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ISOLATION AND CHARACTERIZATION OF *Salmonella Enterica* ASSOCIATED WITH DIARRHEA IN CHICKENS AND DUCKS IN HAI DUONG PROVINCE

Dong Van Quyen[1,2]✉, Pham Thi Lanh[1], Nguyen Kim Oanh[1], Tran Nam Khang[1,3]

[1]*Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.*

[2]*Gradutate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.*

[3]*University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.*

✉To whom correspondence should be addressed. E-mail: dvquyen@gmail.com

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ABSTRACT

Salmonella infections, or salmonellosis, represent a significant threat to poultry health and the global poultry industry, leading to considerable economic losses and serving as a major source of foodborne illnesses in humans and animals. Identifying the specific strains present in local poultry farms is crucial for implementing targeted interventions, including the development of effective biosecurity measures, vaccination strategies, and treatment protocols to mitigate outbreaks. This study focuses on isolating and characterizing *Salmonella* strains associated with diarrhea in chickens and ducks in Hai Duong province, Vietnam. The *Salmonella* strains were initially isolated using a culture-based method, followed by identification and characterization through Matrix-Assisted Laser Desorption Ionization/Time of Flight (MALDI-TOF), PCR amplification of the *invA* gene, and 16S rRNA gene sequencing. As a result, 18 *Salmonella* isolates were obtained, all of which contained the *inv*A gene, indicating its potential significance in *Salmonella* pathogenesis. Analysis of the 16S rRNA gene sequences confirmed that all isolates belonged to the species *Salmonella enterica*, a well-known causative agent of intestinal diseases in humans and animals. Furthermore, phylogenetic analysis revealed that all 18 isolates grouped with *Salmonella enterica* subsp. *enterica* strains from China and Korea, suggesting a close relationship with strains circulating in the broader Southeast Asian region. This regional similarity may be attributed to the movement of poultry and poultry products, facilitating the cross-border spread of *Salmonella*. Our findings underscore the importance of implementing robust biosafety measures throughout the poultry production chain to control the spread of *Salmonella*, thereby enhancing both animal and food safety.

Keywords: 16S rRNA, *inv*A gene, MALDI-TOF, *Salmonella*, Salmonellosis

INTRODUCTION

Salmonella, a causative agent of Salmonellosis, is one of the most common and dangerous pathogens in poultry, inflicting massive losses on the poultry industry worldwide (Thames & Theradiyil Sukumaran, 2020). The risk of *Salmonella* contamination extends beyond the farm, posing a significant threat to human health through improper handling and consumption of poultry products (Shaji *et al.*, 2023; Wibisono *et al.*, 2020). *Salmonella* has adapted to survive in a wide range of habitats and hosts, making it difficult to restrict its spread over time (Foley *et al.*, 2013). Although numerous prevention and control strategies, such as vaccination and medication use, have been implemented, contamination with *Salmonella* remains one of the most critical concerns globally (Wang *et al.*, 2020).

Salmonella is a bacterial genus in the family *Enterobacteriaceae*. They are gram-negative, facultatively anaerobic, non-spore forming, motile and intracellular bacilli with peritrichous flagella (Coburn *et al.*, 2007; Wessels *et al.*, 2021). Biochemically, *Salmonella* spp. are characterized by their ability to produce hydrogen sulfide and catalase, but lack of oxidase enzymes, and their inability to ferment lactose. They are heat labile and can be killed at 70°C, although they can survive in dust and harsh environmental conditions for more than 2 years (El-Saadony *et al.*, 2022b).

Up to date, there are two *Salmonella* species have been identified, including *Salmonella bongori* and *Salmonella enterica*. Particularly, *S. enterica* is divided into 6 subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica* (El-Saadony *et al.*, 2022b; Popa & Papa, 2021). Further, *Salmonella* isolates are usually identified by their serotype based on the determination of cell O antigen (an oligosaccharide component of lipopolysaccharide located in the outer membrane) and H flagellar antigen found in the bacterial flagellum (El-Saadony *et al.*, 2022b; Popa & Papa, 2021). Currently, about 2,500 different serovars of *Salmonella* have been identified (Popa & Papa, 2021).

