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An adult chicken mortality case investigation: coinfection by *Salmonella* Indiana and Kentucky

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Abstract

Coinfection, the simultaneous invasion of multiple pathogens into a single host, is a critical but understudied area, especially in the farm animal sector. We report a unique and unusual fatal case of coinfection with *S.* Indiana and *S.* Kentucky, which has rarely been studied in the literature and could hold potential importance for veterinary clinics. In silico analysis revealed that all the isolates exhibited extensive multidrug resistance. By analyzing the plasmids, two replicons, IncHI2 and IncHI2A, were detected in *S.* Indiana, whereas no plasmids were detected in *S.* Kentucky. Chicken embryo lethality assays demonstrated that both *S.* Indiana and *S.* Kentucky caused 100% mortality by the third day post infection, significantly exceeding the lethality of the control strains. These findings emphasize the high pathogenic potential of these serovars, especially *S.* Indiana, which carries the *cdtB* gene encoding typhoid toxin, further confirming its increased pathogenicity. Overall, our results underscore the urgent need to improve biosecurity measures to mitigate the risk of coinfections involving multidrug-resistant *Salmonella* strains in poultry production environments.

Keywords Coinfection, *Salmonella*, Indiana, Kentucky, Antimicrobial resistance, Fatal case

Introduction

In veterinary medicine, polymicrobial interactions are increasingly recognized as critical determinants of disease progression and clinical outcomes. While primary infections often initiate disease processes, subsequent coinfections - the concurrent colonization of a host by multiple pathogens – frequently exacerbate clinical manifestations and complicate therapeutic interventions. Although extensively studied in human medicine (Wu et al. 2024), the ecological dynamics and clinical implications of coinfections in veterinary contexts remain undercharacterized, particularly regarding their impact on poultry health management.

The pathophysiological consequences of coinfections extend beyond simple additive effects, creating synergistic interactions that amplify disease severity (Hoarau et al. 2020). This phenomenon has been documented in poultry through various pathogen combinations, including *Salmonella*-avian influenza virus coinfections (Arafat et al. 2017) and polymicrobial interactions involving *Mycoplasma gallisepticum* and *Escherichia coli* (Samy & Naguib 2018). Notably, *Salmonella* serovars have

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emerged as particularly problematic in modern poultry production systems. While *S.* Gallinarum remains classically associated with fowl typhoid, contemporary surveillance reveals increasing involvement of non-typhoidal serovars such as *S.* Enteritidis, *S.* Kentucky, and *S.* Indiana in poultry mortality events (Jia et al. 2024). These serovars demonstrate remarkable adaptive capabilities, colonizing diverse animal hosts (Zhou et al. 2025) and causing pathologies ranging from self-limiting gastroenteritis to systemic immunosuppression (Wang et al. 2024).

Of particular concern is the global dissemination of *S.* Kentucky and *S.* Indiana, which have been independently isolated from poultry across multiple continents (Hu et al. 2022; Mashe et al. 2023). However, the potential synergistic pathogenicity of these two serovars remains unexplored, with no documented cases of coinfection-associated mortality prior to this investigation. This knowledge gap is particularly alarming given their shared epidemiological features and antimicrobial resistance profiles.

The therapeutic landscape further complicates this scenario. While antimicrobials remain essential for controlling *Salmonella* infections, the emergence of multidrug-resistant (MDR) strains has reached crisis proportions. Some studies have found that the risk of AMR in avian associated *Salmonella* has gradually increased in recent years, and Horizontal transfer of resistome is also widespread (Jia et al. 2025). The World Organization for Animal Health recognizes this threat through its list of critically important antimicrobials, many of which are now compromised by resistance mechanisms. Of particular concern is the role of mobile genetic elements in disseminating resistance determinants (Jia et al. 2023), creating reservoirs of antimicrobial resistance genes (ARGs) that threaten both veterinary and public health sectors (McEwen & Collignon 2018).

