BMC Veterinary Research

RESEARCH

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Antimicrobial resistance, virulence gene profiles, and phylogenetic groups of *Escherichia coli* isolated from healthy broilers and broilers with colibacillosis in Thailand

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Abstract

Background Multidrug resistance in Escherichia coli has a significant global impact on poultry production. This study aimed to determine the phenotypic and genotypic backgrounds of antimicrobial resistance (AMR) and virulence gene profiles of *E. coli* strains isolated from diseased and healthy broilers. A total of 211 *E. coli* isolates were recovered from diseased ($n=110$) and healthy broilers ($n=101$). All the isolates were subjected to antimicrobial susceptibility testing. A PCR-based technique was applied to screen AMR genes, virulence genes and analyze phylogenetic groups.

Results Phylogenetic groups B1 and D were the most prevalent for *E. coli* isolated from diseased and healthy birds. Among virulence genes, the detection rates of *cva/cvi*, *iutA*, *iucD*, *iroN*, *iss* and *ompT* were considerably greater in *E.coli* strains from diseased birds than in healthy birds. The virulence gene pattern of *hlyF-iutA-iucD-iroN-iss-ompT* (16.4%) was frequently observed in *E.coli* isolated from diseased birds, whereas approximately 22.8% of *E.coli* from healthy birds did not carry any virulence genes. Analysis of AMR profiles revealed that 58.3% of *E.coli* were resistant to multiple classes of antibiotics, and 96.7% carried at least one antibiotic resistance gene AMR genes.

Conclusion The findings of this study demonstrate the variable distribution of phylogenetic groups and virulence genes. *E.coli* strains isolated from broilers had multidrug resistance profiles. The study emphasizes the need for continuous monitoring of AMR emergence in *E. coli* from broilers. This monitoring allows for early detection and implementation of strategies to control the spread of resistant strains.

Keywords Antimicrobial resistance, *Escherichia coli*, Diseased broilers, Healthy broilers

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Background

Most *Escherichia coli* strains are nonpathogenic and benefit the intestinal health of the host. However, some pathogenic strains of *E. coli* can cause symptoms and diseases. In poultry, avian pathogenic *E. coli* (APEC) is a causative pathotype agent of avian colibacillosis that causes extraintestinal infection and colisepticemia, resulting in poor growth performance and significant economic loss in broiler production [1].

APEC can be a primary or secondary bacterial pathogen that causes systemic or local infections, such as airsacculitis, pericarditis, perihepatitis, and avian cellulitis. Several risk factors, such as coinfection with viruses or other bacterial agents and unfavorable sanitary conditions, can reduce the host immune response, resulting in more severe APEC infection in birds of all ages [2].

Different types and numbers of virulence genes (VRGs) can be carried by APEC and nonpathogenic *E. coli* strains [3]. Several APEC virulence-related genes, such as those involved in iron acquisition *(iroN, irp2, and iutA)*, serum resistance (*iss* and *cvaC*), and temperature-sensitive hemagglutination (*tsh)*, are often observed to be associated with the pathogenicity of APEC [4].

When a colibacillosis outbreak occurs, biosecurity measures, good sanitation management, vector control programs, etc., should be implemented to minimize the spread of the pathogen among broiler flocks. The use of antibiotics is important for treating colibacillosis in birds and reducing the severity of illness. However, the extensive and often improper use of various antibiotic classes, including β-lactams, fluoroquinolones, and aminoglycosides, over the past decade has driven the emergence of antibiotic-resistant *E. coli* strains in broilers [5].

These resistant strains can harbor genetic determinants of antimicrobial resistance (AMR), which can be identified in both APEC and commensal *E. coli* strains. The localization of AMR genes on plasmids facilitates the distribution of AMR through horizontal gene transfer, which can promote the rapid spread of AMR to other bacterial populations in different hosts [6]. The pathogenicity of *E. coli* increases because AMR genes and VRGs coexist on the same plasmids; therefore, *E. coli* strains can be virulent and/or resistant to several classes of antibiotics [7].

The spread of multidrug-resistant *E. coli* is a serious challenge for the global livestock industry, including that in Thailand, which is one of the world's largest exporter of broilers [8]. Thai poultry producers recognize the challenges of antibiotic use and AMR, especially in broiler production. Broilers and their products can act as a reservoir for *E. coli* strains carrying multiple AMR and virulence genes. This not only negatively affects broiler production but also serves as a potential zoonotic source of antibiotic resistance and virulence genes for extraintestinal pathogenic (ExPEC) *E. coli* strains, which infect humans through the food chain [9].

