ACADEMIA JOURNAL OF BIOLOGY 2024, 46(3): 17–26
DOI: 10.15625/2615-9023/20228

ISOLATION AND CHARACTERIZATION OF *Escherichia coli* ASSOCIATED WITH DIARRHEA IN CHICKENS AND DUCKS IN HAI PHONG PROVINCE

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Received 29 February 2024; accepted 28 August 2024

ABSTRACT

Colibacillosis is an intestinal tract infection in poultry caused by *Escherichia coli.* It is one of the leading causes of mortality and results in significant economic losses for the poultry farming sector due to its high incidence rate. Recently, *E. coli* has been considered a predominant bacterial pathogen that is responsible for diarrhea and bloodstream infections in chickens and ducks in Vietnam. In this study, *E. coli* strains associated with diarrhea were isolated from chicken and duck feces and intestines collected from poultry farms in Hai Phong province using a Macconkey selective medium. The obtained isolates were initially identified by the MALDI-TOF MS method, screened for hemolytic activity, and finally identified by 16S rRNA gene sequencing. As a result, eight *E. coli* strains exhibiting hemolytic activity were identified. Among them, *E. coli_5*, *E. coli_6*, and *E. coli_8* strains were clustered with *E. coli* O78:H51, whereas *E. coli*_3 was grouped with *E. coli* O78:H4; *E. coli*_4 and *E. coli*_7 were clusterd with *E. coli* O25b: H4; and *E. coli*_9 and *E. coli*_16 were grouped with *E. coli* O26:H11. Our results provide insights into the genetic diversity of *E. coli* strains associated with diarrhea in poultry farms in Hai Phong province. The isolated strains will be further characterized for the development of effective strategies to control colibacillosis in poultry.

Keywords: 16S rRNA, colibacillosis, *Escherichia coli*, hemolytic activity, MALDI-TOF.

Citation: Dong Van Quyen, Pham Thi Lanh, Nguyen Kim Oanh, 2024. Isolation and characterization of *Escherichia coli* associated with diarrhea in chickens and ducks in Hai Phong province. *Academia Journal of Biology*, 46(3): 17–26. https://doi.org/10.15625/2615-9023/20228

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INTRODUCTION

Colibacillosis is one of the main threats in the poultry farming industry (Ghunaim et al., 2014), leading to economic losses of hundreds of millions of dollars in this sector worldwide (Ghunaim et al., 2014). This disease has significantly reduced poultry meat production (about 2% in live weight), egg production (about 20%), and fertility (about 40%) (Dho-Moulin & Fairbrother, 1999; Guabiraba & Schouler, 2015). Remarkably, the disease can cause high mortality rates (up to 53.5%) in chicks (Mellata, 2013).

Colibacillosis may be associated with various disease conditions (Lutful Kabir, 2010). It can act as the primary pathogen or as a secondary pathogen, resulting in a range of disease manifestations in poultry, such as yolk sac infection, omphalitis, respiratory tract infection, swollen head syndrome, septicemia, polyserositis, coligranuloma, enteritis, cellulitis and salpingitis (Lutful Kabir, 2010). In poultry, colibacillosis is characterized by acute form with septicemia leading to death and subacute form with peri-carditis, airsacculitis and peri-hepatitis (Dho-Moulin & Fairbrother, 1999).

Escherichia coli, the main causative agent of avian colibacillosis, belongs to the Enterobacteriaceae family, are rod-shaped, gram-negative bacilli, non-spore-forming, and can grow in aerobic or facultative anaerobic conditions (Adinortey, 2014). *E. coli* strains are classified according to their serotypes, determined by the presence of three major antigens in their bacterial cells: the O, H, and K antigens. To date, there are about 250 O antigens, 89 K antigens, 56 H antigens, and a number of F antigens were found in *E. coli* strains (Fairbrother et al., 1993). Most *E. coli* strains that cause disease in poultry belong to specific serogroups, including O78, O2, and O1, along with other disease-causing serogroups such as O18, O15, and O55 (Younis et al., 2017).

E. coli infection can occur locally or throughout the bodies of chickens and ducks (Dipak Kathayat et al., 2021). Generally, typical signs of *E. coli* infection include edema, redness, swelling, and scaling in the cloaca and/or omphalitis (Kathayat et al., 2021). In addition, when *E. coli* accumulate under the skin around the eyes and head, they can secrete inflammatory fluid and cause swollen head syndrome (SHS) (Nolan et al., 2013).