Of two *Salmonella* species, *Salmonella enterica* is a causative agent of intestinal diseases with high morbidity and mortality rates in poultry, including serovars such as *S. typhi*, *S. dublin*, *S. pullorum*, and *S. gallinarum* (El-Saadony *et al.*, 2022b). Among them, *S. gallinarum* and *S. pullorum* (causing fowl typhoid) are the two most pathogenic serovars in poultry, causing systemic infections in all ages with pathological signs including an enlarged liver, anemia, and intestinal hemorrhage (Chappell *et al.*, 2009), leading to significantly economic losses (El-Saadony *et al.*, 2022b). Virulence assessment of *S. gallinarum* showed that they caused up to 60% mortality in 3-week-old hybrid chickens (Chappell *et al.*, 2009). In Nigeria (2009), an outbreak of *S. gallinarum* was recorded in commercial laying hens, affecting 11,000 birds with a mortality rate of up to 25% (Barrow & Neto, 2011). Fowl typhoid has been largely controlled in North America and Western Europe, although there is still evidence of its circulation in small-scale production and occasional outbreaks in commercial production. Meanwhile, *S. gallinarum* remains a major pathogen in many developing poultry industries, including Asia and South America (Chappell *et al.*,

2009). Additionally, *S. enteritidis* and *S. typhimurium* are the two main causative agents of paratyphoid in poultry, with common pathological signs including inflamed, hemorrhagic, and fluid-filled intestines, folded cecum, and necrotic liver (López-Martín *et al.*, 2016). However, in chickens, gastrointestinal infections, especially in the cecum, by serovars *S. enteritidis* and *S. typhimurium* can persist for several months, rarely causing systemic infections except in newly hatched chicks with few clinical manifestations (Chappell *et al.*, 2009).

Between 2017 and 2020, researchers in Vietnam collected 3,055 samples from broiler farms and households in the Mekong Delta, with 181 samples (5.92%) testing positive for *Salmonella*. The bacteria were identified in chicken feces (7.67%), pest animals (5.98%), and the environment (4.33%). Notably, most *Salmonella* isolates exhibited resistance to multiple antibiotics, including chloramphenicol (62.98%) and tetracycline (55.80%) (Nguyen *et al.*, 2021). Ta et al. (2012) reported high rates of *Salmonella* contamination in poultry samples from wet markets and supermarkets in northern Vietnam, with contamination rates of 51.1% in Hanoi, 45.6% in Hai Phong, 44.7% in Bac Ninh, and 43.8% in Phu Tho. A recent study also identified *Salmonella* in 426 out of 3,700 ducks from Thai Nguyen, Bac Giang, Bac Ninh, Lang Son, and Tuyen Quang provinces (Binh *et al.*, 2020). Despite extensive research in northern provinces, studies on *Salmonella* in Hai Duong are still limited, even though poultry is a major component of the region's agriculture. This study aims to isolate and characterize *S. enterica* from diarrheal poultry in Hai Duong, Vietnam.

MATERIALS AND METHODS

Sampling

Ten suspected *Salmonella*-positive samples (intestinal and fecal material) were obtained from ducks and chickens at five poultry farms in Hai Duong province, with two samples collected from each farm. The samples were kept cold and immediately delivered to the Laboratory of Microbiology at the Institute of Biotechnology, Vietnam Academy of Science and Technology.

Bacterial isolation

A cotton swab was used to collect feces from chicken, duck intestines and mixed with 10 mL of peptone water in a falcon tube. The mixture was then shaken at 150 rpm for 3 hours at 35ºC. The enrichment solution of each sample was diluted with NaCl solution (0.85%) to the appropriate concentration, and 100 µL of the dilution was spread on a Xylose-lysine-deoxycholate (XLD) agar plate (Tan *et al.*, 2022). The plate is incubated overnight at 37ºC. Obtained colonies were streaked several times on new XLD agar plates for purification and then kept at 4ºC for subsequent experiments and in 30% glycerol at -80ºC for long-term storage. The morphology of isolates was investigated by using the Gram staining method.