To reduce the risk of AMR, the use of antibiotics as growth promoters was banned in 2015 in Thailand [10]. Only a few antibiotics have been allowed for therapeutic treatment in broilers with colibacillosis, where the withdrawal time of antibiotics must be considered prior to slaughter. Data on the genetic profiles of antibiotic resistance and virulence factors in *E. coli*, including APEC and non-pathogenic *E.coli* (NPEC) need to be continuously updated and monitored throughout broiler production to develop guidelines for antibiotic usage and improve colibacillosis control in broiler production in Thailand. Therefore, this study aimed to determine the phenotypic and genotypic backgrounds of AMR and its virulence gene profiles in *E. coli* strains isolated from diseased and healthy broilers.

Results

E.coli isolates and their phylogenetic groups

Of the 211 *E. coli* isolates collected from broilers, 110 isolates were obtained from diseased birds while 101 isolates originated from healthy birds. Among these isolates, 198 (93.84%) isolates were assigned to seven different phylogenetic groups, while 13 (6.16%) isolates were assigned to unknown phylogenetic groups. Phylogenetic groups B1 and D were the most common groups identified in *E.coli* isolated from diseased (30% for B1, 34.5% for D) and healthy birds (31.7% for B1, 20.8% for D). The distributions of the phylogenetic groups of *E, coli* isolated from diseased and healthy birds are shown in Table 1.

Table 1 Phylogenetic groups of *E.coli* isolated from diseased ($n=110$) and healthy broilers ($n=101$)

Phylogenetic group	Number of E.coli isolates	
	Diseased broilers *n* (%)	Healthy broilers *n* (%)
A	2 (1.82%)	18 (17.82%)
B1	33 (30.00%)	32 (31.68%)
B2	1 (0.91%)	4 (3.96%)
C	10 (9.09%)	11 (10.89%)
D	38 (34.55%)	21 (20.79%)
E	3 (2.73%)	1 (0.99%)
F	16 (14.55%)	2 (1.98%)
I	2 (1.82%)	4 (3.96%)
Unknown	5 (4.55%)	8 (7.92%)

Distribution of virulence genes

AMR profiles of *E. coli* isolates

Among the 211 *E. coli* isolates, 198 (93.84%) were resistant to at least one antibiotic, and 13 (6.16%) were susceptible to all antibiotics. The percentages of multidrug-resistant (resistant to at least 3 classes of antibiotics)

Table 2 The prevalence of virulence genes of *E.coli* recovered from diseased ($n=110$) and healthy ($n=101$) broilers

VRGs	Number of E.coli isolates		Total isolates (%)	*P*-value*
	Diseased birds *n* (%)	Healthy birds *n* (%)		
astA	20 (18.2)	19 (18.8)	39 (18.5)	1
cva/cvi	39 (35.5)	18 (17.8)	57 (27)	0.005
hlyF	65 (59.1)	48 (47.5)	113 (53.6)	0.10
iutA	94 (85.5)	40 (39.6)	134 (63.5)	< 0.001
iucD	92 (83.6)	38 (37.6)	130 (61.6)	< 0.001
iroN	86 (78.2)	40 (39.6)	126 (59.7)	< 0.001
irp-2	37 (33.6)	42 (41.6)	79 (37.4)	0.26
iss	98 (89.1)	26 (25.7)	124 (58.8)	< 0.001
OmpT	100 (90.9)	49 (48.5)	149 (70.6)	< 0.001
papC	0	0	0	-
tsh	14 (12.7)	11 (10.9)	25 (11.8)	0.83

*Significantly different ($p<0.05$)

