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Research article

Prevalence and characteristics of extended spectrum beta-lactamase-producing *Escherichia coli* isolated from flies in rural Sakon Nakhon, Thailand

Thachawech Kimprasit[a,*], Sugunya Kumla[b], Srisuda Sirilaophaisan[a], Sirapan Sukontasing[c], Eiichi Hondo[d], Atsuo Iida[d], Chatchai Kaewpila[a], Pongsatorn Gunun[a]

[a] *Department of Animal Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus, Sakon Nakhon 47160, Thailand*

[b] *Department of Fisheries, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakon Nakhon Campus, Sakon Nakhon 47160, Thailand*

[c] *Department of Veterinary Technology, Faculty of Veterinary Technology, Kasetsart University, Bangkok 10900, Thailand*

[d] *Laboratory of Animal Morphology, Graduate School of Bioagricultural Sciences, Nagoya University, Aichi 464-8601, Japan*

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Abstract

Importance of the work: *Escherichia coli* that synthesize extended spectrum beta-lactamase (ESBL) could indeed cause major public health issues globally. Flies have been identified as a significant vector for spreading ESBL-producing *E. coli* to humans.

Objectives: To determine the prevalence and characteristics of ESBL-producing *E. coli* in flies from rural Sakon Nakhon.

Materials & Methods: *E. coli* were isolated from flies gathered in Sakon Nakhon's rural areas. Antimicrobial sensitivity profiles, ESBL using the disk diffusion technique and a combination disk assay were all investigated in the microorganism. Furthermore, ESBL-encoded genes were studied using polymerase chain reactions (PCRs) with specific primers for each gene.

Results: From 108 flies, 104 *E. coli* isolates were obtained. Based on the standard disk diffusion assay, the *E. coli* were resistant to cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone at 94.13%, 95.92%, 82.37%, 78.67% and 92.06%, respectively. The ESBL enzymes of *E. coli* were studied that competed with beta-lactam antibiotics. The combination disk method, which included a combination of cefotaxime-clavulanic acid and cefotaxime, and another combination of ceftazidime-clavulanic acid and ceftazidime, showed that all isolates could synthesize ESBL enzymes. Amplification of the *E. coli* ESBL-encoded genes resulted in 51.02% carrying bla_{TEM} and 25.51% carrying bla_{SHV}. The PCR analysis did not detect the genes bla_{VEB}, $bla_{\text{OXA-1}}$ or $bla_{\text{CTX-M}}$.

Main finding: The flies carried ESBL-producing *E. coli*. As a result, the dispersion of ESBL-producing *E. coli* by flies to public venues should be carefully monitored.

* Corresponding author.
E-mail address: thachawech.ki@rmuti.ac.th (T. Kimprasit)

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Introduction

The *Enterobacteriaceae* is a group of normal floral bacteria that attaches to both the human and animal intestines and is important as an indicator that the bacteria of this group, especially *Escherichia coli,* can contaminate several food and agricultural products (Yang et al., 2017). *E. coli* does not usually cause disease; however, if *E. coli* infects an animal in the gastrointestinal tract, urinary tract or a wound, it is able to cause serious health complications in the infected animal (Lee et al., 2018). The type or types of toxin generated determines the severity of the disease and symptoms; *E. coli* can grow and contaminate anywhere, especially in animal husbandry areas and it transfer from animals via cross contamination of animal excreta to food and beverages (Isler et al., 2020).

In humans and animals, beta-lactam drugs are used to treat bacterial infections (Han et al., 2015; Sarrazin et al., 2018). However, as a result of such antimicrobial therapy, antibacterial-resistant bacteria have developed in the human gastrointestinal system (Kelly et al., 1998). The capacity of Gram-negative bacteria to produce beta-lactamase enzyme, which destroys the antibiotics, is the main mechanism in the resistance of beta-lactam antimicrobials. It has been known for decades (Turner, 2005) that *E. coli* develops additional enzyme to break down beta-lactam drugs, known as extended spectrum beta-lactamase (ESBL). Incidences of ESBL-resistant *E. coli* isolated from pet animals, pigs and animal products have been documented globally (Dierikx et al., 2013; Hanhaboon et al., 2015; Alegría et al., 2020; Guo et al., 2021; Kim et al., 2021; Yiğin, 2021). *E. coli* isolated from flies produced ESBL enzymes (Blaak et al., 2014; Wetzker et al., 2019; Punyadi et al., 2021).

