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Prevalence, antimicrobial resistance and phylogenetic analysis of *Salmonella* contamination and transmission in yellow-feathered broiler hatcheries in China

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ABSTRACT

Salmonella is a significant avian pathogen causing infectious diseases in poultry, with hatching playing a crucial role in its transmission. Despite its importance, systematic research on *Salmonella* transmission in hatcheries remains limited. This study evaluates the prevalence and antimicrobial resistance of *Salmonella* throughout all production stages in yellow-feathered broiler hatcheries: laying, egg storage, incubating, hatching, and post-hatch. We found an overall *Salmonella* prevalence of 11.3 %, with the pathogen detected in both chickens and environmental samples. The hatching stage was identified as the most critical for *Salmonella* spread. Moreover, *Salmonella* Pullorum is the predominant serotype (93.97 %). Notably, all *Salmonella* isolates exhibited multidrug resistance, with some resistant to polymyxin B (22.41 %) and tigecycline (12.93 %). Resistance rates were highest for nalidixic acid (100.00 %), sulfamethoxazole (100.00 %), ciprofloxacin (95.69 %), and ampicillin (94.83 %). Additionally, antimicrobial resistance plasmid replicons and virulence genes were identified in these isolates. Whole genome sequencing was performed on 43 *S.* Pullorum isolates, revealing that the majority were ST92 (90.70 %). Phylogenetic analysis classified the isolates into three lineages, with Lineage III being the most predominant (83.72 %). It was found that *Salmonella* isolates from chicks and eggs across various production stages were closely related, and those from the environment also showed significant similarity. This suggests that *Salmonella* in the environment may originate from chicks/eggs and spread to other stages. More attention should be paid to *Salmonella* contamination in yellow-feathered broiler hatcheries, and stringent measures should be taken to control the horizontal spread of *Salmonella*, in addition to blocking the pathway of vertical transmission.

1. Introduction

Salmonella is a significant avian pathogen that can cause a reduction in production performance and death in poultry, resulting in substantial economic losses to the global poultry industry (Caffrey et al., 2021; Wang et al., 2020a). In China, the annual production (head units) of live yellow-feathered broilers is approximately 4.0 billion, comparable to that of white-feathered broilers (Bai et al., 2021). Compared with white-feathered broilers, yellow-feathered broilers exhibit a longer growth cycle and a greater variety of strains and farming methods. Furthermore, each strain varies in body size, growth rate, and disease resistance (Qi et al., 2017), making *Salmonella* prevention and control more challenging. However, there is a notable lack of systematic research on *Salmonella* in yellow-feathered broilers, highlighting the urgent need to enhance such research for improved monitoring within this sector.

Hatcheries, as the upstream stage of the broiler industry chain, serve as a crucial intervention point for controlling *Salmonella* in yellow-feathered broiler production. Vertical transmission is a key route for the spread of *Salmonella*, such as *Salmonella* Pullorum and *Salmonella*

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Enteritidis, in poultry and can lead to their introduction into poultry flocks from infected hatcheries (Shang et al., 2021; Volkova et al., 2011). However, researchers often focus on breeding farms, with a scarcity of systematic studies on hatcheries. Moreover, previous research on hatcheries has predominantly targeted specific aspects, such as chicks or eggs. Actually, hatchery production encompasses various stages, the use of production equipment, and the worker flow, all of which may facilitate *Salmonella* spread. Therefore, comprehensive monitoring of the source and transmission pathways of *Salmonella* contamination in the hatchery is essential.

In China, antibiotic use remains the primary method for preventing and treating Salmonellosis in yellow-feathered broilers. However, the abuse of antimicrobials in the broiler industry has resulted in the emergence of antimicrobial resistance (AMR) bacteria, significantly diminishing the effectiveness of some drugs used in clinical treatments (Talukder et al., 2021). Furthermore, it has been reported that AMR *Salmonella* strains found in human cases are closely linked to the extensive use of antimicrobial agents in livestock and poultry farming (Belachew et al., 2021). Previous studies have described the spread of AMR *Salmonella* in the broiler farm, slaughterhouse, and its downstream retail markets (Samia et al., 2021; Shang et al., 2021; Wang et al., 2020a). However, research on AMR *Salmonella* isolated from yellow-feathered broiler hatcheries is limited. As the upstream stage of the broiler industry chain, hatcheries may serve as a key entry point for studying the spread of AMR *Salmonella* throughout the chain. Therefore, investigating the prevalence and AMR of *Salmonella* in yellow-feathered broiler hatcheries is crucial for identifying specific distribution patterns and developing effective strategies to control and prevent *Salmonella* infections in both humans and animals.

In this study, we conducted longitudinal sampling across all production stages of the yellow-feathered broiler hatchery to identify the main entry points and transmission routes of *Salmonella*. We further assessed the AMR characteristics of the isolates. Additionally, we employed whole genome sequencing (WGS) technology to investigate the relationship between strains at different production stages. Our aim was to reveal the prevalence, AMR, and phylogenetic relationship of *Salmonella* in the yellow-feathered broiler hatchery, providing a reliable reference for precise *Salmonella* control within broiler industry chains and for the purification of yellow-feathered broiler provenance.