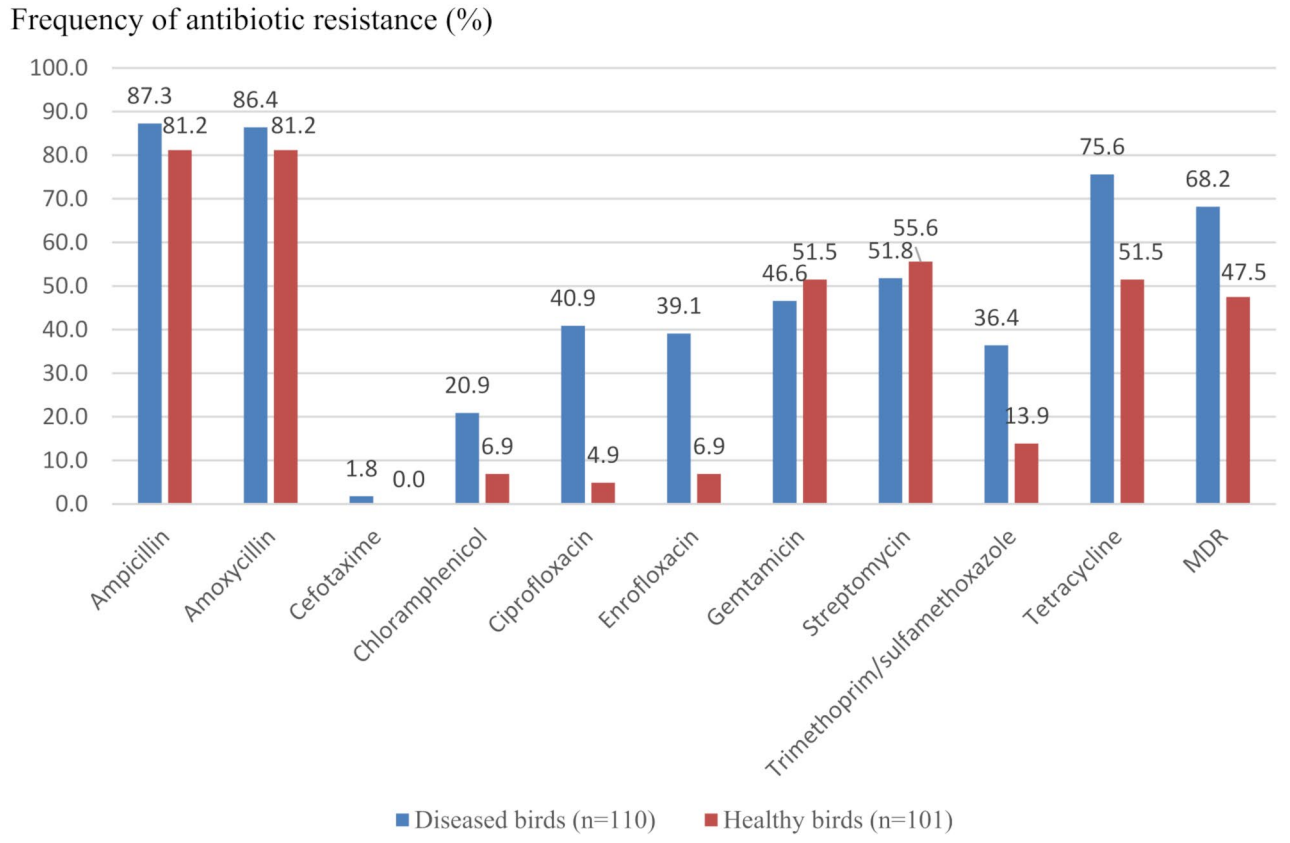

Fig. 1 The percentage of strains showing antibiotic resistance in *E.coli* from diseased and Healthy broilers ($n=211$)

E.coli strains isolated from diseased and healthy broilers were 68.2% and 47.5%, respectively.

A total of 211 *E. coli* isolates showed a high prevalence of resistance to amoxicillin (84.4%), ampicillin (83.9%), tetracycline (63.9%), streptomycin (53.5%) and gentamicin (48.8%) and a low prevalence of resistance to chloramphenicol (14.2%), ciprofloxacin (23.7%), and enrofloxacin (23.7%). A comparison of AMR rates between *E.coli* isolated from diseased and healthy broilers is shown in Fig. 1. No significant differences ($p>0.05$) were observed in the prevalence of resistance of *E.coli* from diseased and healthy birds for amoxycillin (diseased: 87.3%; healthy: 81.2%), ampicillin (diseased: 86.4%; healthy: 81.2%), cefotaxime (diseased: 1.8%; healthy: 0%), gentamicin (diseased: 46.4%; healthy: 51.5%), and streptomycin (diseased: 51.8%; healthy: 55.5%). However, the prevalence of resistance to chloramphenicol, ciprofloxacin, enrofloxacin, sulfa-trimethoprim and tetracycline

were significantly ($p < 0.05$) higher in the *E.coli* strains from diseased birds than in healthy birds. Among the ESBL-producing *E. coli* strains, only two isolates (2/110, 1.8%) recovered from diseased birds were ESBL producers. All *E.coli* isolated from healthy birds were non-ESBL-producing strains.

Furthermore, a comparison of the prevalence of resistance between different phylogenetic groups of *E. coli* revealed that all *E. coli* isolates of phylogenetic group B2 ($n = 5/5$) were susceptible to all the antibiotics examined. All groups except group B2 showed high resistance to amoxycillin and ampicillin (resistance range: 61–100%). Only group B1 and D isolates had low levels of cefotaxime resistance (1.5% and 1.7%, respectively), whereas the other groups were cefotaxime sensitive. A low resistance rate to chloramphenicol (range: 5–23.1%) was found in groups A, B, D, and E; clade I; and unknown. High resistance rates to ciprofloxacin and enrofloxacin were found in group E (75% and 100%, respectively), followed by group D (47.5% and 42.4%, respectively). In contrast, all isolates in group F were susceptible to these antibiotics. The resistance to the aminoglycoside varied among the groups (22.2–76.2% for gentamicin and 20–75% for streptomycin). All phylogenetic groups showed a low prevalence of resistance to sulfa-trimethoprim (range: 5–38.5%), except 75% resistance in phylogroup E. Tetracycline resistance ≥ 50% was detected in phylogroups B1 (75.4%), C (57.1%), D (71.2%), E (100%), F (66.7), and clade I (66.7%). The number of isolates in each phylogenetic group and their antimicrobial resistance rates are shown in Supplemental Fig. 1.