In poultry, different antibiotics are used to control *E. coli* infections (Agunos et al., 2012). However, recent studies have reported the resistance of pathogenic *E. coli* strains toward several antibiotics commonly used in poultry including cephradine (Rahman et al., 2004), tetracyclines (Blanco et al., 1997; Cloud et al., 1985; Irwin et al., 1989), aminoglycoside (Allan et al., 1993), sulfonamide and β-lactam (Blanco et al., 1997; Cloud et al., 1985). In addition, vaccination has been considered an effective approach to control *E. coli* infections in poultry (Dho-Moulin & Fairbrother, 1999). To date, two types of vaccines are used for chickens, namely the live attenuated APEC O78 ΔaroA Poulvac® *E. coli* vaccine and the inactivated Nobilis® *E. coli* vaccine containing F11 fimbrial and FT flagellar antigens (Ghunaim et al., 2014). However, these vaccines lack the protection against different strains (Ghunaim et al., 2014).

In Vietnam, a study on 43 duck farms in 4 districts in Dong Thap province showed that the suspected rate of colibacillosis was 18.4% of isolates, especially, 213 of 214 (99.5%) samples were positive for *E. coli* (Sang et al., 2017). Moreover, several studies showed the considerable infection rate of *E. coli* in chickens and ducks as well as the noticeable resistance to antibiotics of the isolated strains in Vietnam (Nguyen et al., 2021; Nguyen et al., 2015; Sang et al., 2017). Recently, it has been suggested that disease-causing *E coli* strains in poultry could be potential agents for transmission from animals to food, resulting in extraintestinal infections in humans (Liu et al., 2018; Markland et al., 2015). Therefore, this is a dangerous pathogen for the poultry farming industry and public health. This study aimed to isolate and characterize the *E. coli* strains that are associated with diarrhea disease in chickens and ducks in poultry farms in Hai Phong province, Vietnam.

MATERIALS AND METHODS

Sampling

Ten samples of illness chickens and ducks with clinical signs, including edema, redness, swelling, and scaling in the cloaca and/or omphalitis were collected from poultry farms in Hai Phong province, Vietnam in 2023. The samples were kept on ice and immediately delivered to the Lab of Microbiology, Institute of Biotechnology, Vietnam Academy of Science and Technology.

Bacterial isolation

A cotton swab is used to collect feces from each chicken intestinal sample into 10 mL of peptone water for bacterial enrichment. The mixture was then inoculated for 3 hours at 35 ^{o}C, 150 rpm. The enrichment solution was diluted with NaCl solution (0.85%) to the appropriate concentration, and 100 µL of the dilution was spread on a Macconkey selective medium. The medium was incubated overnight at 37 ^{o}C. The obtained colonies were streaked on MacConkey medium several times for purification. The purified colonies were stored at 4 ^{o}C and 30% glycerol at -80 ^{o}C for subsequent analysis.

Identification of isolates using MALDI-TOF

All obtained isolates were quickly screened using the Protein mass spectrometry (Matrix-assisted laser desorption ionization-time of flight) method (Singhal et al., 2015) using a MALDI-TOF Biotyper (Bruker, Bremen, Germany). Isolates were automatically analyzed using Biotyper Compass Explorer software (version 4.1.100) (Bruker, Bremen, Germany). The degree of similarity between the experimental and reference strains was measured on a scale of 0 to 3.0. Results above 2.0 indicate high similarity and can be determined at the genus and species level (Clark et al., 2013).

Hemolysis test

A hemolysis assay was used to examine the virulence of bacterial strains based on their effect on blood cells. Briefly, *E. coli* isolates were grown on blood agar supplemented with 5% washed sheep blood according to Fakruddin et al. (2013) (Fakruddin et al., 2013). The blood agar plates were then incubated at 37 ^{o}C for 24 hours. Colonies that produced clear zones of hemolysis were recorded as hemolysin positive.

DNA extraction

Overnight bacterial inoculum was centrifugated at 4 ^{o}C and 12,000 rpm for 1 minute. The supernatant was removed, and the pellets were used to extract total DNA using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) (Solà-Ginés et al., 2015) following the manufacturer's instructions. The quality of extracted DNA was assessed by electrophoresis on a 1% agarose gel, stained with Ethilium bromide (Merck, Germany), and observed under UV light. The concentration and purity of the DNA were determined using a Nanodrop Lite (Thermo Fisher Scientific, USA).