2. Materials and methods

2.1. Sample collection

From July 2020 to July 2021, a total of 1023 samples were collected from five production stages in the large-scale commercial yellow-feathered broiler hatchery (accommodating >50,000 yellow-feathered broiler embryos) in Guangdong Province, China. The stages include the laying stage, egg storage stage, incubating stage (the stage of incubation of eggs in the incubator from day 1 to day 17), hatching stage (the stage of hatching of eggs in the hatchery from day 17 to day 21), and post-hatch stage (the stage of eliminating weak chicks, vaccinating, and packing). The main sources of samples were dead embryos, sick chicks, environment, meconium, workers' hands/shoes, etc. (Fig. 1). The collected samples were kept in the foam box with ice packs, and it was ensured that the samples arrived at the laboratory within 2 h.

2.2. Salmonella isolation and identification

Upon arrival at the laboratory, the swabs were transferred to 10 mL of BPW (Buffered Peptone Water) and incubated at 37 °C for 8–12 h for pre-culture of bacteria. And then 1 mL of BPW was transferred to 9 mL of

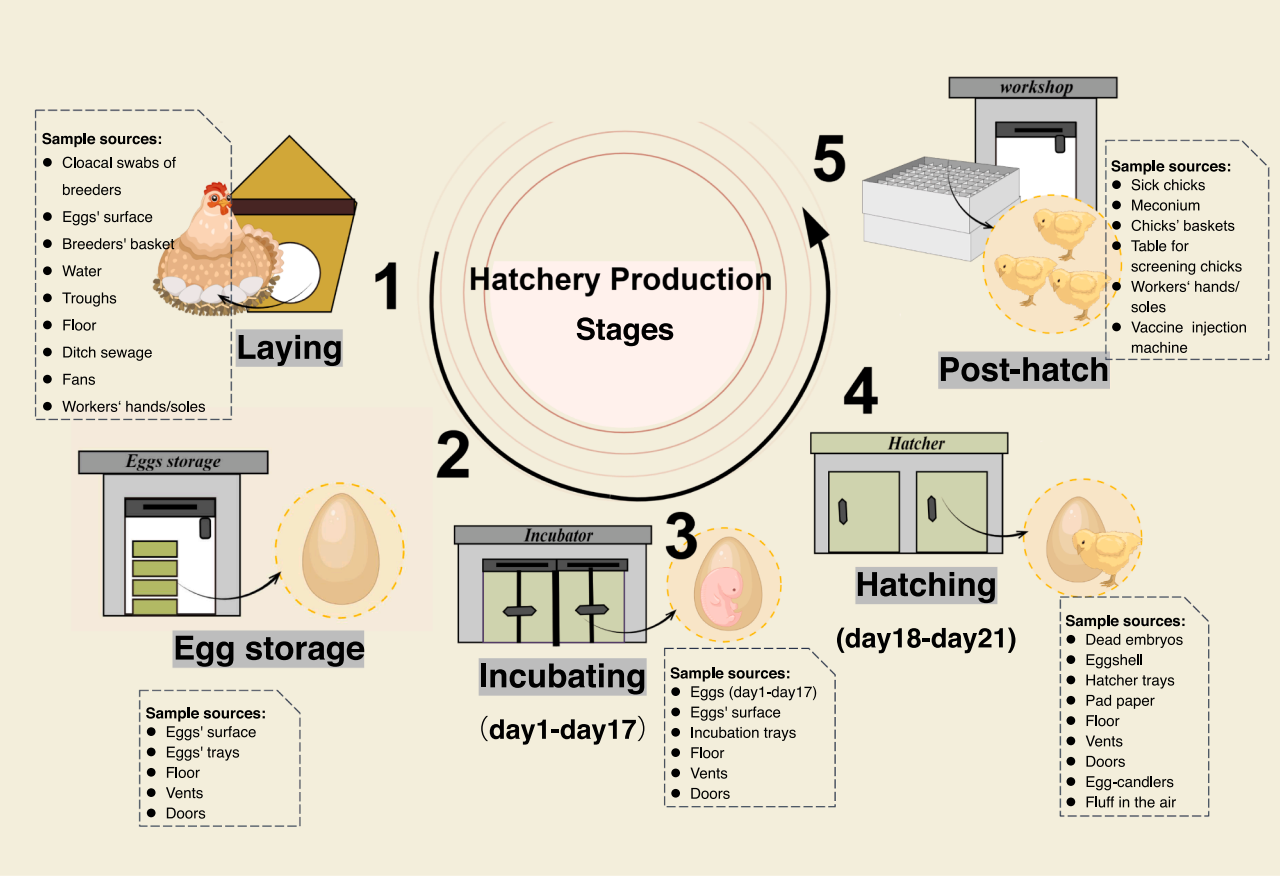

Fig. 1. The main production stages of the large-scale hatchery and sample sources in our study. The figure is by Figdraw. Figure ID: OWSIO2b456.

SC (Selenite Cystine Broth) and incubated at 37 °C for 14–16 h for selective culture of bacteria. At last, the bacterial fluid was inoculated in XLT-4 (Xylose Lysine Tergitol-4 Agar) and incubated at 37 °C for 24 h. During the process, we operated in strict accordance with aseptic requirements, strictly sterilized, and changed the tools for each sample. Genomic DNA was isolated with a Bacterial Genomic DNA kit (Omega, USA) according to the manufacturer's instructions. The obtained supernatant (template DNA) was stored at -20 °C until use. After DNA was extracted by the above method, we detected it by PCR through the *Salmonella*-specific gene *invA* (Lu et al., 2011). The sample separation method was optimized mainly based on the Standard ISO-6579 (International Organization for Standardization, 2002) method (Chen et al., 2020; Ren et al., 2016).