Characterization of antibiotic resistance genes in *E.coli* isolated from diseased and healthy broilers

Among 204 isolates (96.7%), at least one antibiotic resistance gene (ARG) was identified. Of these isolates, 105 isolates were obtained from diseased birds and 99 isolates originated from healthy birds. Antibiotic resistance phenotypes, along with their corresponding antibiotic resistance genes and the distribution of ARGs in *E.coli* strains from diseased and healthy birds, are detailed in Supplemental Tables 1 and 2, respectively. bla_{TEM} was mostly detected in *E.coli* isolated from both diseased (74.6%) and healthy (65.4%) birds. Notably, the two ESBL-producing *E.coli* strains from diseased birds harbored $bla_{\text{CTX-M}}$ (1.8%). The *cmlA* gene was detected only in *E.coli* from diseased birds (26.4%), whereas *catA* resistance gene was identified more frequently in *E.coli* from healthy birds (41.6%) compared to diseased birds (2.73%). Among the plasmid-mediated quinolone resistance (PMQR) genes, *qnrS* was frequently detected in diseased (39.1%) and healthy (15.8%) birds. None of *E.coli* isolates carried *qnrA* gene. Moreover, the prevalence of sulfa-trimethoprim resistant genes in diseased birds including *sul1*, *sul2*, *sul3*, *dfrA1* and *dfrA12* were found in 20%, 2.7%, 27.3%, 1.8% and 12.7%, respectively. In healthy birds, the prevalence of these genes was 10.9%, 18.8%, 55.5% and 0.9%, respectively. The *tetA* was the only tetracycline resistance gene identified in this study. It was found in *E.coli* from diseased birds (53.6%) and healthy birds (28.7%). Among aminoglycoside-resistance genes, *aadA1* gene was the most prevalent, found in both diseased (48.2%) and healthy (39.6%) birds. It was followed by *aadA2* in diseased (28.2%) and healthy (33.6%) birds. The presence of *strA* and *strB* genes encoding streptomycin resistance was observed in *E.coli* from diseased birds (*strA*;17.3%, *strB*; 18.2%) and healthy birds (*strA*; 5,94%, *strB*; 4.94%).

Discussion

Antimicrobial resistance in *E.coli* is a critical problem in broiler production, with public health implications. Avian colibacillosis is a common bacterial disease in global broiler production. This pathogen can be a primary cause, but it often plays a role in secondary bacterial infection, which causes respiratory and systemic infection, resulting in a negative effect on broiler performance. Furthermore, commensal *E.coli*, a natural component of gut microbiota in healthy poultry, can harbor various genetic determinants of resistance to antibiotics. These resistance genes can be shared to pathogenic bacteria, potentially compromising the treatment of human infections [11].

AMR in *E. coli* from diseased and healthy broilers was monitored in this study. High prevalence of resistance to ampicillin (83.9%), amoxycillin (84.4%), tetracycline (63.98%), streptomycin (53.5%), and gentamicin (48.8%), was detected in *E.coli* from diseased and healthy birds. Consistently, high prevalence of resistance to these antibiotics was described by Fancher et al. (2021) [12], who reported high resistance rates to ampicillin, tetracycline, streptomycin, and sulfamethoxazole/trimethoprim in virulent and nonvirulent *E. coli* strains isolated from commercial broilers raised with "no antibiotics ever" production. Several studies have frequently reported a high prevalence of resistance to these antibiotics in pathogenic and commensal *E. coli* strains isolated from poultry [13, 14]. Strict regulations on antibiotic use in broilers and other poultry farming have significantly curtailed the available options for treating bacterial infections in the poultry industry. Amoxicillin, doxycycline, and streptomycin are among the few remaining antibiotics permitted for therapeutic use. However, the continued use of these antibiotics could create selective pressure, leading to a high prevalence of antibiotic resistance.