Bacterial identification using MALDI-TOF method

MALDI-TOF protein mass spectrometry method (Matrix assisted laser desorption ionization - time of flight) with the MALDI-TOF Biotyper (Bruker, Germany) was used to identify the isolated strains. The Biotyper Compass Explorer software (version

4.1.100) (Bruker, Germany) was used to automatically analyze the samples. The similarity level between the experimental strains and reference bacterial strains ranged from 0 to 3.0. Results above 2.0 indicate high similarity and can be determined at the genus and species levels (Tsuchida & Nakayama, 2022). Strains that are highly similar to reference strains will be further identified by molecular biology techniques, including PCR with specific primers for *Salmonella* spp., and subsequently by sequencing and analyzing the 16S rRNA gene of the bacterium (Kang *et al.*, 2017).

DNA extraction

The overnight bacterial inoculum was centrifuged at 12,000 rpm and 4ºC for 5 min. The supernatant was discarded, and the cell pellet was collected and used to extract the total DNA by utilizing GeneJET Genomic DNA Purification Kit Thermo (USA) following the manufacturer's instructions. The quality of the extracted DNA was assessed by electrophoresis on a 1% agarose gel, stained with Ethidium bromide, and observed under UV light. The concentration and purity of the DNA were determined using a Nanodrop spectrophotometer (Thermo Scientific NanoDrop™ 2000, USA).

***invA* gene amplification**

invA gene, a genus specific marker that is used for the detection of *Salmonella* spp. was PCR amplified by using a specific pair of primers with the predicted size of the amplicon of about 404 bp: Forward, 5' – GTATTGTTGATTAATGAGATCCG-3' and Reverse, 5'-ATATTACGCACGGAAACACGTT – 3' (Ranjbar et al., 2017). PCR amplification was performed in a total volume of 25 µL with 12.5 µL master mix (Thermo Scientific), 8.5 µL H_2O, 1 µL forward primer, 1 µL reverse primer, and 2 µL of the extracted DNA sample. The PCR thermal cycle was 94ºC for 3 min and 35 cycles of 94ºC for 30s followed by 50ºC for 30s and 72ºC for 1 min 30s, and a final cycle at 72º for 7 min. The reactions were carried out in a C1000 Thermal Cycler (Bio-Rad, USA). The PCR products were subjected to electrophoresis on a 1% agarose gel, stained with Ethidium bromide, and visualized under UV light.

16S rRNA amplification

One hundred ng of the extracted DNA was used as a template for amplifying the 16S rRNA genes by PCR using a specific pair of primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR amplification was performed as described above. The PCR thermal cycle was 94ºC for 5 min and 35 cycles of 94ºC for 1 min followed by 56ºC for 45s and 72ºC for 1 min, and a final cycle at 72º for 10 min. The reactions were carried out in a C1000 Thermal Cycler (Bio-Rad, USA). The PCR product was purified using the GeneJET PCR Purification Kit (Thermo Scientific, USA) and checked on a 1 % (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light. The DNA concentration and purity were determined using a Nanodrop spectrophotometer (Thermo Scientific NanoDrop™ 2000, USA). The purified PCR products were sequenced by the capillary sequencing system, the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

16S rRNA analysis

The sequencing data was processed and analyzed using BioEdit software (version 7.2.6.1). The obtained gene sequences were compared with the gene sequences of *Salmonella* published on the gene bank (Genbank) using the BLAST tool. MEGA X software was used to calculate genetic distances and construct a phylogenetic tree (Kumar *et al.*, 2018).

RESULTS

Bacterial isolation

A total of 48 isolates were obtained from 10 samples collected from the cecum of diseased chickens and ducks. These isolates exhibited the typical morphological characteristics of *Salmonella* colonies on XLD, a selective medium. The colonies were red (pink) in color, round, convex, smooth (non-wrinkled), and presented with or without black centers, as previously described by Sharma and Katock (Sharma M & Katock RC, 1996).

Bacterial identification by MALDI-TOF method

MALDI-TOF analysis identified 18 out of 48 colonies as *Salmonella*, with scores ranging between 1.97 and 2.40 (Table 1). All of these isolates were confirmed to be Gram-negative, rod-shaped, and motile bacteria (Figure 1). These identified *Salmonella* isolates were subsequently used for further assays as described previously (Kang *et al.*, 2017).