All *Salmonella* isolates were serotyped by slide agglutination with O and H antigen-specific sera according to the Kauffmann-White scheme or by National Food Safety Standard food microbiological examination (Chen et al., 2020).

2.3. Antimicrobial susceptibility test

Minimum inhibitory concentrations (MICs) were determined by the agar dilution method using Mueller-Hinton agar according to the standards of the Clinical and Laboratory Standards Institute (Wang et al., 2020b). A total of 13 antimicrobial agents were tested: ampicillin (AMP), cefotaxime (CTX), imipenem (IPM), streptomycin (STR), gentamicin (GEN), nalidixic acid (NAL), ciprofloxacin (CIP), florfenicol (FFC), chloramphenicol (CHL), sulfamethoxazole (SMZ), polymyxin B (PB), tetracycline (TET), and tigecycline (TGC). *Escherichia coli* ATCC 25922 and ATCC 35218 were used as quality control organisms for this MIC determinations. And the breakpoints for antimicrobials followed interpretive standards provided by CLSI (2022). In addition, an isolate was defined as'multidrug-resistant (MDR)' if it displayed resistance to ≥ 3 different classes of antimicrobials (Tenover, 2006).

2.4. Whole genome sequencing

Representative *S.* Pullorum strains from different times, stages, and sources were selected and underwent WGS and bioinformatics analyses. The selection method is as follows: Firstly, the sampling sources were retained, which exhibited a limited number of isolates (meconium and workers' hands/soles). Secondly, for those sampling sources with a higher number of isolates (dead embryos, sick chicks, and environment), we eliminated the highly similar clonal strains by comparing the sampling time, production stage and the resistance profiles of the subdivided sampling source strains, and highly similar clonal strains were eliminated to ensure the scientific validity of the WGS strains. The strains' raw sequencing data were assembled and evaluated using Trimmomatic v0.36, SPAdes v3.12.0, and QUAST tool 5.0.2. (Bolger et al., 2014, Bankevich et al., 2012, Gurevich et al., 2013). Plasmid typing, antibiotic resistance genes, and virulence genes were screened using RGI (Resistance Gene Identifier) (Alcock et al., 2020), Abricate 1.0.1, and Plasmidfinder databases (Carattoli et al., 2014). Furthermore, MLST v2.11 was used for sequence typing (ST) (Larsen et al., 2012). Based on the core SNP loci of the strains, Gubbins (Croucher et al., 2015) and FastTree (Price et al., 2009) were used to generate the maximum-likelihood phylogenetic tree. Finally, the phylogenetic tree (Price et al., 2009) was visualized and embellished using the iTOL (Letunic & Bork, 2021) online tool. The reference strain LHTF01.1 was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/).

2.5. Statistical analysis

SPSS 26.0 statistical software (v.16.0, SPSS, Chicago, IL, USA) Fisher's exact test was used to analyze the significant differences in *Salmonella* sample isolation. $p < 0.05$ indicated a significant difference.

3. Results

3.1. Prevalence of Salmonella

The overall prevalence of *Salmonella* in the yellow-feathered broiler hatchery was 11.3 % (116/1023), and different prevalences of *Salmonella* among the various production stages could be seen. Specifically, the prevalence during the laying, egg storage, incubating, hatching, and post-hatch stages was 2.1 % (6/288), 0.0 % (0/38), 2.0 % (1/49), 17.2 % (62/361), and 16.4 % (47/287), respectively (Fig. 2). We can note that, during the hatching stage, the prevalence of *Salmonella* increased significantly to 17.2 %, in contrast to the first three production stages. And the prevalence of *Salmonella* remained high in the post-hatch stage (16.4 %). Meanwhile, the *Salmonella* prevalence of chickens/eggs in the laying, egg storage, and incubating stages was 0.0 %, whereas those in the hatching and post-hatch stages were 23.0 % and 19.3 %, respectively (Fig. 2).

In order to identify additional *Salmonella* transmission paths, we also collected environmental source samples. During the laying stage, the most significant source of *Salmonella* contamination was the troughs (33.3 %), followed by the chicken feed (21.4 %) and ditch sewage (13.3 %). During the incubating stage, *Salmonella* was only isolated from the incubation trays (20.0 %). During the hatching stage, the fluff in the air (33.3 %) was the most contaminated with *Salmonella*, followed by the floor (20.0 %) and pad paper (16.7 %). During the post-hatch stage, pad paper (28.1 %) had the highest incidence of contamination, followed by chicks' baskets (11.1 %), tables for screening chicks (11.1 %), workers' soles (11.1 %), and workers' hands (10.0 %) (Fig. 2).

