*Original Article*

# Prevalence of the Cellular and Molecular Antimicrobial Resistance against *E. coli* Isolated from Thai Broilers

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***Abstract***

The antimicrobial resistant characteristics both of the cellular and molecular levels of *Escherichia coli* in broilers were performed by disk diffusion test and polymerase chain reaction technique. Specific primers for the *aadA, aac(3)-IV, cmlA, cat1, tetA, sul1, SHV, CITM, ereA* and *dhfrV* gene were used to detect resistance to gentamicin, chloramphenicol, tetracycline, cephalothin, ampicillin, erythromycin, sulfonamide+trimetroprim. The 30 samples of *E. coli* were found to be 100% antimicrobial disk resistant to tetracycline, ampicillin and erythromycin and revealed a 90%, 93.3% and 73.3% resistant gene to *tetA, CITM* and *ereA*, respectively. While resistance to 73.3% cephalothin and 26.7% sulfonamide+trimethoprim was revealed by disk diffusion tests, the 86.4% *SHV*, 100% *sul1* and 100% *dhfrV* genes were found from PCR techniques. However, no antimicrobial resistance both in the cellular and molecular levels of gentamicin and chloramphenical were found.

**Keywords:** Antimicrobial resistant gene, *Escherichia coli*, poultry, sensitivity test

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# บทคัดย่อ

## ความชุกของการดื้อยาต้านจุลชีพทางเซลล์และทางโมเลกุลของ *อี. โคไล* ที่แยกได้จากไก่เนื้อในประเทศไทย

*สราวุธ มูลจันที*[1] *ปิยะรัตน์ จันทร์ศิริพรชัย*[2] *นิวัตร จันทร์ศิริพรชัย*[1*]

คุณลักษณะการดื้อยาต้านจุลชีพทั้งในระดับเซลล์และระดับโมเลกุลของ *อี. โคไล* ในไก่เนื้อถูกศึกษาด้วยวิธี Disk diffusion test และ Polymerase chain reaction ไพรเมอร์เฉพาะต่อยีน *aadA*, *aac(3)-IV*, *cmlA*, *cat1*, *tetA*, *sul1*, *SHV*, *CITM*, *ereA* และ *dhfrV* ใช้ในการตรวจหาการดื้อต่อเจนตามัยซิน คลอแรมฟินิคอล เตตราซัยคลิน เซฟฟาโลธิน แอมพิซิลลิน อิริโธรมัยซิน ซัลโฟนามายด์และไตรเมธโธปริม *อี. โคไล* จำนวน 30 ตัวอย่าง พบการดื้อร้อยละ 100 ต่อเตตราซัยคลิน แอมพิซิลลิน และอิริโธรมัยซิน และ ร้อยละ 90, 93.3 และ 73.3 พบยีนดื้อ *tetA*, *CITM* และ *ereA* ตามลำดับ ในขณะที่พบการดื้อร้อยละ 73.3 ต่อเซฟฟาโลธิน และร้อยละ 26.7 ต่อซัลโฟนามายด์และไตรเมธโธปริม ด้วยวิธี Disk diffusion tests และร้อยละ 86.4 พบยีนดื้อ *SHV* ร้อยละ 100 พบยีนดื้อ *sul1* และ *dhfrV* ด้วยเทคนิคพีซีอาร์ อย่างไรก็ตามไม่พบการดื้อยาทั้งในระดับเซลล์และระดับโมเลกุลต่อยาเจนตามัยซินและคลอแรมฟินิคอล