Table 1. Representative result of identification of isolated bacteria by MALDI-TOF.

Sample name	Organism (best match)	Score value	Matched pattern	Name
1	*Salmonella* sp	2.21	*Salmonella sp (enterica st Anatum) 11 LAL*	*Salmonella_HaiDuong1*
2	*Salmonella* sp	2.17	*Salmonella sp (choleraesuis) 08 LAL*	*Salmonella_HaiDuong2*
3	*Salmonella* sp	2.02	*Salmonella sp (enterica st Hadar) Sa05 506 VAB*	*Salmonella_HaiDuong3*
4	*Salmonella* sp	2.11	*Salmonella sp (enterica st Enterica) DSM 17058T HAM*	*Salmonella_HaiDuong4*
5	*Salmonella* sp	2.15	*Salmonella sp (enterica st Diarizonae) CIP 82 31T CIP*	*Salmonella_HaiDuong5*
6	*Salmonella* sp	2.48	*Salmonella sp (enterica st Hadar) Sa05_506 VAB*	*Salmonella_HaiDuong6*
7	*Salmonella* sp	2.40	*Salmonella sp (enteritidis) 25089078 (PX) MLD*	*Salmonella_HaiDuong7*
8	*Salmonella* sp	2.38	*Salmonella sp (choleraesuis) 08 LAL*	*Salmonella_HaiDuong8*
9	*Salmonella* sp	2.32	*Salmonella sp (choleraesuis) 08 LAL*	*Salmonella_HaiDuong9*
10	*Salmonella* sp	2.05	*Salmonella sp (enterica st Enterica) DSM 17058T HAM*	*Salmonella_HaiDuong10*

11	*Salmonella* sp	2.21	*Salmonella sp (enteritidis) 25089078 (PX) MLD*	*Salmonella_HaiDuong11*
12	*Salmonella* sp	2.35	*Salmonella sp (enterica st Anatum) 11 LAL*	*Salmonella_HaiDuong12*
13	*Salmonella* sp	2.13	*Salmonella sp (enteritidis) 25089078 (PX) MLD*	*Salmonella_HaiDuong13*
14	*Salmonella* sp	2.22	*Salmonella sp (choleraesuis) 08 LAL*	*Salmonella_HaiDuong14*
15	*Salmonella* sp	1.99	*Salmonella sp (enteritidis) 25089078 (PX) MLD*	*Salmonella_HaiDuong15*
16	*Salmonella* sp	2.11	*Salmonella sp (choleraesuis) 08 LAL*	*Salmonella_HaiDuong16*
17	*Salmonella* sp	2.23	*Salmonella sp (enteritidis) 25089078 (PX) MLD*	*Salmonella_HaiDuong17*
18	*Salmonella* sp	1.97	*Salmonella sp (enteritidis) 25089078 (PX) MLD*	*Salmonella_HaiDuong18*

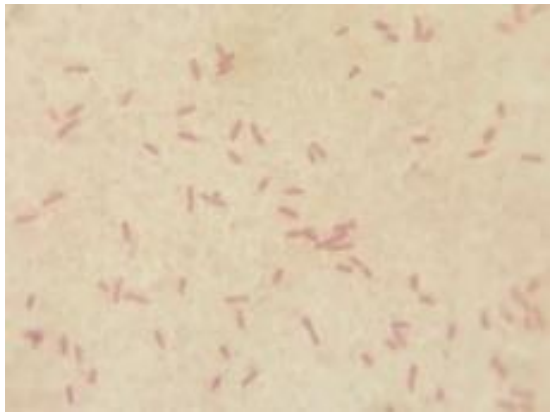

Figure 1. Gram staining of a *Salmonella* strain isolated from poultry farm

Amplification of *invA* and 16S rRNA genes

In this study, the concentration of the extracted DNA samples ranged from 723.5 to 1216.3 ng/µL, with A260/A280 ratios between 1.8 and 1.99, indicating that the samples meet the required standards for successful PCR. The purified DNA samples were then used as templates to amplify *invA* and 16S rRNA genes, which are highly conserved gene regions across *Salmonella* species and bacteria, respectively, using specific primers as described above. As a result, the *invA* genes were amplified from all MALDI-TOF-identified *Salmonella* isolates, producing a band of approximately 404 bp, as predicted (Figure 2A). In addition, the 16S rRNA genes from all the above isolates were successfully amplified, yielding DNA bands of about 1,500 bp on the agarose gel (Figure 2B**)**.