3.2. Serotypes analysis of Salmonella

Six different serotypes were identified among isolates in our study: *S.* Pullorum (109/116, 93.97 %) was the predominant serotype, while a small number of other serotypes were present: *S.* Enteritidis (2/116, 1.72 %), *S.* Typhimurium (2/116, 1.72 %), *S.* Tennessee (2/116, 1.72 %), and *S.* Braenderup (1/116, 0.86 %). *S.* Pullorum exists in various production stages and various source samples, while *S.* Enteritidis, *S.* Tennessee, and *S.* Braenderup were only found in environmental source samples during the laying and post-hatch stages. It is worth noting that the *Salmonella* isolated from sick chicks and dead embryos were all *S.* Pullorum (Table 1).

3.3. Antimicrobial resistance analysis of Salmonella

The AMR of 116 *Salmonella* isolates is as follows (Table 2): The highest resistance was observed against NAL (100.00 %), SMZ (100.00 %), CIP (95.69 %), and AMP (94.83 %), followed by FFC (34.48 %), PB (22.41 %), TET (22.41 %), TGC (12.93 %), STR (11.21 %), CTX (1.72 %), and CHL (1.72 %). IPM (0.0 %) and GEN (0.0 %) had 100 % susceptibility to the *Salmonella* isolates in this study. Furthermore, we also compared the resistance rates of different source isolates. *Salmonella* from different sources exhibit different resistance characterizations. Compared with other sources of samples, the isolates from the environment were resistant to more drugs (11/13, 84.61 %), such as CTX and CHL. And more resistant strains of PB and TET in the cloacal swabs of sick chicks and meconium were identified (Fig. 3).

The MDR rate of *Salmonella* isolates in this study was 100 % (Table 3). Isolates multi-resistant to AMP, NAL, CIP, and SMZ took up 35.34 %, this is the predominant resistance pattern. A total of five isolates showed resistance to six classes of antibiotics. Interestingly, four of these five *Salmonella* isolates showed resistance to PB, and two isolates showed resistance to TGC. However, TGC and PB are both types of line of defense drugs used in human clinical settings.

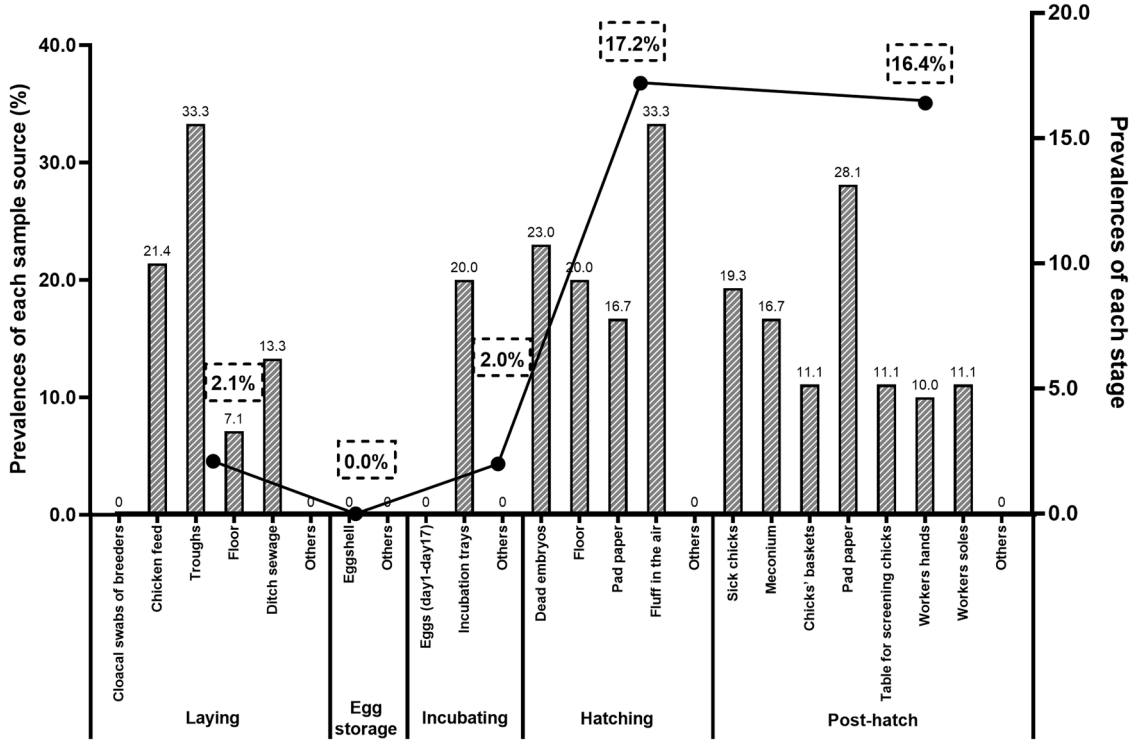

Fig. 2. The prevalences of *Salmonella* at different production stages and different sample sources. The difference in the prevalences of *Salmonella* in each production stage was statistically significant. ($p < 0.001$). The difference in the positive rates of *Salmonella* in each source was statistically significant. (Laying: $p < 0.001$. Hatching: $p = 0.019$. Post-hatch: $p < 0.001$.).

Table 1
Serotype distribution of *Salmonella* isolates (n= 116).