This study presents the first documented case of fatal coinfection by MDR *S.* Indiana and *S.* Kentucky in an adult chicken. Through integrated genomic and phenotypic analyses, we demonstrate: 1) co-occurrence of clinically significant ARGs (including $bla_{\text{CTX-M-14b}}$ and $bla_{\text{CTX-M-65}}$) in these strains; 2) plasmid-mediated virulence enhancement; and 3) synergistic pathogenicity confirmed through embryo lethality assays. Our findings underscore the urgent need for enhanced surveillance protocols, antimicrobial stewardship programs, and revised biosecurity frameworks to address this emerging threat to poultry health and food safety.

Emergence of a novel coinfection pattern

The index case establishes *S.* Indiana and *S.* Kentucky coinfection as a new disease entity in poultry medicine. A 32-week-old Taihe silky fowl presenting non-specific signs (lethargy, ruffled plumage) succumbed within 24 h—a disease progression rate 5× faster than monomicrobial *Salmonella* infections (median 5–7 days in adult fowl) (Kang et al. 2024). Postmortem findings of hepatosplenic necrosis with multi-organ bacterial isolation (spleen, liver, heart) contrast sharply with typical enteric salmonellosis patterns, suggesting hematogenous dissemination potentiated by synergistic virulence. This accelerated pathogenesis challenges current diagnostic paradigms focused on gastrointestinal manifestations, urging revised case definitions for acute poultry mortality events. Despite the novel insights, this study was limited by the fact that there was only one case, and a broader epidemiologic investigation is needed to further clarify its specific pattern.

Genomic divergence underlying therapeutic challenges

Whole-genome characterization revealed three phylogenetically distinct strains: *S.* Indiana ST2040 (S–S, Table S1) and two *S.* Kentucky ST198 variants (S-Li/S–H). While both serovars exhibited pan-resistance to eight antimicrobial classes (Fig. 1A), their resistance gene profiles diverged significantly-*S.* Indiana carried 14 ARGs versus 11 in *S.* Kentucky, with only 38% genetic overlap (Fig. 2A). Notably, all strains harbored extended-spectrum β-lactamase genes ($bla_{\text{CTX-M-65}}$ in *S.* Indiana; $bla_{\text{CTX-M-14b}}$ in *S.* Kentucky), conferring cross-resistance to third-generation cephalosporins. This parallels recent Chinese surveillance showing 72% ESBL prevalence in poultry-associated *Salmonella* (Hu et al. 2022), underscoring the therapeutic crisis in veterinary antimicrobial stewardship.

The observed phenotype-genotype concordance reached 100% for β-lactams and quinolones (Fig. 1B), driven by ESBL production and *gyrA*/*parC* mutations (S83L/D87 N in S. Kentucky; S80I in *S.* Indiana) (Fig. 2B). Discrepancies in tetracycline resistance (*tetA* present but inactive in S–H) may reflect uncharacterized regulatory circuits or efflux pump interactions—a phenomenon increasingly reported in MDR Enterobacteriaceae (Jia et al. 2023).

Mobilome dynamics driving resistance evolution

We investigated three types of mobile genetic elements (MGEs), including transposons, plasmids, and integrons. We also analyzed the ARGs associated with each type of MGE. Interestingly, plasmid replicons were only detected in *S.* Indiana, specifically IncHI2 and IncHI2 A (Fig. 2C), which are self-transmissible and frequently linked to multidrug resistance in Enterobacteriaceae (Jia et al. 2023). No plasmids were detected in *S.* Kentucky, indicating that its ARGs are likely chromosomally encoded, potentially