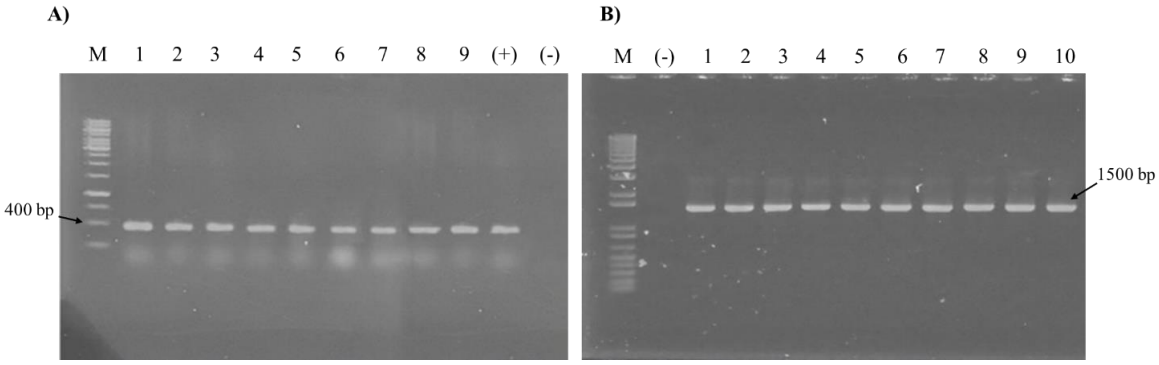

Figure 2. A) The *inv*A gene PCR product of representative *Salmonella* strains (1 - 9) isolated from poultry farms with (+): positive control, plasmid carries the *invA* gene, (-): negative control, M: Thermo Scientific™ GeneRuler™ 1kb DNA ladders; and B) The 16S rRNA PCR products from isolated bacterial strains (1 - 10) on 1% agarose gel with (-): negative control (B); M: Thermo Scientific™ GeneRuler™ 1kb plus DNA ladders.

16S rRNA sequencing and phylogenetic tree analyses

The sequence analysis revealed that all 18 *Salmonella* isolates in this study exhibited 99.99% to 100% similarity with each other and 99.0% to 100% sequence identity with the 16S rRNA sequences of *Salmonella enterica* subsp. *enterica* (GenBank accession numbers CP022963.1, EU073018.1, and MW282043.1). The 16S rRNA sequences of all 18 isolates were then aligned and used to construct a phylogenetic tree together with other *Salmonella* sequences available in the GenBank. *Yersinia pestis* strain NCTC 5923 (accession number NR025160.1) was used as an outgroup. The NJ tree analysis clearly clustered these 18 isolates in one group together with other *Salmonella enterica subsp. enterica* isolated from chickens in China (CP022963.1; EU073018.1; MW282043.1; OQ915456.1; MT968431.1) and Korea (NR116126.1) (Figure 3).