Third and fourth generation cephalosporins are classified as highest priority critically important antimicrobials (HPCIA) in the WHO list [15], the use of which should be restricted in food-producing animals to reduce and

prevent the emergence of cephalosporin resistance in bacterial pathogens of animals. Most of our isolates were identified as non-ESBL-producing *E. coli* strains and showed low prevalence resistance to cefotaxime (1.8%), which is attributable to a lack of selective pressure due to the restriction of cephalosporin use in Thai broilers and poultry production.

Broilers are important reservoirs of quinolone-resistant *E. coli* strains that can be transferred to humans and are a serious public health problem [16]. Quinolones are also classified as the highest priority critically important antimicrobials (HPCIA) in the WHO list [15]. In response to this concern, enrofloxacin, a fluoroquinolone antibiotic previously used for colibacillosis treatment in poultry, has been banned for disease treatment in Thai broilers for many years. In our study, the overall resistance of *E. coli* strains from diseased and healthy broilers to enrofloxacin was 23.7% lower than that of the Thai APEC strains reported in other studies, in which approximately 30% of the APEC strains isolated from different regions of Thailand were resistant to enrofloxacin [17, 18]. This rate was also lower than those reported for APEC strains from Algeria (86.27%) [19] and China (96.1%) [20].

Although our findings indicate a relatively lower prevalence of resistance, it remains unclear whether the ban on enrofloxacin in Thailand has directly contributed to this resistance phenotype in *E. coli*. Long-term monitoring is necessary to fully understand the impact of the ban on resistance trends. Nonetheless, this study provides valuable data that can contribute to future evaluations of the long-term effects of the enrofloxacin ban on fluoroquinolone resistance in *E. coli*.

Furthermore, in this study, *E. coli* strains isolated from diseased broilers exhibited significantly higher prevalence resistance rates to ciprofloxacin and enrofloxacin compared to those from healthy broilers ($p < 0.001$). Quinolone resistance rates among *E. coli* strains varied. A study from Brazil reported that non-APEC strains displayed greater resistance to enrofloxacin than did APEC strains [21]. However, high prevalence of resistance to enrofloxacin (92.3%) was observed in APEC strains from broilers in Algeria [22]. This is likely attributable to the increased exposure of diseased broilers to antibiotics, including fluoroquinolones, even after their ban in Thai broiler farms. The continued use of other antibiotics, such as β-lactams, may exert selective pressure or contribute to the co-selection of resistance mechanisms.

Both susceptible and resistant *E. coli* strains carried multiple AMR genes in this study. Among β-lactam resistance genes, bla_{TEM} is frequently observed in both *E. coli* isolated from diseased and healthy broilers. The bla_{TEM} family encodes the β-lactamase enzyme, which hydrolyzes the β-lactam rings of penicillin and cephalosporins [23]. Most of the bla_{TEM} genes and their variants are often associated with broad-spectrum β-lactamases or extended-spectrum β-lactamases (ESBLs), which are derived from narrow-spectrum β-lactamases ($bla_{\text{TEM-1}}$, $bla_{\text{TEM-2}}$) [24]. However, a high prevalence of bla_{TEM} in our study was observed in non-ESBL-producing *E. coli* strains, which is consistent with other studies on non-ESBL-producing *E. coli* isolates from broiler farms in Spain [25] and non-ESBL isolates from clinical cases in the Philippines [26]. In contrast, several studies have demonstrated that bla_{TEM} is frequently identified in ESBL-producing *E. coli* and *Enterobacteriaceae* in southeastern Austria [27]. Data from Egypt [28] showed the dominance of bla_{TEM} in ESBL-producing *E. coli* recovered from chickens, as well as broilers from different parts of Indonesia [29]. The detection of bla_{TEM} in both non-ESBL and ESBL-producing *E. coli* isolates suggests the possibility of different TEM types due to the gene's evolution, leading to variants encoding either narrow-spectrum or broad-spectrum β-lactamases [30]. Therefore, further investigation of the characteristics of bla_{TEM} in non-ESBL *E. coli* from this study is warranted.

A wide distribution of PMQR in *E. coli* and gram-negative bacteria in human and animal sources has been reported in Thailand [31, 32]. Our study found that *qnrS* was frequently identified in *E.coli* from diseased (39.1%) and healthy (15.8%) broilers. In Taiwan, *qnrS* was the major PMQR determinant, followed by *aac(6')-Ib-cr*, which was detected in *E. coli* strains isolated from swine and chicken [33]. *E. coli* isolates from broiler feces on commercial farms in Japan harbored *qnrS* and *aac(6')-Ib-cr* [34]. Similarly, these two PMQR-determinants are frequently carried by ciprofloxacin-resistant *E. coli* isolated from layer chickens in Korea [35]. The prevalence of diverse PMQR types across studies may be influenced by region-specific selective pressures, including antibiotic practices in livestock farms. This can create distinct residue environments that impact the selection and maintenance of PMQR genes. Additionally, the expression of *qnr* genes can be regulated by fluoroquinolone concentration in the environment [36]. However, chromosomal-mediated quinolone resistance can occur through mutations in the quinolone resistance determining region (QRDR) of topoisomerase II and IV. Therefore, this mechanism should be investigated in quinolone-resistant *E. coli* strains that do not possess plasmid-mediated quinolone resistance (PMQR) genes.

