeply explored.

With the increasing use of antibiotics, bacterial resistance has become increasingly severe. Clinically, quinolones and third-generation cephalosporins are often used to treat *Salmonella* infections (Fardsanei et al. 2018). In this study, the 21A strain has shown resistance to varieties of antibiotics, especially ampicillin and ciprofloxacin, but sensitivity to cephalosporins, which is consistent with reports of clinically multidrug-resistant *Salmonella* in China (Xu et al. 2022). For *S.* Enteritidis in food animals, slaughterhouses, and retail markets, ampicillin and tetracycline have the highest resistance rates among antibiotics (Wang et al. 2020a; Tang et al. 2022). In addition, Balbin et al. (2020) collected *Salmonella* from different environments exposed to human activities and found that its drug resistance rate was high. *Salmonella* isolated from natural environments was also found to exhibit high resistance rates; for example, as high as 100% resistance to sulfamethoxazole was found, mainly because the majority of antibiotics currently in use have been extracted from the natural environment, and also due to the fact that human activity in the environment affects the genetics and variation of microbial communities, leading to the emergence of new resistance mechanisms.

Moreover, the use of antibiotics and disinfectants was increased in response to the efforts to ameliorate

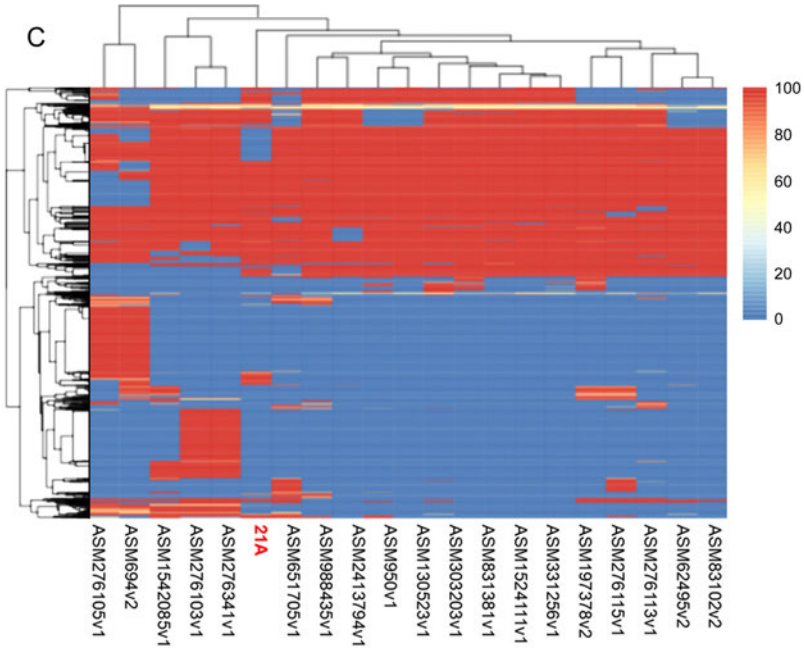


Fig. 8. C. Core and pan genome analysis.

the effects of COVID-19. Exposure of microbes to antibiotics and disinfectants contributes to their ability to evolve mechanisms that will increase antimicrobial resistance (Lobie et al. 2021). According to a previous study (Lu et al. 2023), compared with those in 2018, the antibiotic resistance of *Salmonella* isolates from December 2020 to April 2021 was found to have increased, and an increased number of gene mutations associated with resistance had developed. Therefore, it is crucial to continuously monitor the prevalence and infection of *Salmonella* in the livestock industry and the environment to prevent the development and spread of multidrug-resistant bacteria. At the same time, treating infectious diarrhea should be combined with the results of antimicrobial susceptibility testing in clinical practice. In this study, patients were initially treated with empirical therapy using levofloxacin upon hospital admission, but antimicrobial susceptibility testing of the strain showed resistance to levofloxacin. Therefore, clinical treatment should be closely integrated with antimicrobial susceptibility testing to avoid increasing the proportion of multi-resistant bacteria due to excessive use of antibiotics in clinics.