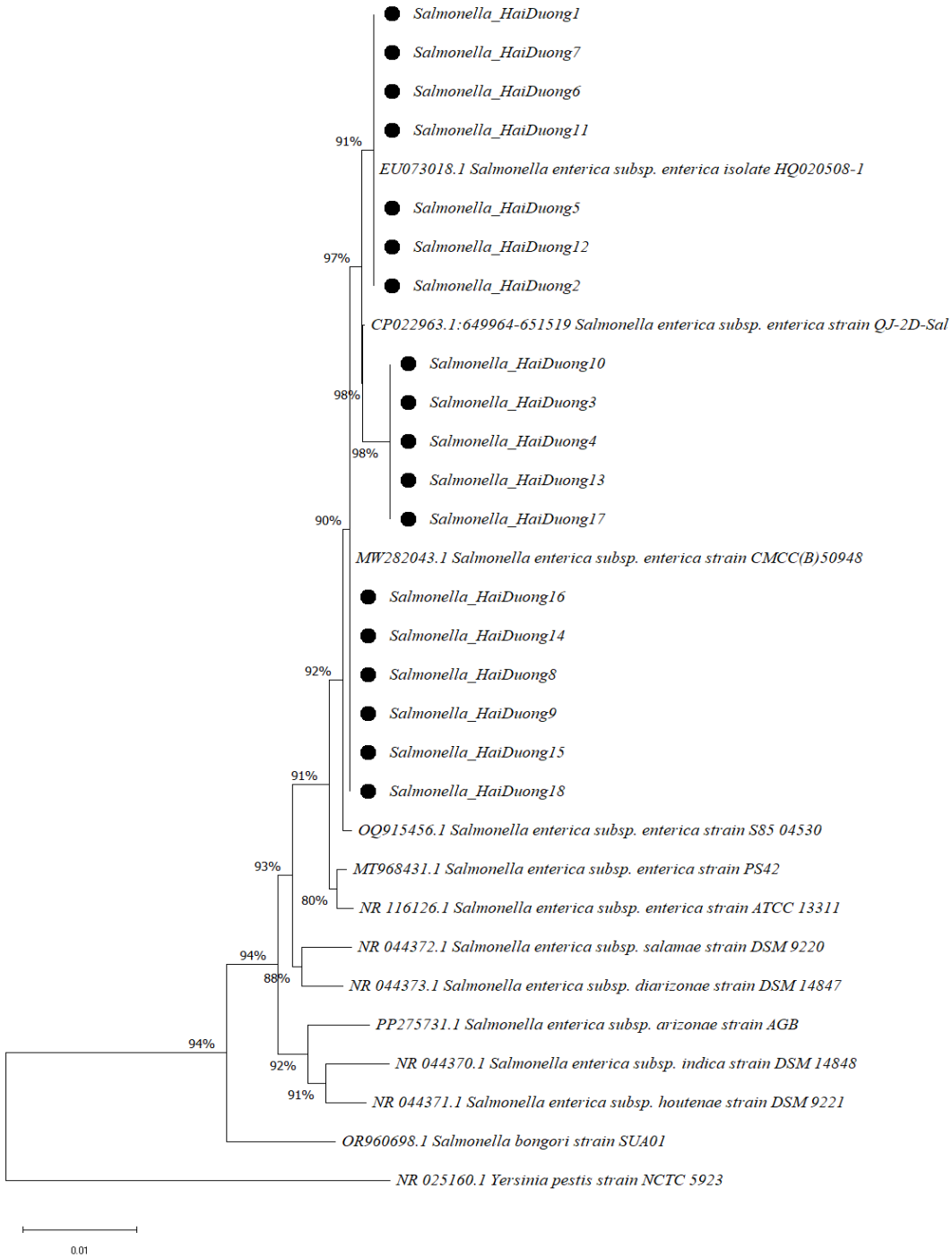

Figure 3. A phylogenetic tree based on 16S rRNA sequences of *Salmonella* isolates in this study (*Salmonella_HaiDuong* 1 - 18) and other *Salmonella* strains from GenBank. *Yersinia pestis* strain NCTC 5923 was used as the outgroup.

DISCUSSION

Salmonella is a significant zoonotic infectious agent, and chickens and ducks are one of its primary hosts (Wang *et al.*, 2020). Every year, *Salmonella* infections represent a significant threat to the poultry sector in developing countries, particularly Vietnam. Identifying and classifying the *Salmonella* strains in the region is essential for developing effective preventive measures and controlling outbreaks. In the present study, 18 *Salmonella* isolates were isolated from diarrheal chickens and ducks in Hai Duong province, underscoring the pathogen's significant role in poultry-associated illnesses and the potential risks to food safety. These 18 isolates were confirmed as *Salmonella enterica* through MALDI-TOF analysis, *invA* gene amplification, and 16S rRNA gene sequencing, highlighting the prevalence of this pathogen in local poultry farms. Our findings are consistent with previous studies, which indicated that *Salmonella* remains a persistent and pervasive threat to poultry production (Binh *et al.*, 2020).