Tetracycline is a broad-spectrum antibiotic widely used to treat colibacillosis and other bacterial diseases in broiler production. Our study also importantly identified the presence of *tetA* in *E. coli* isolates from both healthy and diseased broilers. The *tet* family is associated with efflux pump resistance in gram-negative bacteria [37]. In recent studies, high frequencies of *tetA*-carrying *E. coli* were observed in samples from broilers, layer and

broiler breeders, swine, and the environment [38–40]. In this study also found *E.coli* isolates exhibiting phenotypic tetracycline resistance but lacking the *tet B*, *tet C* and *tet D* genes investigated. This suggests the presence of other tetracycline resistance genes such as *tetM* or *tet G*, which were not examined in our study. The existence of these genes in *E. coli* has been documented in a previous study [41].

In broiler production, aminoglycosides and sulfa-trimethoprim are rarely used, whereas chloramphenicol was prohibited. The prevalence of phenotypic resistance to these antibiotics and their corresponding resistance genes in *E.coli* strains was detected in this study. It is possible that the extensive use of other antibiotic groups, such as β-lactams, facilitated cross- or co-resistance mechanisms to these antibiotics [23].

Notably, 13 *E. coli* isolates exhibited sensitivity to all antibiotics, but 9/13 isolates carried at least one ARG. This result confirmed that phenotypically susceptible bacterial strains can be potential reservoirs for ARGs.

Consistent with other studies on poultry in China and South Korea [42, 43], this study revealed diverse distribution of phylogenetic groups in *E. coli* strains isolated from diseased and healthy broilers. We found that phylogenetic group B1 was the predominant phylogenetic group in *E.coli* from both diseased and healthy broilers. This finding contrasts with another study that reported a strong association between *E. coli* in group B1 and *E.coli* strains from healthy laying chickens and broilers [44]. In contrast to group B1, the prevalence of group D among the *E.coli* from diseased broilers was greater than *E.coli* from healthy broilers, which is consistent with the findings from Brazil and Iran [45, 46] reported that *E.coli* isolated from chicken with colibacillosis lesions, classified as APEC strains, have a potential association with group D strains, which can cause septicemia or swollen head syndrome. Furthermore, APEC plays a role as a potential bacterial zoonotic agent that causes extraintestinal infections, such as urinary tract infections, meningitis, and septicemia, in humans [47]. While our study revealed a low prevalence of group B2 in *E.coli* isolates, previous research suggested that group B2 in the APEC strains was closely genetically related to the human ExPEC strains, raising concerns about poultry as a potential source of zoonotic infections [47, 48]. According to distribution of phylogenetic groups across different countries, the diversity of *E. coli* might depend on geographic location and bird ages [43, 49].

Eleven virulence genes were identified in *E. coli* isolated from diseased and healthy broilers. The prevalence of *papC*, which is a chromosomally encoded virulence factor associated with adhesion properties, was not detected in all *E.coli* isolates in this study. Our results are comparable to those of studies from Algeria and Jordan [50, 51], which reported a low frequency of detection of *papC* in APEC strains. A high frequency of *astA*, which encodes a heat-stable toxin, in avian fecal *E. coli* strains was detected in a previous study [48]; this gene might be used as an indicator for non-pathogenic *E.coli* strains or to distinguish between intestinal *E. coli* and APEC strains [3]. However, a study in northern Iran detected *astA* in both ExPEC and APEC strains [52]. In contrast to these findings, our study revealed the presence of *astA* in *E.coli* from both diseased and healthy broilers, with no association between *astA* presence and *E.coli* strains. In addition, *tsh* is involved in adhesion and protease activity during the early stage of infection of host cells. This gene was detected in *E.coli* from both broiler groups in our study, which is inconsistent with other findings showing the presence of *tsh* only in APEC strains [53, 54]. This inconsistency suggests that further investigation is needed.