16S rRNA amplification

The qualified DNA extraction was used as a template to amplify the full length of the 16S rRNA gene by PCR using a specific pair of primers (F-5'-AGAGTTTGATCMTGG-3' and R-5'-TACCTTGTTACGACT-3'). The PCR products were then checked by electrophoresis on a 1% agarose gel, stained with Ethidium bromide (Merck, Germany), and visualized under UV light. The PCR product was then purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA). The DNA concentration and purity were determined using a Nanodrop Lite (Thermo Fisher Scientific, USA).

16S rRNA sequencing and analysis

The bacterial 16S rRNA gene sequences were sequencing by the Sanger method using an ABI prism 3100 Sequencer automatic DNA sequencer (Applied Biosystems, USA). The sequencing data was processed and analyzed using BioEdit software (version 7.2.6.1). The obtained gene sequences were

compared with the 16S rRNA sequences of *E. coli* strains that are available on the Genbank (NCBI) using the BLAST tool. Genetic distances and phylogenetic trees were generated using MEGA X software (Kumar et al., 2018). The tree was replicated in 1000 replicates in which the association with taxa clustered together in the bootstrap test.

RESULTS

Bacterial isolation

After culturing on MacConkey selective medium overnight, we obtained many colonies. The *E. coli* colonies displayed small, round, glossy, and pink-colored morphologies that were selected as described in a previous study (Geletu et al., 2022). 30 colonies (designated as A1-A30) with the above characteristics isolated from the collected samples were then purified and stored at 4 ºC for subsequent experiments and in 30% glycerol at -80 ºC for long-term storage.

Identification of bacterial isolates by MALDI-TOF

In our study, 15 of 30 isolates were identified as *E. coli* by MALDI-TOF with the score values ranging from 2.3-2.51 to the reference strain *E. coli* (Table 1) whereas the remaining isolates belonged to *Klebsiella* spp. All obtained *E. coli* strains were utilized for further experiments.

Table 1. Representative result of identification of isolated bacteria by MALDI-TOF. All the isolates (A1-A7) were identified as *Escherichia coli*

Sample name	Sample ID	Organism (best match)	Score value	Organism (second-best match)	Score value
A1 (+++) (A)	1 (standard)	*Escherichia coli*	2.49	*Escherichia coli*	2.41
A2 (+++) (A)	2 (standard)	*Escherichia coli*	2.50	*Escherichia coli*	2.40
A3 (+++) (A)	3 (standard)	*Escherichia coli*	2.48	*Escherichia coli*	2.41
A4 (+++) (A)	4 (standard)	*Escherichia coli*	2.41	*Escherichia coli*	2.36
A5 (+++) (A)	5 (standard)	*Escherichia coli*	2.51	*Escherichia coli*	2.50
A6 (+++) (A)	6 (standard)	*Escherichia coli*	2.45	*Escherichia coli*	2.38
A7 (+++) (A)	7 (standard)	*Escherichia coli*	2.41	*Escherichia coli*	2.37

Hemolysis test

The hemolysis assay showed that 8 out of 15 *E. coli* strains (53.3 %) exhibited the ability to produce alpha-hemolysin (α-hemolysis) (Fig. 1A**)**, a cytolysin that forms pores and acts as a virulence factor in both intestinal and extraintestinal pathogenic strains of *E. coli* (Burgos & Beutin, 2010). These strains consisted of A3 (*E. coli*_3), A4 (*E. coli*_4), A5 (*E. coli*_5), A6 (*E. coli*_6), A7 (*E. coli*_7), A8 (*E. coli*_8), A9 (*E. coli*_9) and A16 (*E. coli*_16). In contrast, the remaining strains showed no hemolytic activity (γ-hemolysis) (Fig. 1B**)**.