Analysis of the CARD database revealed that the 21A strain carried different resistance genes, which were associated with its high-level multi-resistance phenotype. 21A was found to contain the *aac(6')-Iaa* gene associated with aminoglycosides; however, this gene is usually transcriptionally silent and rarely has transcriptional activity, so its presence would not typically confer resistance to aminoglycoside antibiotics in *Salmonella*. The *blaTEM-1* gene confers ampicillin resistance to *Salmonella*; the *bla* gene is known to control resistance to β-lactam antibiotics through hydrolyzing their β-lactam rings, thereby inactivating this class of antibiotics (Tang et al. 2022). One of the main reasons for multi-resistance was found to be the overexpression of intrinsic and acquired efflux pumps. The primary efflux pump in strain 21A was found to be the resistance-nodulation-division superfamily (RND), including *acrB*, *baeR*, *mdtB*, and *mdtC*, which encode secondary transporter proteins responsible for the efflux of a variety of antibiotics with different structures (Zwama and Nishino 2021). The *acrB* gene encodes the AcrB protein, mainly associated with quinolone antibiotic resistance (Johnson et al. 2020). The *mdtB*/*mdtC* genes encode the transmembrane heteropolymers MdtB/MdtC, which are responsible for the efflux of β-lactam, neobiotin, tetracycline, aminoglycoside, and other antibiotics (Abi Khattar et al. 2019). BaeR (which is the translation product of the gene *baeR*) is a DNA-binding regulatory factor in the cytoplasm, which forms the BaeSR two-component signal transduction system to regulate the resistance to ciprofloxacin with the membrane-associated histidine kinase BaeS.

Moreover, a direct regulatory effect involves both the BaeSR two-component system and the AcrB efflux pump system. They interact with each other to influence drug resistance and toxicity by affecting the phosphorylation states of *Salmonella* proteins (Qi et al. 2022). The BaeSR two-component system also regulates the MdtABC efflux pump, which detects several specific envelope-damaging compounds (including indoles, zinc, copper, and certain flavonoids) and induces the MdtABC efflux pump to expel these inducible compounds, as well as certain antibiotics and bile-salt

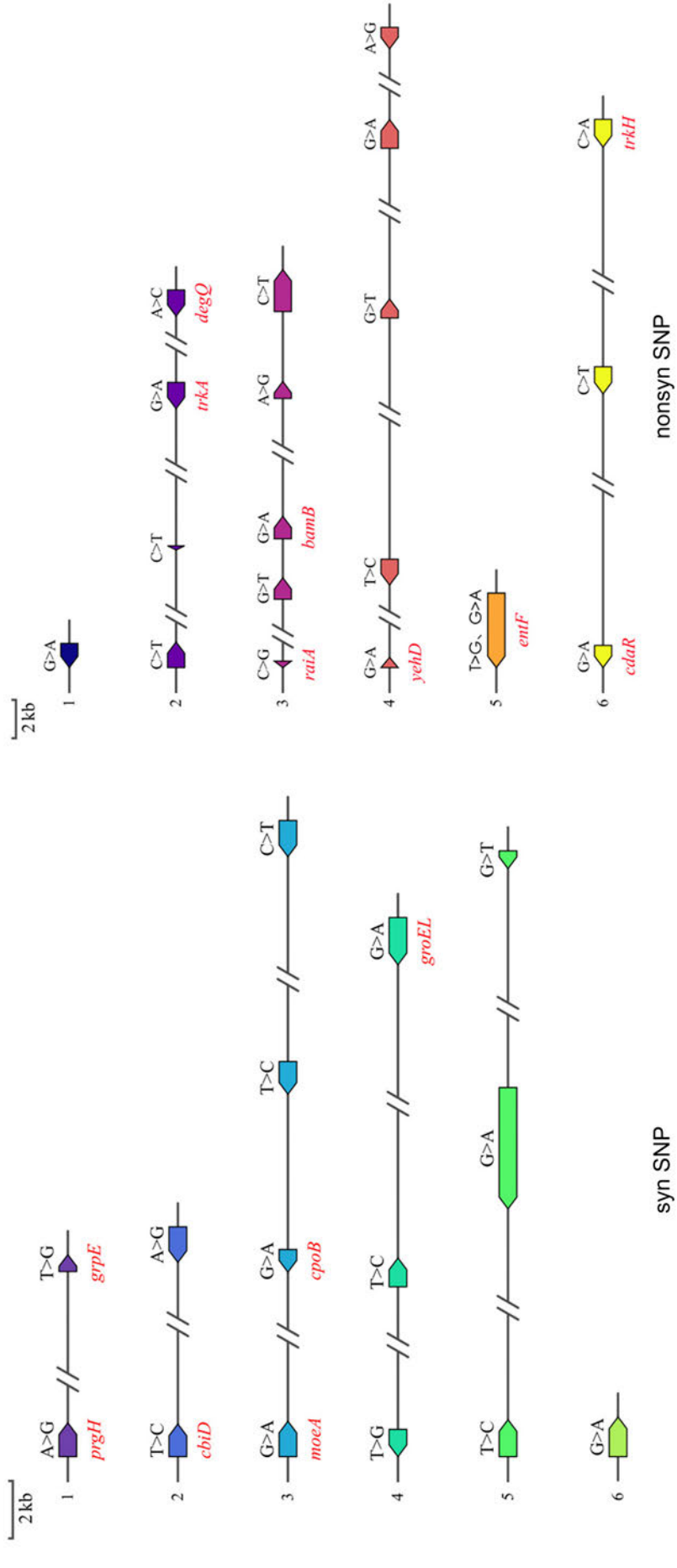


Fig. 9. SNP distributions in the chromosome gene cluster of strain 21A.

derivatives, thereby maintaining bacterial envelope homeostasis (Abi Khattar et al. 2019). Aside from these findings, 21A also expresses other multi-resistant bacterial transporters, including the major facilitator superfamily (MFS), where EmrAB has been shown to confer resistance to neomycin and nalidixic acid, and MdtK conferred resistance to norfloxacin and doxorubicin (Gu et al. 2021).