More than 300 different ESBL types in Gram-negative bacteria have been discovered (Yezli et al., 2015, Parajuli et al., 2016). Of the ESBL genes, the *bla* are the most prevalent that lead to antibiotic resistance in pathogenic bacteria worldwide (Dirar et al., 2020). Studies have revealed that there has also been a rise in the frequency of ESBL-producing *E. coli* containing the *bla* gene types during the past few years including bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-1}}$ and bla_{VEB} (Seenama et al., 2019; Al-Ouqaili et al., 2020).

Flies are another important vector that might allow transmission between animals and humans (Blaak et al., 2014; Geden et al., 2021) because they are able to move among carcasses, excrement, animals and animal products. Furthermore, flies have been recorded to travel up to 7 km (Nazni et al., 2005). Thus, the prospect of microbes being transferred from flies to humans and other animals by bodily contact or via feces or both might be a viable alternative indirect transmission pathway (Overdevest et al., 2011; Dierikx et al., 2013).

Current knowledge suggests that the study is still limited on the ESBL enzymes from *E. coli* isolated from animal flies in Thailand. Consequently, the goals of this study were to isolate *E. coli* from flies on livestock farms and in fresh markets, a slaughterhouse and a retail food shop in rural areas of Sakon Nakhon province, Thailand where human residences are nearby livestock farms and to determine their ability to produce ESBL enzymes as the base information for managing excessive antimicrobial usage in industrial animal production.

Materials and Methods

Flies sampling and identification

The equation of Thrusfield (2007) was applied to determine the sample size as shown in Equation 1:

$$n = z^{2}\, p\,(1-p) / i^{2} \qquad (1)$$

where *n* is the determined sample size, *z* is the appropriate degree of confidence (1.96), *i* is the standard sampling error (10%) and *p* is the 50% estimated prevalence. Flies were collected using nontoxic sticky flypaper from two swine farms, two dairy farms, two fresh markets, one slaughterhouse and one retail food shop located in a rural area of Sakon Nakhon province, Thailand and harvested within 24 h after placement. Distances among sampling sites were less than a radius of 5 km. The flies were stored in sterile containers and species identification was performed under a microscope following the guideline of Sawaby et al. (2018). The procedures were approved and authorized by the Institutional Animal Care and Use Committee Rajamangala University of Technology Isan (Approval no. 9/2565).

Isolation and detection of E. coli

The flies were inoculated in tubes containing phosphate-buffered saline and transferred to sterile filter bags. The single fly in each bag was homogenized using a stomacher machine at 230 rpm for 5 min. The supernatant from the homogenate was streaked over eosin-methylene blue agar for *E. coli* isolation and then incubated at 37 °C for 18–24 h. The metallic sheen on a colony was used as a distinguishing feature and Gram staining was used to examine its morphology. IMViC (indole, methyl red, Voges-Proskauer (VP) and citrate) assays (Antony et al., 2016) were performed on the green metallic sheen colonies with Gram negative and rod morphologies applied to assess their biochemical properties. Colonies that tested positive for indole and methyl red but negative for citrate and VP were selected and kept for further study in a culture medium broth containing 10% glycerol at -80 °C.

Detection of extended spectrum beta-lactamase using agar disk diffusion assay

The standard disk diffusion assay was used to determine the antimicrobial susceptibility profiles of the isolated *E. coli* according to the guidelines of the Performance Standard for Antimicrobial Susceptibility Testing M100-S24 (Clinical and Laboratory Standards Institute, 2014). Five antimicrobial agents were investigated: cefpodoxime (10 µg), cefotaxime (30 µg), aztreonam (30 µg), ceftazidime (30 µg) and ceftriaxone (30 µg). The control bacteria were *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603. The zone diameters were measured after 18 h of incubation at 37 °C. The strains resistant to the five beta-lactam drugs were identified as the expected ESBL-producing isolates.

Detection of extended spectrum beta-lactamase using combination disk assay

Following CLSI guidelines, the bacteria resistant to the antimicrobial agents used in the disk diffusion method were subjected to a phenotypic confirmatory test using a combination disk assay (Clinical and Laboratory Standards Institute, 2014). Diameter zones were measured for 30µg/mL of cefotaxime, 30µg/mL of ceftazidime, 30/10 µg/mL of cefotaxime plus clavulanic acid and 30/10µg/mL of ceftazidime plus clavulanic acid. Isolates resistant to cefotaxime or ceftazidime or both and with a reduction zone diameter ≥ 5mm with a disk of clavulanic acid were defined as ESBL-producing strains.