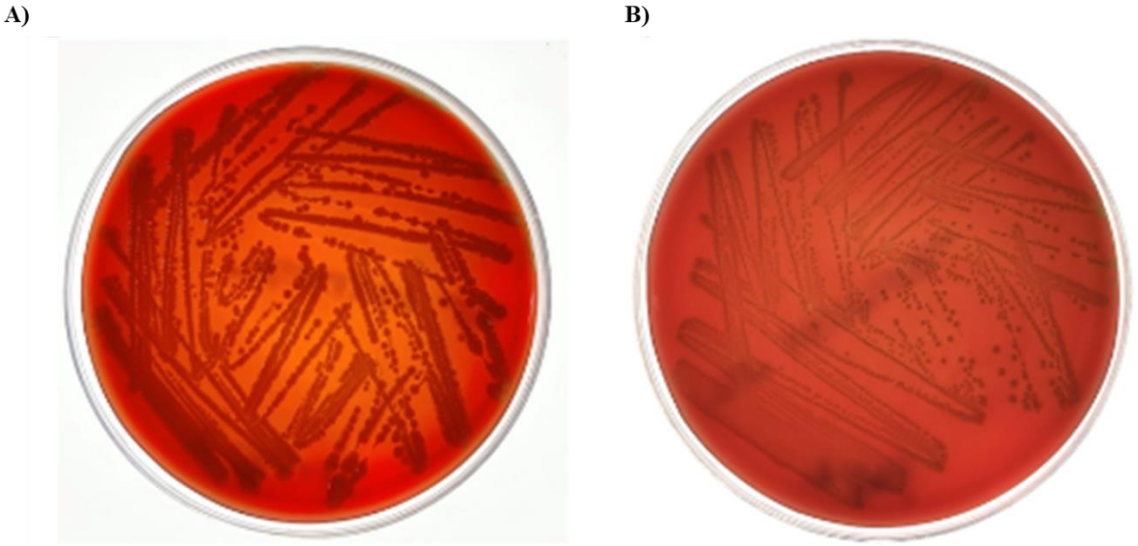

Figure 1. Hemolysis activity of *Escherichia coli* strains on blood agar: A) α-hemolysis, B) γ-hemolysis

DNA extraction and 16S rRNA amplification

Eight *E. coli* strains that exhibited α-hemolysis were then utilized for DNA extraction. The quality and quantity of the extracted DNA samples were confirmed by agarose gel electrophoresis (Fig. 2A**)** and NanoDrop Lite, respectively. The DNA concentration of the samples ranged from 767.8 to 1246.7 ng/µL, with A260/280 values between 1.80-1.94. This result indicated that the extracted DNA samples were good enough for subsequent analysis. Further, the 16S rRNA genes from all the purified DNA samples were successfully amplified, resulting in DNA bands with the size of about 1,500 bp appearing in the agarose gels (Fig. 2B).

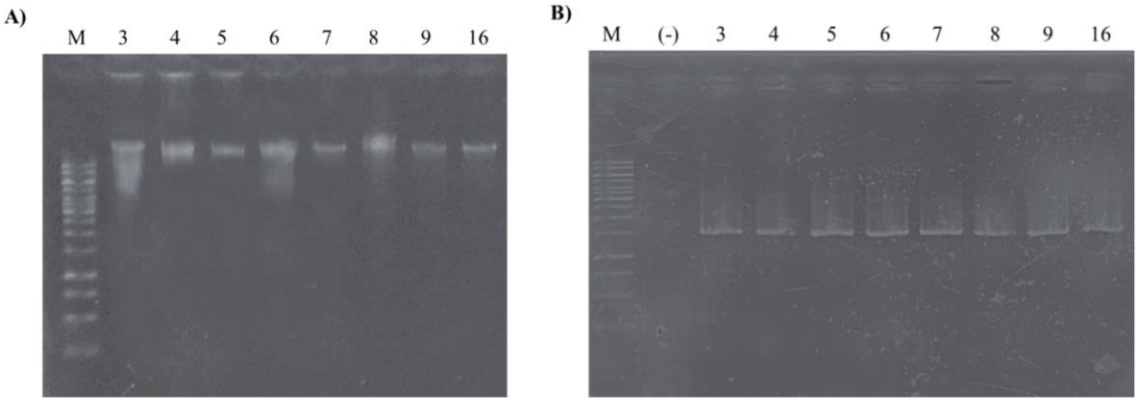

Figure 2. Total DNA extracted of *Escherichia coli* isolated from poultry farms (A) and the 16S rRNA PCR products from isolated bacterial strains on 1% agarose gel (B); M: Thermo Scientific™ GeneRuler™ 1 kb DNA ladders, (-): negative control