**คำสำคัญ:** ยีนดื้อยาต้านจุลชีพ *เอสเชอริเชีย โคไล* สัตว์ปีก การทดสอบความไวของเชื้อ

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### *Introduction*

Colibacillosis is an infectious disease caused by *Escherichia coli* (*E. coli*). Colibacillosis is common and can be seen in poultry flocks worldwide especially in intensive farming systems and causes major economic losses (Chansiripornchai, 2009). Colibacillosis affects many systems of poultry. Cellulitis is frequently seen in the skin infection. Anyhow, the major clinical sign of *E. coli* infection in birds is a respiratory disease which in cases of severe infection can lead to septicemia and death. Colibacillosis in poultry frequently occurs after vaccination against respiratory diseases such as Newcastle Disease and infectious bronchitis. Avian colibacillosis primarily affects broiler chickens between the ages of 4 and 5 weeks and 0.25% to 10 % mortality can be found in poultry flocks after infection (Shane, 1981). Mortality, the cost of treatment and the decrease in feed conversion efficiency result in a significant cost to the poultry industry (Chansiripornchai and Sasipreeyayan, 2002). Other clinical signs of colibacillosis are respiratory distress, reduced appetite and poor growth. The lesions seen at post mortem are airsacculitis, pericarditis, perihepatitis, peritonitis, salpingitis, synovitis, panophthalmitis, coligranuloma, omphalitis and yolk sac infection. The predisposing factors of colibacillosis can be influenced by management and environmental conditions such as temperature, humidity and high concentrations of ammonia. Drinking water and dust in poultry houses also contributes to respiratory stress. The disease can transfer to embryonic infection.

Colibacillosis can be prevented and controlled using antibiotics to treat the bacterial infection and to eliminate some predisposing causes especially Mycoplasmal and other bacterial infections. However, vet practitioners have a limited choice of antimicrobials for use in the poultry industry, due to antimicrobial resistance issues and human health concerns. Moreover, the repeated and unsuitable use of antibiotics has led to an increasing rate of antimicrobial resistance (Chansiripornchai, 2009). Performing antimicrobial sensitivity test priors to application will help promote drug efficacy. In Thailand, Chansiripornchai et al. (1995) reported that more than 80% of isolates were resistant to nalidixic acid, oxolinic acid, sulfamethoxasole+trimethoprim, sulfadiazine, oxytetracycline, tetracycline, kanamycin, novobiocin and erythromycin and antibacterial resistance was low to the third generation of the quinolone group such as norfloxacin, danofloxacin and enrofloxacin during 1990-1995. The selection pressure exerted by antibiotics will promote the antimicrobial resistant rate. The antimicrobial resistant characteristics at the cellular level are encoded by specific genes that can be expressed in a suitable environment. *E. coli* has bacterial diversity because it survives as a common flora in the gastrointestinal and respiratory tract of chickens and other animals. Recently, molecular techniques especially polymerase chain reaction (PCR) have been widely used to study the antimicrobial resistant genes. Herein, we study the prevalence of

antimicrobial resistant characteristics both in the cellular and molecular levels by using an antimicrobial disc sensitivity test and PCR techniques from the E. coli that were isolated from Thai broilers.

## *Materials and Methods*

***Sampling collection and E. coli identification:*** The thirty samples of cloacal swabs were isolated from broiler farms in Central (Ayudhya, Ratchaburi and Saraburi provinces) and Eastern (Chonburi and Prachinburi provinces) of Thailand that had a history of colibacillosis. The sampling farms had been free from antimicrobial application for at least 7 days. The cloacal swabs were cultured on 5% sheep blood and MacConkey agar (Oxoid, Hampshire, UK) and incubated for 18 to 24 hrs at 37°C. The colony with the typical color and appearance of *E. coli* was picked and streaked again on blood agar plates, then re-streaked on EMB agar (Oxoid, Hampshire, UK). The green metallic sheen isolates were considered to be *E. coli* and the presumptive colonies were biochemically tested with TSI, OF, LIA, CIT, Urea, Indole, MR and Motility (Bauer et al., 1974). The E. coli isolates were stored in tryptic soy broth (Oxoid, Hampshire, UK) with 15% glycerol at -20°C.

***Antimicrobial susceptibility tests:*** All isolates of the *E. coli* samples were tested for sensitivity following the Bauer Kirby disk diffusion (Bauer et al., 1966). Briefly, a McFarland 0.5 standardized suspension of bacteria was swabbed over the surface of an agar plate, and paper disks containing single concentrations of each antimicrobial agent were placed onto the inoculated surface (Oxoid, Hampshire, UK). Following overnight incubation, the diameters of the zones produced by the antimicrobial inhibition of bacterial growth were measured and the isolate was interpreted as either susceptible, intermediate or resistant to a particular drug according to preset criteria (NCCLS, 2002).