Detection of extended spectrum beta-lactamase encoded genes

Polymerase chain reaction (PCR) has been used to amplify the encoded genes of ESBL (*bla* $_{VEB}$, *bla* $_{SHV}$, *bla* $_{OXA-1}$, *bla* $_{TEM}$ and *bla* $_{CTX-M}$). DNA extraction kits were used to extract the bacterial DNA (InvitrogenTM; USA). Then, ESBL gene amplification was performed in a reaction volume of 50 µL. Each reaction combination corresponded to the Takara Bio Inc., Japan, procedure. The target genes were amplified using the thermal cycle (GeneAmp®, PCR System 9700; Applied Biosystems; Singapore) under ideal cycling conditions: initial heat activation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min each, annealing temperatures that varied for each primer according to Table 1 for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Genomic DNA from previously examined isolates of *E. coli* that had previously tested positive for the relevant genes served as positive controls.

Results

Prevalence of flies and E. coli on flies

For the experiment, a sample size of 96 was anticipated. However, up to 108 flies were captured for the study to enhance the accuracy, with 43 from swine farms, 29 from dairy farms, 16 from fresh markets, 10 from a slaughterhouse and 10 from a retail food shop. Of these, 88 flies were identified as *Musca domestica* (common housefly), while the remaining 20 flies were *Chrysomya megacephala* (Oriental latrine fly), as shown in Table 2. With the exception of three flies from swine farm A and one fly from dairy farm A, 104 *E. coli* isolates (96.30%) were isolated from all fly samples.

Table 1 Primers used for amplification of extended spectrum beta-lactamase genes

Target gene	Primer	Primer sequence (5'-3')	Annealing temperature (°C)	Reference
bla $_{TEM}$	TEMF	ATGAGTATTCAACATTTCCGTG	55	Garcia et al. (2008)
	TEMR	TTACCAATGCTTAATCAGTGAG		
bla $_{SHV}$	SHV S1	ATTTGTCGCTTCTTTACTCGC	55	Garcia et al. (2008)
	SHV S2	TTTATGGCGTTACCTTTGACC		
bla $_{CTX-M}$	CTX-M/F	TTTGCGATGTGCAGTACCAGTAA	51	Edelstein et al. (2003)
	CTX-M/R	CGATATCGTTGGTGGTGCCATA		
bla $_{OXA-1}$	OXA-1F	TTTTCTGTTTGGGTTTT	52	Bert et al. (2002)
	OXA-2R	TTTCTTGGCTTTTGTGCTTG		
bla $_{VEB}$	VEBF	CGACTTCCATTTCCCGATGC	55	Hanhaboon et al. (2015)
	VEBR	CGACTTCCATTTCCCGATGC		

Table 2 Number of fly samples, number of isolated *E. coli*, their origins and fly identification

Origin	Number of flies	Fly Identification	Number of isolated *E. coli*
Swine farm A	10	*Musca domestica*	7
Swine farm B	33	*Musca domestica*	33
Dairy farm A	9	*Musca domestica*	8
Dairy farm B	20	*Musca domestica*	20
Slaughterhouse	10	*Chrysomya megacephala*	10
Fresh market A	6	*Musca domestica*	6
Fresh market B	10	*Chrysomya megacephala*	10
Retail food shop	10	*Musca domestica*	10
Total	108	-	104

Extended spectrum beta-lactamase-producing E. coli prevalence

Standard disk diffusion was used to screen the antimicrobial sensitivity profiles of all 104 *E. coli*. As shown in Table 3, 98 *E. coli* were resistant to the antimicrobial disks in the current study, with varying percentages depending on the targeted drugs: cefpodoxime (94.13%), ceftazidime (95.92%), aztreonam (82.37%), cefotaxime (78.67%) and ceftriaxone (92.06%).

A phenotypic confirmatory test using the combination disk method was carried out on all *E. coli* that were resistant to the antimicrobials used in the disk diffusion assay. All the isolates (100%) produced ESBL enzymes against a combination of cefotaxime-clavulanic acid and cefotaxime as well as another combination of ceftazidime-clavulanic acid and ceftazidime. Then, the ESBL-encoded genes in *E. coli* were investigated further.

Extended spectrum beta-lactamase-encoded genes in E. coli isolates able to produce extended spectrum beta-lactamase

In total, 98 ESBL-producing *E. coli* isolates were examined for their ESBL genes, consisting of genes encoded by *bla* $_{\text{CTX-M}}$, *bla* $_{\text{TEM}}$, *bla* $_{\text{SHV}}$, *bla* $_{\text{VEB}}$ and *bla* $_{\text{OXA-1}}$. The results revealed that 50 isolates (51.02%) carried the CTX-M gene and 25 isolates (25.51%) carried the SHV gene. None of the isolates examined had the TEM, VEB or OXA-1 genes. All the isolates evaluated had ESBL genes and were resistant to all the antimicrobials.