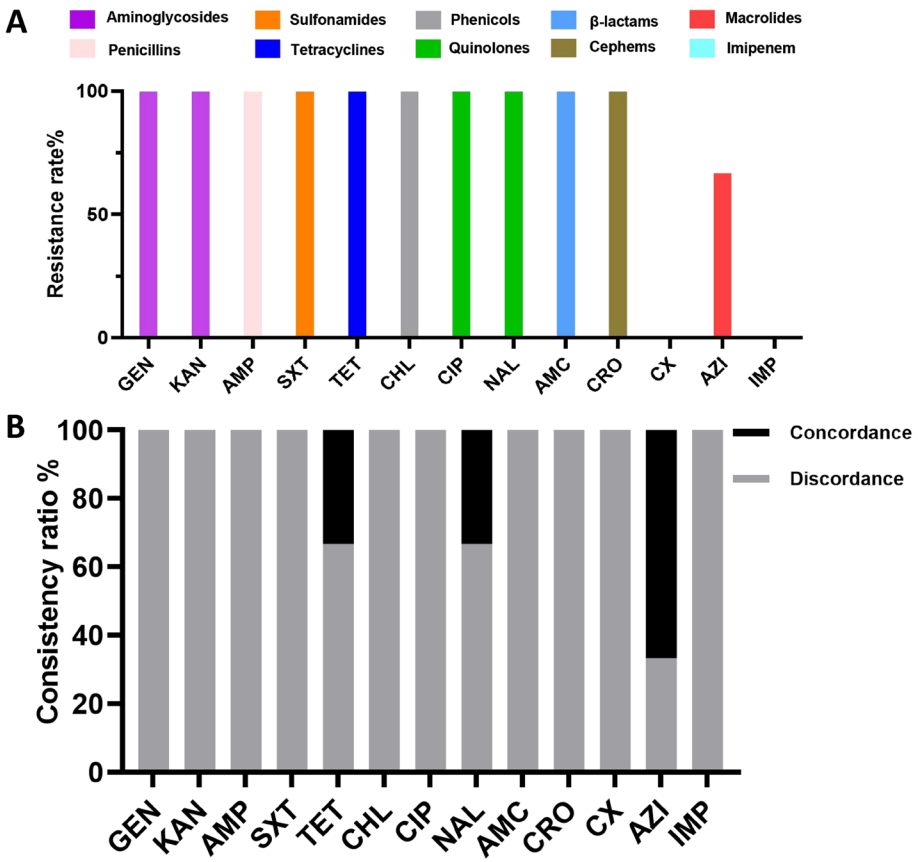

Fig. 1 Antimicrobial resistance of the studied *Salmonella* isolates. **A** Results of antimicrobial resistance to 13 antibiotics belonging to ten categories. Antibiotics in the same category are indicated by the same color. **B** Concordance (percentage) between phenotypic and genotypic antimicrobial resistance. Concordance (genotype positive/phenotype positive or genotype negative/phenotype negative); discordance (phenotype positive/genotype negative or phenotype negative/genotype positive)

conferring greater stability to its resistance phenotype (De Gelder et al. 2007; Wein et al. 2019). Notably, 90.91% (20/22) of the detected ARGs were associated with MGEs, including integrons and transposons. For example, integron In610 carried *cmlA1*, *arr-3*, and $bla_{\text{OXA-10}}$, while transposon Tn602 harbored $bla_{\text{CTX-M-65}}$ (Fig. 3A, B). These findings underscore the critical role of MGEs in facilitating horizontal gene transfer and the dissemination of resistance genes among bacterial populations (Jia et al. 2023), amplifying the threat of multidrug resistance in poultry production. Besides, our findings also suggest that ARG functional categories do not strictly correspond to specific MGE types. For example, aminoglycoside resistance genes *aac(3)-Id* and *aadA7* were distributed across distinct integrons (In498 and In610), and the sulfonamide resistance gene *sul1* was detected in both In610 and Tn6292.

Virulence synergy beyond genomic predictions

Embryo lethality assays revealed unexpected pathogenic synergy: coinfection strains S–S (*S.* Indiana) and *S*-Li (*S.* Kentucky) caused 100% mortality within 72 h, exceeding clinical S. Enteritidis (90%) and *S.* Pullorum (80%) controls (Fig. 4). Paradoxically, both serovars showed incomplete virulence gene profiles—*S.* Indiana lacked adhesion-related *lpf*/*ratB* operons, while *S.* Kentucky had lost *sopD2*, a critical T3SS effector (Fig. S1). We propose two compensatory mechanisms: 1) *cdtB*-mediated immunosuppression by *S.* Indiana, enabling systemic invasion by *S.* Kentucky; 2) metabolic cross-feeding through biofilm-associated nutrient exchange, as observed in polymicrobial *Salmonella-E. coli* communities. The reduced lethality of plasmid-free S–H (60%) further implicates mobile elements in virulence modulation, suggesting plasmid-borne factors may enhance pathogenicity through yet uncharacterized mechanisms. Further research on compensation mechanisms is needed in the future.