***Antibiotic resistant gene detection:*** Template DNAs for PCR were extracted by the boiling method described previously (Millar et al., 2000). Fresh cultures of *E. coli* isolates were suspended in 1 ml of PBS and boiled at 95°C for 10 min. After centrifugation at 14,000x g for 2 min, the supernatants were collected and stored at -20°C until use. The resistant genes of *E. coli* were investigated using multiplex PCR assay. PCR primers specific to these genes were designed based on the gene sequence information in the GenBank database and in previous published studies (Van et al., 2008). Conserved sequences of each gene were selected and a set of primers was used for each gene (Table 1). PCR reactions for multiplex PCR were performed in a total volume of 25 µl containing DNA template, 10x PCR buffer, 10 mM dNTPs, forward and reverse primers and Taq DNA polymerase (Promega, USA). The PCR condition was followed by 30 cycles of denaturation at 94 amplification at 72°C for 10 min. Amplicons were visualized by electrophoresis at 80v in 2% agarose gel.

**Table 1** *Escherichia coli* antimicrobial resistant genes and primer sequences used for PCR identification

| Gene | Antibiotic type | Primers* | Primer 5′-3′ | Annealing temperature (°C) | PCR products (bp) |
|---|---|---|---|---|---|
| *aadA* | Gentamicin | aadA(F) | TGATTTGCTGGTTACGGTGAC | 55 | 284 |
| | | aadA(R) | CGCTATGTTCTCTTGCTTTTG | | |
| *cmlA* | Chloramphenicol | cmlA(F) | CCGCCACGGTGTTGTTGTTATC | 55 | 698 |
| | | cmlA(R) | CACCTTGCCTGCCCATCATTAG | | |
| *aac(3)-IV* | Gentamicin | aac(3)-IV(F) | CTTCAGGATGGCAAGTTGGT | 55 | 286 |
| | | aac(3)-IV(R) | TCATCTCGTTCTCCGCTCAT | | |
| *cat1* | Chloramphenicol | CATI(F) | AGTTGCTCAATGTACCTATAACC | 55 | 547 |
| | | CATI(R) | TTGTAATTCATTAAGCATTCTGCC | | |
| *tetA* | Tetracycline | tet(A)(F) | GTGAAACCCAACATACCCC | 57 | 887 |
| | | tet(A)(R) | GAAGGCAAGCAGGATGTAG | | |
| *Sul1* | Sulfonamide+ Trimethoprim Cephalothin | Sul1(F) | TTCGGCATTCTGAATCTCAC | 47 | 822 |
| | | Sul1(R) | ATGATCTAACCCTCGGTCTC | | |
| SHV | | blaSHV(F) | TCGCCTGTGTATTATCTCCC | 52 | 768 |
| | | blaSHV(R) | CGCAGATAAATCACCACAATG | | |
| CITM | Ampicillin | CITM(F) | TGGCCAGAACTGACAGGCAAA | 47 | 462 |
| | | CITM (R) | TTTCTCCTGAACGTGGCTGGC | | |
| *ereA* | Erythromycin | ereA(F) | GCCGGTGCTCATGAACTTGAG | 52 | 419 |
| | | ereA(R) | CGACTCTATTCGATCAGAGGC | | |
| *dhfrV* | Trimethoprim | dhfrV(F) | CTGCAAAAGCGAAAAACGG | 47 | 432 |
| | | dhfrV(R) | AGCAATAGTTAATGTTTGAGCTAAAG | | |

*F: forward primer, R: reverse primer

## *Results*

The antimicrobial susceptibility tests revealed no antimicrobial resistant to gentamicin and chloramphenicol. High percentages of antimicrobial resistance were found in ampicillin, erythromycin, tetracycline and cephalothin. Also, no antimicrobial resistant genes related to gentamicin and chloramphenical were found in any tested isolates. On the contrary, ampicillin, erythromycin and

tetracycline reveal 100% of specific antimicrobial resistant genes. Cephalothin showed 73.3% antimicrobial resistance by disk diffusion tests and 86.4% of specific antimicrobial resistant genes were found in these isolates. Sulfonamide+trimethoprim revealed 26.7% of antimicrobial resistance and 100% of *sul1* and *dhfrV* genes were found that were related to sulfonamide+trimethoprim resistant characteristics, respectively (Table 2).