The virulence of *Salmonella* is associated with the combination of chromosome and plasmid factors. In the present study, the virulence genes of strain 21A were identified and functionally annotated. The VFDB indicated that these genes were mainly involved in transmission and infection, including motility, catalytic activity, metabolism, toxins, virulence regulation, and secretion. Specific genes that help *Salmonella* attach to intestinal villi can also induce interbacterial attachment and promote biofilm formation. The *invA* gene was predicted to exist in strain 21A, which was found to be highly conserved in *Salmonella* and could be used as a specific biomarker for *Salmonella* identification. As a component of the TTSS, *invA* is located in SPI-1 and induces *Salmonella* to invade intestinal mucosal epithelial cells (Mohammed 2022). The SPIs were both found to be required for virulence, encoding the majority of the virulence factors, and shown to be closely associated with the TTSS, allowing *Salmonella* to colonize the host by attaching to and invading the cells and bypassing their defense mechanisms. Four SPIs have been annotated in strain 21A, namely SPI-1, -2, -3 and -4. SPI-1 encodes TTSS-1, effector proteins, and associated transcription factors, enabling it to invade into epithelial cells (Lou et al. 2019; Lerminiaux et al. 2020). SPI-2 has been associated with systemic infection and the intracellular accumulation of *Salmonella* (Jennings et al. 2017). SPI-3 can be used as a virulence marker for the detection of *Salmonella*, such as the *mgtC* gene, which was detected in strain 21A and subsequently has become a virulence marker for the purposes of *Salmonella* screening. Another gene, the *pagC* gene, has been identified. Studies have shown that the detection rate of the *pagC* gene in *Salmonella* extracted from the feces of diarrheal children was 100% (Yue et al. 2020). It can act by inhibiting the proliferation of *Salmonella* within macrophages and can be used in food production to detect whether *Salmonella* is in a viable but nonculturable state (Yue et al. 2020). The absence of this gene in strain 21A may be associated with the state of the bacteria at the time of isolation and detection. In addition, specific genes were detected in strain 21A, including the serum resistance gene *rck*, stress adaptation gene *sodCl* and the *spv* genes (*spvB*, *spvC*, and *spvR*), which are associated with plasmid transmission. The *spv* genes have been shown to inhibit the type I interferon response and neutrophil chemotaxis through inhibiting autophagy and can also destroy the integrity of intestinal epithelial cells to increase intestinal permeability, thereby facilitating the translocation of *Salmonella* (Wang et al. 2019; Sun et al. 2020;).

The rapid development of WGS technology has reduced the time and costs required for sequencing. Its powerful functions and rapidly implemented methods have proven to be convenient for studying bacterial epidemiology, providing the means for rapid tracking and tracing of the spread of pathogens (Wu and Hulme 2021). In the present study, strain 21A was shown to belong to ST11 *S.* Enteritidis. According to reports published by the US Centers for Diseases Control and Prevention and European Food Safety Authority, *S.* Enteritidis was identified as the most common serovar of nontyphoidal salmonellosis, and ST11 *S.* Enteritidis was also shown to be one of the common subtypes of invasive *Salmonella* in certain Asian and African countries (Aung et al. 2022). ST11 was revealed as the primary prevalent type of *S.* Enteritidis in China, and has been shown in clinically infected people, animals, and food (Yan et al. 2021; Chen et al. 2022;). In 2021, a food poisoning incident with severe symptoms of gastroenteritis broke out in Beijing, China. The 20 strains of *S.* Enteritidis isolated from those afflicted with food poisoning were all ST11. The patients in this food poisoning incident were young people with an average age of 24.1 years, and the clinical symptoms were mainly diarrhea and high fever (Zhang et al. 2021). These findings were consistent with the results presented in the present study. In this food poisoning incident, an ST11 *S. enterica* 27A strain had been previously isolated (Xu et al. 2023). It shares the same genotype, serotype, and plasmid replicon as the 21A strain in this study, but the clinical symptoms caused by the two strains have slight differences. Patients infected with 27A strain mainly exhibited diarrhea as the primary symptom and were discharged after 2 days of antibiotic treatment.