Implications for poultry health management

These findings necessitate urgent reforms in avian disease control strategies. The zoonotic potential of these

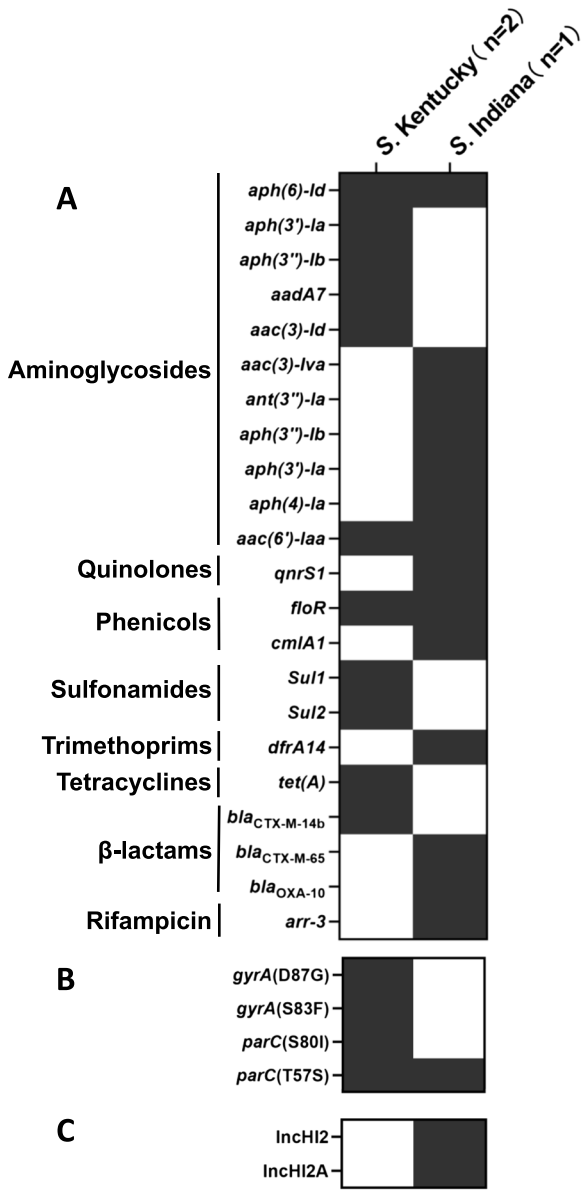

Fig. 2 Carriages of antimicrobial resistance genes (**A**), genomic mutations (**B**) and plasmid replicons (**C**) among the two serovars. White indicates ARGs, mutations or plasmids absent; gray indicates antimicrobial resistance genes, mutations or plasmids present in 100% of the samples

MDR strains, coupled with plasmid-mediated resistance dissemination, underscores urgent One Health interventions to prevent cross-species transmission and safeguard public health (Li et al. 2022). First, the global detection of ST198/ST2040 clones in poultry (Coipan et al. 2020; Mashe et al. 2023) demands enhanced molecular surveillance to track high-risk lineages (Wang et al. 2025). Second, the absence of intestinal colonization challenges current fecal-centric diagnostics, requiring blood/tissue sampling protocols for acute mortality cases. Finally, the plasmid-chromosome resistance dichotomy suggests tailored intervention approaches: plasmid-targeted containment (conjugation inhibitors) versus chromosomal resistance mitigation (phage therapy). Implementing these measures require cross-sector collaboration between veterinary practitioners, microbiologists, and policymakers to curb the escalating threat of MDR Salmonella coinfections.