Serovar (Serogroup)	Laying Sample source (n)	Egg storage Sample source (n)	Incubating Sample source (n)	Hatching Sample source (n)	Post-hatch Sample source (n)	Total, n[a] (%)
S. Pullorum	chicken feed (2)	–	Incubation trays (1)	Floor (1) Dead embryos (56) pad paper (2) Fluff in the air (3)	Sick chicks (26) Chick baskets (3) meconium (4) Pad paper (8) Workers' hands (2) Workers' soles (1)	109 (93.97)
S. Typhimurium	–	–	–	–	Meconium (1) Chick baskets (1)	2 (17.21)
S. Enteritidis	ditch sewage (2)	–	–	–	–	2 (17.21)
S. Tennessee	Troughs (1) floor (1)	–	–	–	–	2 (17.21)
S. Braenderup	–	–	–	–	Table for screening chicks (1)	1 (0.86)
Total						**116**

[a] n number of isolates.

3.4. Whole genome sequencing and bioinformatics analyses

Phylogenetic tree analysis was performed based on SNPs of *S.* Pullorum isolated at various sampling dates, production stages, and sampling sources (Fig. 4). The *S.* Pullorum isolates were distributed in 3 different lineages, named Lineage I, Lineage II, and Lineage III here. There were 4 (9.30 %) and 3 (6.98 %) isolates in Lineage I and Lineage II, respectively. The dominant cluster in this study was Lineage III, which had a total of 36 (83.72 %) isolates. Among them, Lineage I was ST2151, Lineage II and Lineage III were ST92, and isolate FHC-103 was an unknown ST type. It is noteworthy that the dominant Lineage III was isolated from five time periods, three production stages, and five sampling sources (Fig. 4).

We further compared the SNPs of *S.* Pullorum isolated from different sources of samples. Significant cross-contamination was found to exist at the hatching and post-hatch stages, with isolated samples originating from dead embryos, sick chicks, meconium, workers' hands/soles, and other environmental source samples (Fig. 4, branches marked in red). Specifically, many isolates from animal sources such as sick chicks, meconium, and dead embryos of different stages were closely related (SNP ≤5). At the same time, the isolates from environments such as chicken feed and workers' hands/soles were closely related.

All the isolates were identified with various AMR genes, which were consistent with the resistance phenotype. Six plasmid replicons were detected among the isolates in this study, including ColRNAI, ColpVC, Col440I, IncFII(S), IncN, and IncX1. And all isolates were carried with the Col and IncFII(S), whereas 41 (95.3 %) isolates were carried with the IncX1 and 2 (4.6 %) were carried with the IncN.

4. Discussion

Salmonella contamination has historically posed a significant

Table 2
Minimum Inhibitory Concentration (μg/mL) Distribution of 116 *Salmonella* isolates (n= 116). AMP (ampicillin), CTX (cefotaxime), IPM (imipenem), STR (streptomycin), GEN (gentamicin), NAL (nalidixic acid), CIP (ciprofloxacin), FFC (florfenicol), CHL (chloramphenicol), SMZ (sulfamethoxazole), PB (polymyxin B), TET (tetracycline), and TGC (tigecycline).

Antibiotic	Minimum inhibitory concentration (μg/mL) distribution of 116 *Salmonella* isolates													Resistant Breakpoint	Number of resistant strains	Resistance % (n= 116)
	<0.125	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512			
AMP							1	2	3						110	≥32	110	94.83
CTX	113			1							1			1		≥4	2	1.72
IPM				113	3											≥4	0	0.00
STR							37	3	49	13	13	1				≥64	14	12.07
GEN	0	92	1	17	4	1	1									≥16	0	0.00
NAL															116	≥32	116	100.00
CIP	5				92	19										≥1	111	95.69
FFC						4	71		40				1			≥16	41	35.34
CHL							114					1	1			≥32	2	1.72
SMZ															116	≥512	116	100.00
PB				6		84	7	19								≥4	26	22.41
TET						90						8	18			≥16	26	22.41
TGC	5			88	8	1	13	1								≥2	15	12.93

challenge in the Chinese broiler industry, particularly in the production of yellow-feathered broilers. The hatchery, as a vital component of the broiler production chain, plays a crucial role in preventing *Salmonella* contamination in this sector. In this study, we found the total prevalence of *Salmonella* in hatcheries was 11.3 %, which is higher than the prevalence reported in previous studies on broiler farms (Zhao et al., 2020) and breeder farms (Barua et al., 2013). In addition, the *Salmonella* prevalences in dead embryos and sick chicks were recorded at 23.0 % and 19.3 %, respectively, surpassing figures reported for hatcheries raising white-feathered broilers (Ha et al., 2018; Oloso et al., 2019; Shang et al., 2021). These results indicate a severe level of *Salmonella* contamination in yellow-feathered broiler hatcheries, highlighting the urgent need for more in-depth and comprehensive research on the epidemiology of *Salmonella* in this context.

In the present study, we found that *Salmonella* contamination occurred at multiple stages of the hatchery. Firstly, during the laying stage, there were high isolation rates of *Salmonella* in chicken feed and troughs, indicating that these may have been the initial source of contamination. *Salmonella* infects hens first, subsequently spreading vertically to chicks or eggs. The risk of further spread to downstream industries cannot be ignored. At the same time, *Salmonella* has been detected in various environmental source samples, including workers' hands and soles, at different stages of production. To prevent the spread of *Salmonella*, it is crucial to strengthen daily management practices related to these sources.