16S rRNA sequencing and phylogenetic analysis

The result of 16S rRNA sequence analysis showed that eight isolated *E. coli*

strains had 99.99–100% sequence similarity with each other and 99.96–100% sequence similarity with those of other *E. coli* strains on GenBank (NCBI) (data not shown). Further, 16S rRNA sequences of eight isolated *E. coli* strains and those of five *E. coli* strains available in GenBank (NCBI) were used to construct a phylogenetic tree with *Salmonella enterica* subsp. *enterica* serovar *Paratyphi* A str ATCC 9150 as an outgroup (Fig. 3). In the phylogenetic tree, the three strains, *E. coli*_5, *E. coli*_8, *E. coli*_6 were clustered into the same group as the *E. coli* O78:H51 strain APEC E19025 isolated from the liver of *Gallus gallus* in Korea, with a bootstrap of 83%. In contrast, *E. coli*_3 was in the same clade as *E. coli* O78:H4 strain APEC E12049 also isolated from *Gallus gallus* in Korea with a bootstrap of 100%. Additionally, two strains of *E. coli*_4 and *E. coli*_7 were in the same branch as *E. coli* O25b:H4-ST131 strain 2882 isolated from humans in the USA and the *E. coli* ATCC 8739 strain isolated in China. Meanwhile, the two strains of *E. coli*_9 and *E. coli*_16 were in the clade of the *E. coli* O26:H11 strain isolated from cattle in Belgium formed a separate branch (Fig. 3).

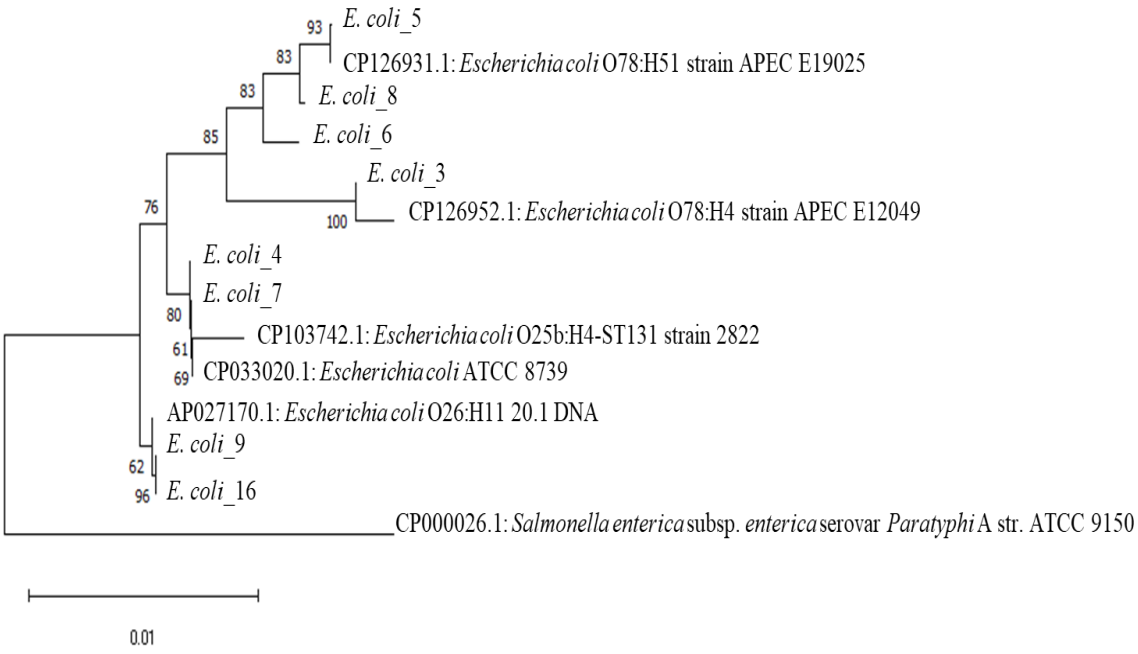

Figure 3. Phylogenetic tree based on 16S rRNA sequences of isolated *Escherichia coli* strains and *Escherichia coli* strain sequences from Genbank. *E. coli_3*, *E. coli_4*, *E. coli_5*, *E. coli_6*, *E. coli_7*, *E. coli_8*, *E. coli_9* and *E. coli_16* are isolates obtained from this study. *Salmonella enterica* subsp. *enterica* serovar *Paratyphi* A str ATCC 9150 was used as the outgroup