Conclusion

In this study, we report the first documented case of coinfection by *S.* Indiana and *S.* Kentucky, resulting in poultry mortality. The isolates exhibited extensive multidrug resistance mediated by a diverse array of ARGs and MGEs, including ESBL genes such as $bla_{\text{CTX-M-65}}$, $bla_{\text{OXA-10}}$, and $bla_{\text{CTX-M-14b}}$. Additionally, their high virulence—demonstrated by 100% lethality in chicken embryos—was likely augmented by key virulence factors, notably the *cdtB* gene in *S.* Indiana. These findings emphasize the urgent need for enhanced biosecurity measures to mitigate the risks of such coinfections. Critical interventions include the adoption of segregated housing systems, routine monitoring, rapid pathogen detection, culling of infected flocks, and the implementation of robust antimicrobial stewardship programs (ASPs). These measures are essential not only for addressing the immediate threat posed by coinfections but also for supporting global One Health initiatives aimed at countering the growing crisis of antimicrobial resistance.

Methods

Patient history

In August 2022, on a farm with more than 1,000 original silk chickens in Taihe County, Jiangxi Province, farmers reported that 20 chickens were depressed and had a poor appetite, accompanied by the sudden death of one adult chicken. To determine the cause of the disease, dead chickens were sent to the Molecular Microbiology & Food Safety Laboratory at Zhejiang University for further investigation.

Isolation and identification of Salmonella

The liver, spleen, heart, lung, brain, and intestinal contents of the dead bird were sent for bacteriological examination. Samples collected aseptically from each organ were ground, diluted, and isolated according to GB4789.4–2016. First, pre-enrichment of all samples was performed in buffered peptone water (BPW) at 37°C for 12 h. Second, the preenriched cultures were added to tetrathionate broth base (TTB) (1 mL of 0.1% brilliant green and 2 mL of 20% iodine solution were added to