Discussion

Multiple antimicrobial drugs for treating *E. coli* infections, including beta-lactam and third-generation cephalosporin medications, have been demonstrated to be resistant to *E. coli*. ESBL-generating *E. coli* are becoming more common globally (Kim et al., 2021; Yiğin, 2021; Zhang et al., 2021), prompting researchers to explore particular enzymes in each resistance epidemic. *E. coli* that produce ESBL are a serious public health issue because they have the potential to raise fatality rates. ESBL-producing *E. coli* samples have been explored in Thailand from a variety of sources (Runcharoen et al., 2017; Bubpamala et al., 2018; Seenama et al., 2019). However, the study of ESBL-producing *E. coli* isolated from flies, on the other hand, is still limited. The findings from the current investigation revealed a large incidence of ESBL-producing *E. coli* in house flies and oriental latrine flies from various origins around livestock farms in Sakon Nakhon province, Thailand. If farm flies can travel outside the farm, they might help transmit ESBL-producing *E. coli* from farms to public places, contaminating food intended for human consumption. As a result, it is indeed critical to keep a focus on the spread of resistant bacteria in animals, as it might expand to humans via the food chain.

The ESBL-producing *E. coli* rate was 94.23% in the current study, which is considered a high rate compared to several European nations. ESBL-producing *E. coli* contamination in meat

Table 3 Antimicrobial percentages of antimicrobial-resistant *E. coli* isolated from flies from different origins

Sampling site (N)	Antimicrobial				
	Cefpodoxime	Ceftazidime	Azetronam	Cefotaxime	Ceftriaxone
Swine farm A (7)	85.71	100.00	85.71	57.14	85.71
Swine farm B (33)	84.85	84.85	75.76	69.70	75.76
Dairy farm A (8)	87.50	87.50	87.50	87.50	100.00
Dairy farm B (20)	95.00	95.00	60.00	65.00	85.00
Fresh market A (6)	100.00	100.00	100.00	100.00	100.00
Fresh market B (10)	100.00	100.00	90.00	70.00	90.00
Slaughterhouse (10)	100.00	100.00	100.00	100.00	100.00
Retail food shop (10)	100.00	100.00	60.00	80.00	100.00
Average	94.13	95.92	82.37	78.67	92.06

N = number of isolated *E. coli*, where total samples = 104 has been reported to be limited or nonexistent in several regions. For example, only one isolate of ESBL-producing *E. coli* was found in 732 meat samples in Denmark, according to Jensen et al. (2006). Another experiment in Sweden revealed no ESBL-producing *E. coli* in 419 samples of poultry meat (Tham et al., 2012). In Zurich, Switzerland, Geser et al. (2012) reported no ESBL-producing *E. coli* detected in 104 pork and beef meat samples.

The high prevalence of ESBL-producing *E. coli* in flies isolated from livestock farms and neighboring areas in the current study may have resulted from the improper use of antimicrobials in the treatment of sick animals or even as a growth stimulant in feedstuffs, leading to the development of antimicrobial resistant bacteria. The use of antimicrobial drugs in animal husbandry has been associated with drug-resistant *E. coli* in several studies; because drug resistance genes are frequently found on mobile genetic elements, the transmission of one bacterial resistance to another bacterium is possible (Diallo et al., 2013; Caruso, 2018; Li et al., 2019).

It is important to find genes associated with ESBL enzymes. The interpretation of the current results is limited whilst using the standard disk diffusion of screening. ESBL enzymes may not be detected due to a loss of sensitivity or specificity. The current study found that the bla_{TEM} and bla_{SHV} enzymes could be created at a rate of 51.02% and 25.51%, respectively. The genes producing TEM, VEB or OXA-1 were not detected among the tested isolates. Compared with a study in the Netherlands (Blaak et al., 2014), the $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ genes harbored in *E. coli* originated from flies and farmyard manures in bird farms . Furthermore, a study in Thailand contradicts the current study. With the exception of the bla_{SHV} and bla_{VEB} genes, the genes that produce bla_{TEM} and $bla_{\text{CTX-M}}$ were found in *E. coli* isolated from sick animals brought to Kasetsart University's Animal Teaching Hospital. (Hanhaboon et al., 2015). Nonetheless, the current findings suggested that flies obtain ESBL-producing *E. coli,* necessitating further investigation into the importance of flies in the spreading of ESBL-producing *E. coli* in public areas. In addition, *E. coli* or other pathogenic bacteria or both should be isolated from additional insect vectors associated with animal husbandry to track the dissemination of antimicrobial-resistant bacteria and the mechanisms by which they propagate in the environment.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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