Previous studies have shown a higher prevalence of *Salmonella* during the laying stage compared to the hatching stage (Fei et al., 2017). However, our findings indicate that *Salmonella* prevalence was relatively low during the laying, egg storage, and incubation stages, while a significantly higher level was observed during the hatching stage. Notably, despite the elimination of sick chicks after hatching, *Salmonella* was still detected in the healthy chicks at the post-hatch stage (Fig. 2, Meconium). Additionally, while fumigation was proceeding daily, *Salmonella* was still detected in the environment (such as hatching trays, fluff in the air, and the floor) of the hatching stage. These results suggest that *Salmonella* may spread to the environment upon chicks hatching and then horizontal transmission to other chicks via fluff in the air or other obscure environmental media.

Further investigation into the serotypes of the isolates revealed that *S.* Pullorum was present at all stages of hatchery production and was the sole serotype associated with the mortality of chicken embryos and the illness of chicks. This finding indicates that *S.* Pullorum is the predominant *Salmonella* serotype in yellow-feathered broiler hatcheries. Similar conclusions have been reported in previous studies (Wang et al., 2020a; Xu et al., 2020). Conversely, *S.* Enteritidis was predominant in the white-feathered broiler hatcheries (Shang et al., 2021; Zamil et al., 2021). To effectively prevent *Salmonella* in the yellow-feathered broiler hatcheries, greater attention should be given to the predominant serotype, *S.* Pullorum, and its prevalence in this sector deserves our more in-depth study.

AMR in *Salmonella* of poultry origin has emerged largely due to the widespread use of antimicrobials (McDermott et al., 2018). In this study, all isolates were MDR strains, exhibiting high resistance rates to AMP, NAL, CIP, and SMZ. Notably, the AMR rates for these four drugs were higher than those reported between 1962 and 2019 (Sun et al., 2021). In most hatcheries across China, day-old chickens receive a single dose of ampicillin to mitigate the risk of Salmonellosis before transfer to farms. Additionally, the frequent use of antimicrobials in upstream egg-laying farms exacerbates AMR acquisition in *Salmonella* during the hatching stage. The study investigated the AMR of isolates from different sources. The strains isolated from the environment showed resistance to most of the antimicrobials tested, which could be attributed to the adaptive evolution of *Salmonella* under environmental pressure (Müller et al., 2022). Furthermore, the presence of isolates with identical MDR profiles across various production stages and sources suggests potential horizontal spread during the production process of yellow-feathered broiler

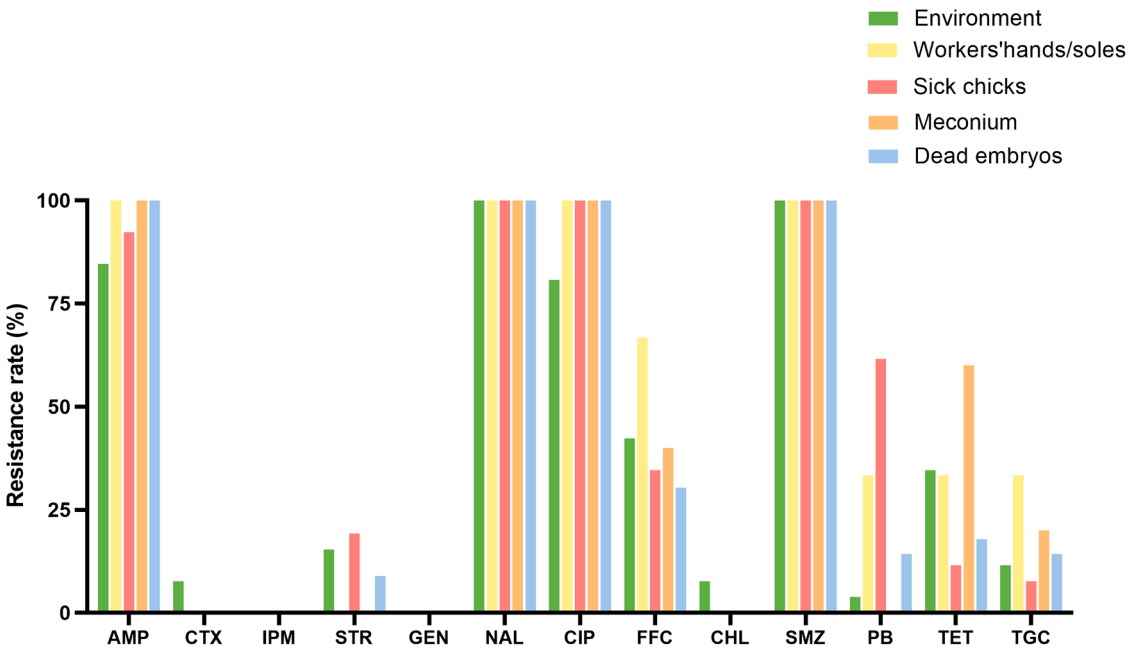

Fig. 3. Resistance rate among *Salmonella* from different sample sources. AMP (ampicillin), CTX (cefotaxime), IPM (imipenem), STR (streptomycin), GEN (gentamicin), NAL (nalidixic acid), CIP (ciprofloxacin), FFC (florfenicol), CHL (chloramphenicol), SMZ (sulfamethoxazole), PB (polymyxin B), TET (tetracycline), and TGC (tigecycline).