Phylogenetically, the NJ tree analysis convincingly grouped all these 18 isolates in one group along with other *S. enterica* subsp. *enterica* originated from chicken in China (CP022963.1, EU073018.1, MW282043.1, OQ915456.1, MT968431.1) and Korea (NR116126.1), suggesting that these strains are closely related to those circulating in the broader Southeast Asian region. This regional similarity could be due to the movement of poultry and poultry products, which facilitates the spread of *Salmonella* across borders. The high sequence similarity (99.0-100%) among the isolates further indicates that these strains are highly conserved, which might reflect limited genetic diversity within the local *Salmonella* population.

The occurrence of *Salmonella* has previously been reported in poultry farms, poultry meat, eggs (Mohammed *et al.*, 2022; Wang *et al.*, 2020; Zhao *et al.*, 2022), and even in healthy poultry (Mohammed *et al.*, 2022) or in poultry feed sources (Zhao *et al.*, 2022). Furthermore, several previous studies have indicated that *Salmonella* obtained from poultry can exhibit single or multiple antibiotic resistance, representing a potential source of transmission of these resistant germs to humans and posing a public health risk. Specifically, resistance has been predominantly observed to ampicillin, kanamycin, tetracycline, streptomycin, erythromycin, and colistin (Mohammed *et al.*, 2022; Wang *et al.*, 2020; Zhao *et al.*, 2022; Yang *et al.*, 2019). These data further emphasize the need to research the *Salmonella* strains circulating in poultry in Vietnam that can lead to effective strategies to mitigate the impact of these bacteria.

Salmonella virulence - associated genes are generally located in distinct genomic areas dispersed throughout the genome, known as *Salmonella* pathogenicity islands (SPIs), which allow bacteria to avoid the host immune system while exerting pathogenicity (Shen *et al.*, 2023). *InvA* gene, a critical virulence factor encoded by *Salmonella*, located within the *Salmonella* Pathogenicity Island 1 (SPI-1) and plays a pivotal role in the bacterium's ability to invade host cells (Kim & Lee, 2017; Ranjbar *et al.*, 2017). More specifically, *invA* is essential for the formation of the type III secretion system (T3SS), a molecular apparatus that injects effector proteins into host cells, facilitating bacterial entry and establishment of infection. Due to its ubiquitous presence and conserved nature

among *Salmonella* strains, the *invA* gene has become a valuable target for rapid and accurate detection of *Salmonella* in various samples (Ranjbar *et al*., 2017). In this study, the *invA* gene was detected in all 18 *Salmonella* isolates, indicating its potential significance in *Salmonella* pathogenesis, which is consistent with previous reports (Shen *et al*., 2023; Zhang *et al*., 2018; Yang *et al*., 2019).

The presence of pathogenic *Salmonella* strains in local poultry farms presents a significant risk for the transmission of salmonellosis to humans, particularly through contaminated poultry products. Effective control of *Salmonella* at the farm level - through the implementation of enhanced hygiene practices, regular monitoring, and potentially vaccination is essential for mitigating the spread of this pathogen (Wang *et al*., 2020). The observed prevalence of *Salmonella enterica* subsp. *enterica* in poultry farms in Hai Duong province highlights the critical need for robust biosafety and biosecurity measures throughout the poultry production chain. Given that *Salmonella enterica* is a well-established causative agent of both localized and systemic infections in poultry, controlling its spread is vital for preventing outbreaks that could result in significant economic losses and pose a substantial threat to public health. Future research should focus on employing whole-genome sequencing to gain deeper insights into the genetic characteristics and potential virulence factors of the isolated strains. Additionally, investigating the antibiotic resistance profiles of these strains is imperative, given the growing concern over antimicrobial resistance in *Salmonella*. Expanding the geographical scope of the study to include additional provinces would also provide a more comprehensive understanding of *Salmonella* distribution and diversity across Vietnam.

CONCLUSION

This study highlights the persistent presence of *Salmonella enterica* subsp. *enterica* in poultry farms in Hai Duong province, emphasizing the ongoing risk to both poultry and public health. The findings underscore the importance of continued surveillance, improved biosecurity, and advanced molecular techniques in managing salmonellosis. Addressing these challenges is essential for ensuring the safety of poultry products and protecting public health.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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