DISCUSSION

The present study aimed to isolate, identify, and characterize pathogenic *E. coli* strains obtained from poultry farms in Hai Phong province, Vietnam. The results revealed a diverse range of *E. coli* strains, with significant implications for both veterinary and public health. Utilizing a MacConkey selective medium, colonies displaying distinct morphologies were selected, consistent with a previous study (Geletu et al., 2022). Further identification using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) revealed that 15 out of 30 isolates were identified as *E. coli*, with the remaining isolates belonging to *Klebsiella* spp. In this study, we found that 8 out of these 15 strains exhibited

hemolysis activities. This virulence factor is implicated in both intestinal and extraintestinal pathogenic strains of *E. coli*, underlining the potential clinical significance of these isolates (Burgos & Beutin, 2010). In addition, the 16S rRNA sequence analysis indicated that they belong to *E. coli* O78:H51, *E. coli* O78:H4, *E. coli* O25b:H4, and *E. coli* O26:H11 strains. Our result was consistent with previous studies which showed that 53.3% of the isolated *E. coli* strains from chicken specimens produced hemolysis on blood agar plates (Zahid et al., 2016). Additionally**,** Shivani & Jindal (2018) showed that 34% of uropathogenic *E. coli* strains were found to produce hemolysin (Jindal & Shivani, 2018). Especially, it has been reported that 44.6% of clinical isolates of *E. coli* obtained from non-enteric infections were hemolytic (Fakruddin et al., 2013). All *E. coli* strains including *E. coli* O78:H51, *E. coli* O78:H4, *E. coli* O25b:H4, and *E. coli* O26:H11 strains were previously detected in poultry farms (Khattab, 2019; Nguyen et al., 2021; Solà-Ginés et al., 2015; Yousef et al., 2023).

Among them, *E. coli* O78 strains are known to cause a large variety of clinical syndromes in a wide host range including humans (Babai et al., 1997). These strains were previously detected and isolated from poultry farms in Vietnam (Nguyen et al., 2021; Nguyen et al., 2015), and other countries in the world (Ronco et al., 2017; Younis et al., 2017). Especially, *E. coli* O78:H4 strain contributed considerably to the increase of colibacillosis cases observed on Nordic poultry farms from 2014 to 2016 (Ronco et al., 2017).

Furthermore, the human extraintestinal pathogenic *E. coli* group O25b: H4 was found in our study. Similarly, this strain was previously detected in poultry by Mora et al. 2010, Cortés et al., 2010 (Cortés et al., 2010; Mora et al., 2010). Notley, *E. coli* O25b: H4-B2-ST131 poultry isolates in Spain showed a multi-drug resistance in broiler farms and contained a high number of virulence-associated genes (Solà-Ginés et al., 2015). Importantly, enterohaemorrhagic *E. coli* (EHEC) O26:H11 was also isolated in our study. They can cause diarrhea and haemolytic uraemic syndrome (HUS) - a leading cause of acute renal failure in children and is mainly caused by EHEC expressing Shiga toxins (Stx) 1 and/or 2 (Bielaszewska et al., 2007). The identification of pathogenic *E. coli* strains, including those associated with extraintestinal infections, raises concerns about potential transmission to humans through the food chain. The multi-drug resistance observed in certain strains emphasizes the need for continuous monitoring and characterization. Notably, the presence of *E. coli* O26:H11, associated with diarrheal diseases and hemolytic uremic syndrome, underscores the importance of further characterization and surveillance to assess the potential public health risks associated with these strains.

Hence, this study demonstrates the presence of different pathogenic *E. coli* strains in illness chickens and ducks at poultry farms in Hai Phong province. The *E. coli* population among poultry animals could be transmitted into the community through the food chain. More epidemiological studies are necessary to identify clonal groups with potential relevance to public health in order to develop effective strategies to increase the survival of poultry animals and reduce the risk of human infection.

CONCLUSION

In conclusion, this study provides a comprehensive analysis of pathogenic *E. coli* strains isolated from poultry farms in Hai Phong province, Vietnam. The combination of selective culturing, MALDI-TOF identification, hemolysis assays, and genetic analyses revealed a diverse array of hemolytic *E. coli* strains with potential public health implications. Further epidemiological studies are warranted to elucidate the transmission dynamics and assess the risk of human infection, facilitating the development of effective strategies to mitigate these risks in both poultry and human populations.

Acknowledgements: This study was financially supported by the Ministry of Agriculture and Rural Development, Vietnam.

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