Six virulence genes, namely, *cva/cvi*, *iutA*, *iucD*, *iroN*, *iss*, *ompT* were detected at significantly greater frequencies in *E.coli* isolated from diseased than in healthy broilers. The plasmid-mediated virulence genes *cva/cvi*, *hlyF*, *iutA*, *iucD*, *iroN*, and *iss* are commonly located on the ColV plasmid, which is a large plasmid carrying many virulence genes, and often occur in APEC strains [55–57]. In addition to plasmid-mediated virulence factors, chromosomal OmpT is frequently detected in *E.coli* recovered from diseased birds. *ompT*, encoding an outer membrane protease or OmpT, is located on the outer membrane of *E. coli* and other gram-negative bacteria. OmpT plays a role in proteolytic activity, hydrolyzing antimicrobial peptides produced by host cells as a defense mechanism against pathogens [58]. Therefore, *ompT* is likely involved in pathogenicity and is predominantly found in APEC strains.

Conclusions

Our study highlighted the diversity of phylogroups and distribution of virulence genes in *E. coli* isolates from healthy and diseased broilers with colibacillosis in Thailand. Interestingly, our findings revealed low prevalence of resistance to cephalosporins in *E. coli* strains isolated from broilers. This is positive for preserving these critical antibiotics for human medicine. However, high rates of multidrug resistance to other antibiotics were observed in *E.coli* isolates form diseased and healthy broilers. This result suggests that although the use of antibiotics in livestock production has been restricted in the last several years, continued monitoring of the prevalence of avian pathogenic and nonpathogenic *E. coli* strains and AMR is essential. This updated data will promote antibiotic stewardship, reducing the inappropriate use of antibiotics with high resistance rates (such as amoxicillin) and encouraging the adoption of alternative strategies like

farm biosecurity and improved chicken health for colibacillosis control in broiler production. Additionally, developing vaccines targeting prevalent virulence genes offers a promising approach to minimize inappropriate antibiotic use and control colibacillosis in Thai broiler farms in the future. Moreover, tracking the emergence of new *E. coli* strains and their AMR mechanisms is crucial, as the spread of AMR from food-producing animals to humans and the environment poses a significant public health risk.

Methods

Sample collection

During 2019–2022, 211 *E. coli* isolates were recovered from diseased ($n = 110$) and healthy ($n = 101$) broiler chickens raised on 27 commercial broiler farms in central Thailand. Necropsy and diagnostic sampling were performed at the Kampheangsean Veterinary Diagnostic (KVDC) Unit, Faculty of Veterinary Medicine, Kasetsart University, Kampheangsean Campus, Nakorn Pathom. Dead or moribund birds with depression, ruffled feathers, or respiratory distress were necropsied, and tissue samples were collected from visceral organs, such as the heart, liver, or air sac, with fibrinous serositis lesions. Among diseased birds, the number of *E. coli* isolates per farm ranged from 1 to 10, depending on the number of birds with fibrinopurulent lesions. A single colony was randomly selected from each positive sample to confirm *E. coli* isolation. Among healthy birds, we employed simple random sampling to select 100 *E. coli* isolates from our existing bacterial stock of 650 isolates. This stock originated from cloacal swabs collected from healthy broilers raised on three participating farms with established health monitoring programs. A single swab was collected per bird and cultured for *E. coli*. Isolates were confirmed by randomly selecting 3–5 colonies from a positive sample. The confirmed *E.coli* colonies were stored at -80 °C for further experiments. The birds were placed in a gas-filled container using carbon dioxide at high concentration for euthanasia following AVMA guidelines for the euthanasia of animals (2020) [59]. All samples were aseptically collected and delivered to the microbiology laboratory for bacterial isolation and identification within 1 h of sample collection.

E. coli isolation and identification

Samples were cultured on blood agar and MacConkey agar (HI-media®) and incubated at 37 °C for 24 h. Typical colonies were restreaked on eosin methylene blue agar and incubated for 24 h at 37 °C. Suspect colonies on eosin methylene blue agar were identified by gram-staining and biochemical tests, such as indole, urease, motility, and triple-iron sugar, according to clinical veterinary microbiology guidelines (2014) [60]. Pure colonies were collected and stored in LB broth supplemented with 20% glycerol and stored at – 80 °C until use.

Antimicrobial susceptibility testing and detection of ESBL strains

All *E. coli* isolates were subjected to antimicrobial susceptibility testing using the Kirby–Bauer disk diffusion method, according to CLSI (2018) guidelines 4th edition [61]. We used nine antibiotics. The antibiotic concentration and their clinical breakpoints were as follows: ampicillin (10 µg, ≤ 13 mm), amoxicillin (10 µg, ≤ 13 mm), gentamicin (10 µg, ≤ 12 mm), enrofloxacin (5 µg, ≤ 16 mm), ciprofloxacin (5 µg, ≤ 15 mm), chloramphenicol (30 µg, ≤ 12 mm), tetracycline (30 µg, ≤ 11 mm), sulfatrimethoprim (25 µg, ≤ 10 mm), and streptomycin (10 µg, ≤ 11 mm). *Pseudomonas aeruginosa* ATCC 27,853, *Staphylococcus aureus* ATCC 25,923, and *E. coli* ATCC 25,922 were used as reference bacterial strains. The breakpoint for enrofloxacin in chicken-derived *E. coli* was established. Breakpoints for the other tested antibiotics followed those established for human-derived *E. coli*.