Table 3
Resistance profiles of *Salmonella* at different production stages. (*n*= 116).

Serial number	Number of drug-resistant types	Resistance mode	Production stages	Number of isolates (*n* = 116)	Percentage (%)
1	3	AMP + NAL + CIP + SMZ	Hatching, Post-hatch	41	35.34
2	4	AMP + NAL + CIP + FFC + SMZ	Laying, Hatching, Post-hatch	19	16.38
3	4	AMP + NAL + CIP + SMZ + PB	Hatching, Post-hatch	8	6.90
4	4	AMP + NAL + CIP + SMZ + TET + TGC	Hatching, Post-hatch	6	5.17
5	5	AMP + NAL + CIP + FFC + SMZ + PB	Hatching, Post-hatch	5	4.31
6	4	AMP + NAL + CIP + SMZ + TET	Hatching, Post-hatch	5	4.31
7	5	AMP + STR + NAL + CIP + SMZ + PB	Hatching, Post-hatch	5	4.31
8	5	AMP + NAL + CIP + FFC + SMZ + TET + TGC	Hatching, Post-hatch	4	3.45
9	4	AMP + STR + NAL + CIP + SMZ	Hatching	3	2.59
10	5	AMP + NAL + CIP + FFC + SMZ + TET	Hatching, Post-hatch	3	2.59
11	6	AMP + STR + NAL + CIP + FFC + SMZ + PB	Hatching, Post-hatch	2	1.72
12	6	AMP + NAL + CIP + FFC + SMZ + PB + TET + TGC	Hatching, Post-hatch	2	1.72
13	3	NAL + STR + SMZ	Laying, Post-hatch	2	1.72
14	5	AMP + CTX + NAL + CIP + FFC + CHL + SMZ + TET	Post-hatch	1	0.86
15	5	AMP + NAL + CIP + SMZ + PB + TET	Hatching	1	0.86
16	5	AMP + NAL + CIP + SMZ + PB + TET + TGC	Hatching	1	0.86
17	5	AMP + NAL + CIP + SMZ + PB + TGC	Hatching	1	0.86
18	4	AMP + NAL + FFC + SMZ	Laying	1	0.86
19	3	AMP + NAL + SMZ	Laying	1	0.86
20	6	AMP + STR + NAL + CIP + FFC + CHL + SMZ + TET	Post-hatch	1	0.86
21	5	NAL + CIP + FFC + SMZ + PB	Post-hatch	1	0.86
22	4	NAL + CIP + SMZ + TET	Post-hatch	1	0.86
23	4	STR + NAL + CIP + SMZ + TET	Incubating	1	0.86
24	5	CTX + STR + FFC + NAL + SMZ	Laying	1	0.86
MDR				**116**	**100.0**

hatcheries. Therefore, monitoring the transmission pathways of AMR *Salmonella* throughout production is essential to prevent further spread to downstream industries.

In consideration of public health, it is also urgent to monitor the resistance profile of *Salmonella* in the hatchery. In the present study, we identified certain isolates resistant to TGC and PB, which are considered "last defense" drugs (PB, TGC, and IPM) for human clinical treatment. It should be noted that these antimicrobials are banned in poultry and livestock production (Yang et al., 2022). However, other colistin and tetracyclines antimicrobials are still commonly used in clinical practice to prevent and treat bacterial infections in poultry and livestock production. The emergence of "last defense" resistant isolates may result from either gene horizontal transfers from other strains or synergistic resistance to similar antimicrobials. Therefore, enhancing surveillance