In contrast, patients infected with the 21A strain, in addition to diarrhea, also experienced dizziness, nausea, and fever and were discharged after 5 days of antibiotic treatment. Both 27A and 21A strains contain various antibiotic resistance genes and virulence genes, with 27A specifically containing the unique fimbrial adhesin gene *sefR*. The phylogenetic tree analysis revealed that the genetic characteristics of drug resistance in *Salmonella* exhibited geographical variations, and the genetic relationships among the various *Salmonella* strains were associated with geographical location. Cao et al. (2023) conducted a phylogenetic analysis on 197 strains of clinical *Salmonella* from China, the United States, Europe, and Africa and identified four main clades. The Chinese isolates were located in clade II, with the common resistance genes being *aph(3')-IIa*, *blaCTX-M-55* and *blaTEM-1B.* By contrast, the Africa isolates were mainly

located in clade III, with the common resistance genes being *aph(3")-Ib* and *aph(6)-Id*. This confirmed that, for *Salmonella* isolated from humans, significant geographical differences exist in genome and drug resistance.

Moreover, similar findings were identified in *Salmonella* isolated from domestic animals (Li et al. 2021). In addition, the phylogenetic tree analysis showed that strain 21A from humans was closely associated with strains from animals and foodstuffs, which was consistent with a previously published study, which reported that human diseases caused by *Salmonella* were linked with *Salmonella* isolated from farms or foods (Pan et al. 2019). The genomic similarities that were noted between strain 21A and the SJTUF series strains from Shanghai, China, were attributed to the fact that they shared the same biological characteristics and evolutionary environment. The strain 21A (from the clinic) was found to be closely associated with SJTUF11565, 11653 (from animals), and SJTUF11561 (from foodstuffs), which strongly supported the notion that animals infected with *Salmonella* can infect humans through the food chains and cause human *Salmonella* infection. Therefore, it is necessary to ensure food safety by continuously monitoring the prevalence and spread of *Salmonella* in food, and employing enhanced management, surveillance and control measures in farms and slaughterhouses where chickens, ducks, pigs, etc., are raised.

In conclusion, the present study screened and isolated *S.* Enteritidis from the feces of patients with food poisoning, and the 21A strain was subjected to epidemiological monitoring, drug resistance detection and WGS analysis, revealing its drug resistance, virulence, and molecular evolution characteristics. The strain 21A was found to be a multidrug-resistant bacterium, with its genome encoding multiple drug-resistance genes and virulence genes. Identifying and annotating these genes were of great significance in furthering our understanding of drug resistance and the pathogenic mechanism of *S.* Enteritidis. In addition, the genetic evolutionary characteristics of *Salmonella* ST11 were explored using MLST-based minimum spanning tree and SNP-based phylogenetic tree analyses. It was found that the prevalence of *Salmonella* ST11 was both diverse and closely associated with different hosts. These findings have contributed to our understanding of the phylogenetics of *S.* Enteritidis, which should be helpful in terms of developing novel strategies for the prevention and treatment of infections caused by this pathogen.

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Abbreviations

cgMLST – core genome multilocus sequence typing
CLSI – Clinical and Laboratory Standards Institute
CTAB – Cetyltrimethylammonium bromide
InDel – insertions and deletions
M cells – microfold cells
MLST – multilocus sequence typing
SBG – Selenite Brilliant Green
SNP – single-nucleotide polymorphism
SPI – *Salmonella* pathogenicity island
SS culture – *Salmonella-Shigella* culture
ST – sequence type
TSBG – Tryptic soy broth supplemented with glucose
TTSS – Type III secretion system
wgMLST – whole-genome multilocus sequence typing
WGS – whole-genome sequencing
wgSNP – whole-gene single-nucleotide polymorphism

Availability of data and material

The nucleotide sequence of the chromosome and plasmids of *S.* Enteritidis isolate 21A have been deposited in GenBank under accession numbers CP120837-CP120839.

All raw data and supporting materials related to this paper can be obtained from the corresponding author.

Ethical statement

The study was approved by the Ethics Committee at Fenyang College of Shanxi Medical University (2021014). All research subjects signed an informed consent. All methods were performed in accordance with relevant guidelines and regulations. All investigators adhered to the principles expressed in the Declaration of Helsinki.

Funding

This study was supported by Fundamental Research Program of Shanxi Province (Grant No. 20210302123397; Grant No. 2022030 21212351), Key R&D Projects of Introducing High-Level Scientific and Technological Talents in Lvliang City (Grant No. 2021RC-1-4), the Project of Lvliang City Science and Technology Program (Grant No. 2020SHFZ29), Science and Technology Innovation Project of Colleges and Universities in Shanxi Province (Grant No. 2020L0749), the National College Students' Innovation and Entrepreneurship Training Program (Grant No. 20221569), and Special Fund for Key Disciplines of Fenyang College of Shanxi Medical University (Grant No. 2022B14).

Author contributions

Benjin Xu conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Zhuru Hou conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Ling Liu conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Rongrong Yan performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Jinjing Zhang performed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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