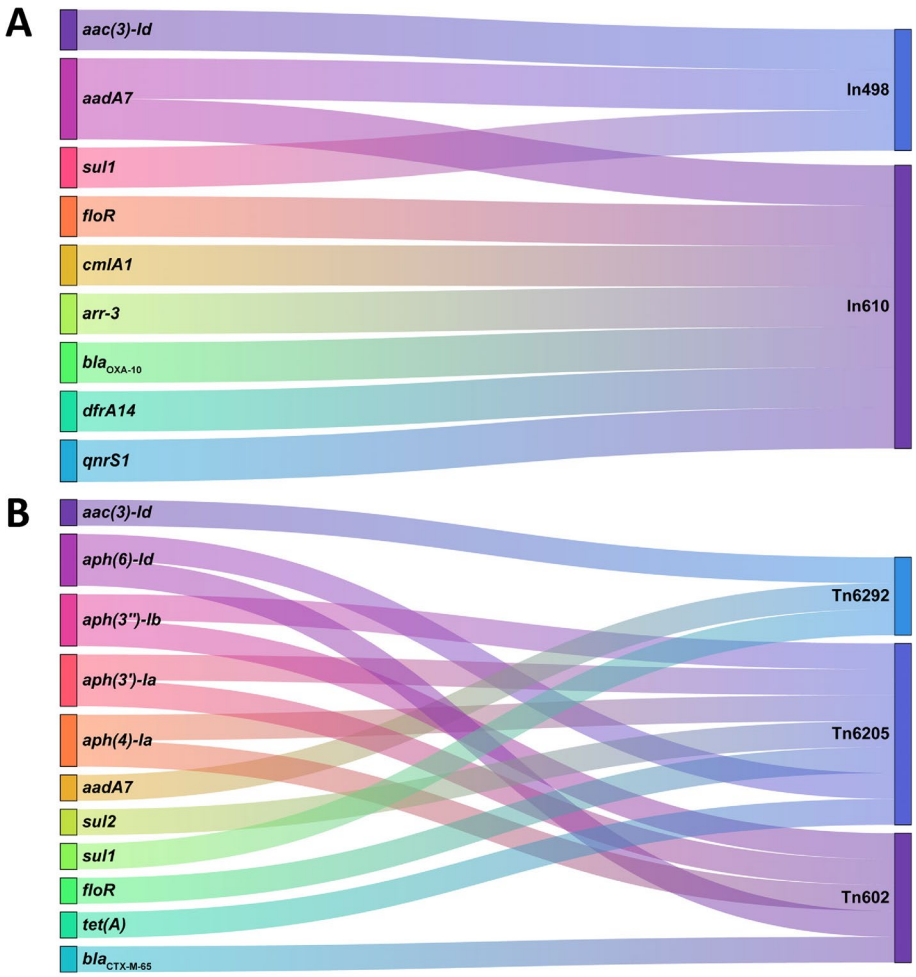

Fig. 3 Colocalization of ARGs with transposons and integrons. **A** ARGs associated with integrons. **B** ARGs associated with transposons

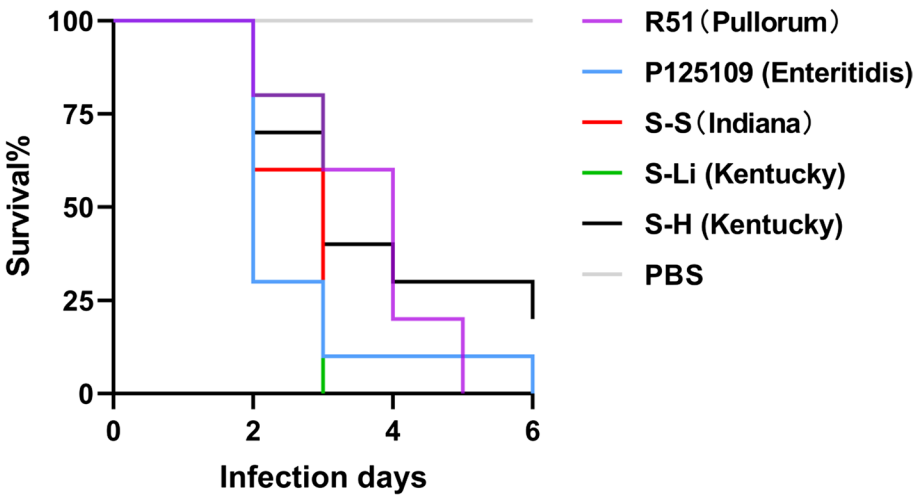

Fig. 4 The virulence of the recovered *Salmonella* strains was examined *via* survival curves. Lethality of isolates, in this case, to chick SPF embryos. Strain S-S represents *S.* indiana, whereas strains S-Li and S-H represent *S.* kentucky. *Salmonella* Enteritidis P125109 and *Salmonella* Pullorum R51 were used as positive controls, $n = 10$

100 mL TTB before use) for selective enrichment under the same conditions. Finally, each sample was isolated on xylose lysine deoxycholate (XLD) medium after 24 h at 37°C. All three types of bacterial media were procured from Beijing Luqiao Technology Company. The isolates were identified through the matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) technique (Bruker, Germany) as previously described.

Antimicrobial susceptibility test

Bacterial isolates were subjected to antimicrobial susceptibility assessment via the broth microdilution method, as mentioned previously (An et al. 2025). A total of 13 antibiotics from 10 categories were selected for the testing process, including ampicillin (AMP), amoxicillin and clavulanate potassium (AMC), gentamicin (GEN), kanamycin (KAN), tetracycline (TET), ciprofloxacin (CIP), nalidixic (NAL), chloramphenicol (CHL), ceftriaxone sodium (CRO), cefoxitin sodium (CX), trimethoprim and sulfamethoxazole (SXT), azithromycin (AZI), and imipenem (IMP). The concentration range refers to previous papers published by our laboratory (Jiang et al. 2019). The results were interpreted on the basis of the Clinical and Laboratory Standards Institute standards guidelines (CLSI, 2022). *Salmonella* isolates resistant to more than three classes of antimicrobials were defined as multidrug-resistant isolates.