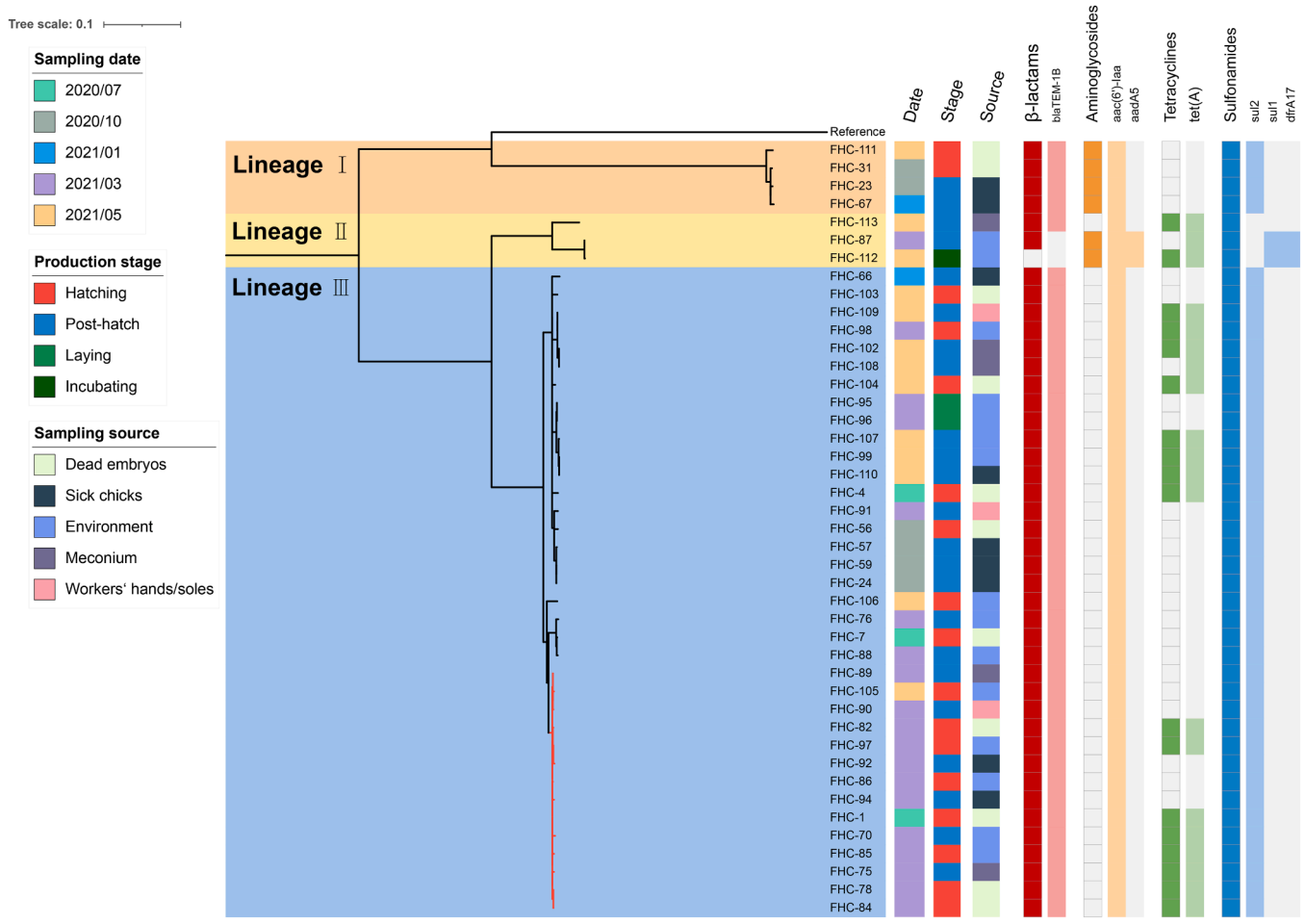

Fig. 4. Phylogenetic relationships of 43 *Salmonella* isolated from different sampling dates, production stages, and sampling sources based on cgSNP. (Branches marked in red mean that the SNP distance between them is ≤5).

of *Salmonella* resistance in yellow-feathered broiler hatcheries is essential, alongside raising public health awareness to mitigate potential threats from cross-contamination and antibiotic misuse.

To further explore the characteristics of *Salmonella* in the hatchery, we conducted WGS to analyze its evolutionary relationship during production. Our findings indicate that *Salmonella* isolates from chicks and eggs at different stages were closely related, and those from the environment also showed significant similarity. This demonstrates that cross-contamination occurs among chicks and eggs in the hatchery, as well as between these and environmental factors. The hatching stage appears critical for cross-contamination. Environmental isolates, such as fluff in the air, troughs, and pad paper during the laying, hatching, and post-hatch stages, exhibited close genetic relationships (SNP ≤10) and carried similar resistance genes and plasmid replicons. *Salmonella* in the environment may come from dead embryos and sick chicks, and spreads into the environment during the hatching stage and subsequently through the worker's hands/soles to the post-hatch stage. Therefore, implementing robust monitoring and control measures is essential to mitigate the horizontal transmission of *Salmonella*. In addition, the isolates carried some AMR plasmid replicons: *IncN* and *IncX1*. These isolates could horizontally transfer AMR plasmid replicons to other recipient bacteria through conjugation, making *Salmonella* with T4SS a potential AMR gene reservoir. At the same time, all serotypes contained virulence genes encoding for nonfimbrial adherence, survival in macrophages, enterotoxin, invasion, magnesium uptake, and secretion systems (Zuo et al., 2020). The presence of these genes heightens the risk of *Salmonella* spread and infection in the yellow-feathered broiler industry's downstream processes.

5. Conclusions

Our findings suggest that in addition to vertical transmission, horizontal transmission is also an important route of *Salmonella* transmission in hatcheries, as demonstrated by phenotype comparisons and WGS analyses. *Salmonella* transmission occurred through various media during daily production, leading to potential cross-contamination. Additionally, we systematically reveal the distribution of resistance genes and plasmid replicons in MDR *Salmonella* in yellow-feathered broiler hatcheries. These findings provide comprehensive insights to understand *Salmonella* in yellow-feathered broiler hatcheries.

Ethical statement

Samples were collected and processed in accordance with Chinese regulations on poultry inspection. Prior to the sampling of animal specimens, including chicken cloacal swabs, sick chicks and dead embryos, the consent of the hatchery proprietor had been obtained. The study was approved by the Animal Ethics and Morality Committee of the College of Veterinary Medicine, South China Agricultural University.

CRediT authorship contribution statement

Canji Wu: Writing – original draft, Software, Data curation, Conceptualization. **Yuhui Deng:** Software, Data curation. **Zeluan Chen:** Writing – original draft, Data curation. **Junhao Peng:** Investigation, Data curation. **Peizhi Wu:** Investigation, Data curation. **Jinger Chen:** Investigation, Data curation. **Pengju Chen:** Project administration.

Ming Liao: Supervision. **Chenggang Xu:** Project administration. **Jianmin Zhang:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no competing interests.

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