All *E. coli* isolates were screened for ESBL production using the disk diffusion method [61]. The positive isolates from the screening test were subjected to phenotypic confirmatory tests using cephalosporins or clavulanic acid combination disks. Susceptibility testing and determination of inhibitory zone diameters were performed and evaluated according to CLSI guidelines, 2018.

DNA extraction, oligonucleotide primers, design, and DNA sequencing

Bacterial genomic DNA was extracted from *E. coli* isolates using the boiled whole-cell lysate method [62]. All primers used are listed in Supplemental Table 3. Positive PCR products obtained from virulence genes, AMR genes, and phylogenetic group analysis were purified using MEGAquick-spin Plus (iNtRON Biotechnology, South Korea) and confirmed by DNA sequencing (Bionics, Republic of Korea). Sequence data were confirmed by comparison with the GenBank database using the Basic Local Alignment Search Tool (BLAST) software.

Determination of virulence genes and AMR genes of *E. coli* isolates

Since reports on the detection of virulence genes in *E. coli* strains found in Thailand are still limited, this study aimed to identify virulence-associated genes that have been frequently reported in *E. coli* strains from various regions and are linked to different virulence characteristics. We identified 11 virulence genes, namely, *astA*, *cva/cvi*, *hlyF*, *iucD*, *OmpT*, *papC*, *tsh*, *iroN*, *irp-2*, *iss*, and *iutA*, as previously described [13]. PCR was performed in a final volume of 25 µL, containing 12.5 µL of 2× DreamTaq Green PCR Master Mix (Thermo Scientific), 0.2 µM

each primer, and 10 ng of DNA template. The thermal cycling conditions for virulence genes were as follows: initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 2 min, and extension at 72 °C for 7 min; and a final extension at 72 °C for 7 min. PCR amplicons were analyzed by gel electrophoresis with 2% (w/v) agarose gels. Suspected PCR-positive isolates were confirmed by DNA sequencing. All *E. coli* isolates were examined for the presence of 32 ARGs, corresponding to seven antibiotic classes. PCR was performed as previously described [63]. Positive controls for each resistance gene, as previously described [63], were included in each PCR.

Phylogenetic group analysis

The phylogenetic groups of *E. coli* were determined using the Clermont *E. coli* phylogenetic typing protocol [64]. The *E. coli* strains were classified into 8 groups, namely, A, B1, B2, C, D, E, F, and *E. coli* clade I, depending on the presence of four target genes (*ChuA*, *yjaA*, *arpA*, and *trpA*) and a DNA fragment (TspE4.C2).

Statistical analysis

The statistical analysis data were analyzed with R version 4.2.3 [65]. The correlation between antimicrobial resistance, phylogenetic groups, and virulence gene profiles in *E.coli* isolates from diseased and healthy broilers were calculated by Fisher's exact test. The results with $P<0.05$ were considered to indicate statistical significance.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04626-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Acknowledgements

The authors would like to acknowledge Kamphaeng Saen Veterinary Diagnostic Center (KVDC), Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen campus for support of this research.

Author contributions

Nuananong Sinwat designed the study and interpreted the experimental results. Sudtisa Laopiem conducted all laboratory procedures and managed the data collection. Kriangkrai Witoonsatian, Sittinee Kulprasetsri, Pun Panomwan, Chutima Pathomchai-umporn, Raktipon Kamtae and Thaweesak Songserm participated in sample collection. Pichai Jirawattanapong participated in the statistical analysis. Sudtisa Laopiem wrote the first draft of the manuscript. Nuananong Sinwat and Thaweesak Songserm edited the manuscript and provided feedback. Nuananong Sinwat revised the manuscript. All the authors have read and approved the final manuscript.

Funding

This work was financially supported by the Kasetsart Veterinary Development Funds Faculty of Veterinary Medicine, Kasetsart University, and partially funded by the National Research Council of Thailand (NRCT) Project ID N42A660897.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All procedures for sample collection from diseased and healthy broilers in this study were approved by the KASETSART UNIVERSITY Institutional Animal Care and Use Committee (ACKU62-VET-040) and found to be in accordance with the guidelines of animal care and use established by the Ethical Review Board of the Office of National Research Council of Thailand (NRCT) for the conduct of scientific research. The committee approved and permitted the animal care and use to be conducted as outlined in the research study and animal use protocol.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 28 March 2024 / Accepted: 24 February 2025
Published online: 08 March 2025

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