DNA extraction and whole-genome sequencing

The genomic DNA of three purified *Salmonella* isolates was extracted *via* the FastPure Bacterial DNA Isolation Kit (Vazyme, China) according to the manufacturer's instructions and assayed for concentration and purity by a Nanodrop 1000 instrument (Thermo, USA). Genomic DNA of acceptable quality was sequenced by Novogene (Beijing, China) on an Illumina NovaSeq 6000 platform.

Bioinformatic analysis

The raw data were subjected to *a* quality check with the FastQC toolkit V. 0.12.1 (Leggett et al. 2013), while the adaptor was removed *via* Trimmomatic (Bolger et al. 2014). The genome was subsequently assembled via SPAdes V. 3.12.0 with default parameters (Antipov et al. 2016), the serovar of the isolates was *subsequently* predicted via SISTR V. 1.1.1 (Yoshida et al. 2016), and the STs were analyzed *via* MLST V. 2.22.0. The detection of ARGs, virulence factors, and plasmids was performed *via* ABRicate V. 1.0.1 against ResFinder v4.0, VFDB, and PlasmidFinder V. 2.1.1, respectively, while the scan of point mutations was conducted *via* Staramr software against PointFinder V.1.9 (Bharat et al. 2022).

Chicken embryo lethality assay

Specific pathogen-free (SPF) embryos were obtained from Zhejiang Lihua Agricultural Technology Co., Ltd. The eggs were incubated in an autorotating egg incubator for 11 d at 37.8 °C and 60–65% relative humidity. Individual colonies of *Salmonella* were selected in Luria–Bertani medium and incubated overnight at 37°C on a shaker at 220 rpm. The bacterial mixture was subsequently transferred to fresh Luria–Bertani medium and incubated for 2–2.5 h at a dilution ratio of 1:33, ensuring that the optical density (OD) was registered at OD_{600} = 0.1. The *Salmonella* suspension was then further diluted, adjusting the concentration of the inoculum to 5×10^3 colony-forming units per milliliter. Finally, a precise volume of 200 μL from the bacterial mixture was delicately introduced into 16-day-old SPF embryos. The eggs were allowed to warm daily to monitor mortality for up to 6 d.

Abbreviations

ARGs	Antimicrobial resistance genes
AMR	Antimicrobial resistance
ASPs	Antimicrobial stewardship programs
MGEs	Mobile genetic elements
SPF	Specific pathogen-free
BPW	Buffered peptone water
TTB	Tetrathionate broth base
XLD	Xylose lysine deoxycholate
OD	Optical density
AMP	Ampicillin
AMC	Amoxicillin and clavulanate potassium
Gen	Gentamicin
KAN	Kanamycin
TET	Tetracycline
CIP	Ciprofloxacin
NAL	Nalidixic
CHL	Chloramphenicol
CRO	Ceftriaxone sodium
CX	Cefoxitin sodium
SXT	Trimethoprim and sulfamethoxazole
AZI	Azithromycin
IMP	Imipenem
CLSI	Clinical and Laboratory Standards Institute standard guidelines
ESBLs	Extended-spectrum β-lactamases
MALDI-TOF MS	Matrix-assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-025-00166-x.

Supplementary Material 1.

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Not applicable.

Authors' contributions

Q.C., C.J. and H.Z. contributed equally to this work. Conceptualization, M.Y.; methodology, Q.C., C.J. and X.W.; software, Q.C., H.Z., C.J. and C.H.; validation, Q.C., X.K. and Y.H.; formal analysis, Q.C. and C.J.; data curation, H. Z and C. J; writing-original draft preparation, Q.C.; writing-review and editing, Q.C., H.A., H.Z., C.J., Y.L., F.H., and M. Y.; visualization, Q.C., H.A. and H.Z.; supervision, M.Y.; project administration, M.Y.; funding acquisition, M.Y. All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets presented in this research can be found in GenBank under BioProject no. PRJNA930160.

Declarations

Ethics approval and consent to participate
The protocols of the animal studies were approved by the Committee of the Laboratory Animal Center of Zhejiang University (ZJU20220295).

Consent for publication
All the authors have consented to the publication of the manuscript.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Author Min Yue and Fang He were not involved in the journal's review or decisions related to this manuscript.

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