**Table 2** Antimicrobial susceptibility tests and the presence of the antimicrobial resistance gene from PCR detection in 30 samples of *E. coli* isolates

| Antimicrobials | Genes | No. of antimicrobial resistance of disk diffusion test (%) | No. of positive gene detection in positive antimicrobial resistant samples (%) |
|---|---|---|---|
| Gentamicin | *aadA* | 0 (0) | 0 |
| Gentamicin | *aac(3)-IV* | 0 (0) | 0 |
| Chloramphenicol | *cmlA* | 0 (0) | 0 |
| Chloramphenicol | *cat1* | 0 (0) | 0 |
| Tetracycline | *tetA* | 30 (100) | 27 (90) |
| Sulfonamide +Trimethoprim | *Sul1* | 8 (26.7) | 8 (100) |
| Sulfonamide +Trimethoprim | *dhfrV* | 8 (26.7) | 8 (100) |
| Cephalothin | *SHV* | 22 (73.3) | 19 (86.4) |
| Ampicillin | *CITM* | 30 (100) | 28 (93.3) |
| Erythromycin | *ereA* | 30 (100) | 22 (73.3) |

## Discussion

In this study, 30 isolates of *E. coli* were identified and studied from broilers. The antibiotic susceptibility tests revealed one hundred percentages antimicrobial resistance to the ampicillin, erythromycin and tetracycline that are frequently used in the Thai broiler industry. This is as opposed to, a zero percentage of antimicrobial resistance of gentamicin and chloramphenical, these 2 kinds of antimicrobial agents are less applied in Thai poultry industry. Because gentamicin presents only an injection preparation it is not practically used in the broiler industry and chloramphenical had been banned from Thai livestock industry for more than 10 years. Cephalothin is not frequently used in the Thai broiler industry but it is in the same group and mechanism of action as ampicillin so higher percentages of antimicrobial resistance can be expected. Compared to the current results, Chansiripornchai et al (1995) reported the antimicrobial resistant profiles of *E. coli* in the Thai poultry industry of ampicillin, erythromycin and tetracycline showing an equal or higher percentage of antimicrobial resistance following 42, 100 and 96, respectively. In the current results, sulfonamide+trimethoprim showed a lower antimicrobial resistance than the previous report (83.52%). This result accords to the reduction of sulfonamide+trimethoprim application in Thai broiler industry. The results of antimicrobial resistant genes are in accord with the antimicrobial sensitivity test. More than 73% of antimicrobial resistant genes were found in each specific antimicrobial resistant isolate. Also, no antimicrobial resistant genes were found in any isolates that showed the negative results of antimicrobial resistant to the disks of antimicrobial agents. Anyhow, some isolates that showed antimicrobial resistance to the sensitivity disks did not occupy the specific antimicrobial resistant genes. This means that there are more antimicrobial resistant genes that are responsible for the expression of antimicrobial characteristics. Compared to the previous report, the prevalence of antimicrobial resistant genes in poultry *E. coli* is quite varied. It may depend on the geographical distribution and antimicrobial usage in each area. Van et al. (2008) reported the antimicrobial resistant genes (*tetA, aadA, cmLA, aac3, cat1, sul1* and *dhfrV*) of *E. coli* isolated from Vietnam were 81.0, 81.0, 61.9, 23.8, 14.3, 27.1 and 19.1%, respectively. While, Costa et al. (2009) reported the antimicrobial resistant genes (*tetA, aadA, cmLA* and *sul1*) of *E. coli* isolated from Protugal were 41.1, 70.6, 8.6 and 23.5%, respectively. In conclusion, *E. coli* isolated from Thai broilers showed a variation on antimicrobial resistant characteristics both at cellular and genetic levels. There are many genes involved in the genetic expression of antimicrobial characteristics. These characteristics mainly belong to the antimicrobial application in the current situation. More antimicrobial resistant profiles should be studied both at the cellular and molecular level to suggest to practitioners effective antimicrobial applications and to lessen the suffering of infected poultry.

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