Original Article

Characteristics of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Escherichia coli* Associated with Antimicrobial Use in captive wild animals

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Abstract

Antimicrobial agents are a cause of the increase in antimicrobial-resistant (AMR) bacteria. We investigated the prevalence and characteristics of extended-spectrum beta-lactamase (ESBL)-producing bacteria in captive wild animals before and after exposure to antimicrobial agents. Sixty-six fecal samples were collected from captive animals (spotted deer, Rusa deer, geese, chickens, rabbits, and guinea pigs) at the Mahasarakham University Mini Zoo in Thailand on three sampling occasions: October 2021, January 2022, and April 2022. Using MacConkey agar supplemented with cefotaxime, ESBL-producing *Escherichia coli* (ESBL-Ec) was isolated and subjected to antimicrobial susceptibility tests and genetic analysis. The results showed a high prevalence of ESBL-Ec in the captive wild animals, with 86.4% of the samples tested positive. After deer were exposed to antimicrobials (i.e., oxytetracycline and streptomycin), the resistance rates of tetracycline and sulfamethoxazole/trimethoprim were increased as the prevalence of ESBL-Ec was increasing. ESBL-Ec isolates from some captive animals were resistant to multiple drug classes over time. CTX-M subfamily ESBL genes were found in all isolates, with the CTX-M-1 group (i.e., CTX-M-15, CTX-M-55, and CTX-M-79 genes) being the most prevalent. In conclusion, this study found a high prevalence of ESBL-Ec in captive wild animals and observed variations in AMR profiles and ESBL gene distributions. The findings highlight the potential impacts of antimicrobial use on the emergence and spread of ESBL-producing bacteria in captive animal populations and emphasize the need for targeted interventions and management practices to mitigate this issue.

Keywords: captive wildlife, CTX-M-15 genes, *Escherichia coli*, Thailand, zoo

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Introduction

Extended-spectrum beta-lactamases (ESBLs) are enzymes produced by certain bacteria that confer resistance to a broad range of beta-lactam antibiotics, including cephalosporins, penicillin, and aztreonam (Rawat and Nair, 2010). The emergence and dissemination of ESBL-producing bacteria have become a significant public health concern, primarily due to their ability to cause infections that are difficult to treat (Muñoz-Miguel *et al*., 2018). While the link between antimicrobial use and the development of ESBLs in human and livestock settings has been extensively studied (Boonyasiri *et al*., 2014; Mahazu *et al*., 2022), there is a paucity of research exploring the characteristics of ESBL-producing bacteria associated with antimicrobial use in captive wild animals.

Captive wild animals, such as those housed in zoological parks and conservation centers, are often subjected to medical interventions that involve the administration of antimicrobial agents (Silva *et al*., 2021). These interventions aim to treat infections, prevent disease outbreaks, and ensure the overall well-being of the animals (Rodrigues da Costa and Diana, 2022). However, using antimicrobials in these settings may inadvertently contribute to the emergence and spread of ESBL-producing bacteria among captive wild animal populations. In fact, a study by Catry *et al*. (2018) revealed that an increase in antimicrobial prescriptions led to a significant increase in antimicrobial-resistant (AMR) bacteria identified in human patients. Therefore, understanding the characteristics of ESBL-producing bacteria associated with antimicrobial use in captive wild animals is crucial for both animal welfare and public health. This knowledge can shed light on the transmission dynamics, genetic determinants, and potential reservoirs of ESBLs within captive wild animal populations.

In October 2021, we identified ESBL-producing bacteria in the fecal samples of captive wild deer and other animals at the Mini Zoo located on the Mahasarakham University campus in Maha Sarakham, Thailand. Approximately a month later, a subset of the deer population showed clinical signs of foot and mouth disease (FMD), leading to the administration of antimicrobial treatment. A keeper administered antimicrobial agents to the deer through their drinking water to control secondary bacterial infections. The use of antimicrobials for treatment could influence antimicrobial selective pressure and the development of AMR bacteria in animal guts (Kimura *et al*., 2017); therefore, this study aimed to investigate the characteristic features of ESBL-producing bacteria isolated from captive wild animals before and after exposure to antimicrobial agents. A comprehensive analysis of bacterial isolates examined the genetic determinants of ESBLs and their phenotypic profiles. By elucidating the underlying factors contributing to ESBL production in this context, our findings can inform the development of targeted interventions and management practices to mitigate the impact of antimicrobial use on captive wild animal populations.

Materials and Methods

Study area and sample collection time: The Mahasarakham University (MSU) Mini Zoo (16.2440° N, 103.2490° E), situated in the MSU Museum on the Kham Riang campus in Maha Sarakham province, Thailand, houses a variety of animals, including 67 deer, ten chickens, six rabbits, five geese, and five guinea pigs. Additionally, the Mini Zoo is situated in close proximity, approximately 0.8 km away, from nearby small communities and paddy fields. This Mini Zoo, depicted in Fig. 1, consists of different animal enclosures. The 30 m x 16 m cage accommodates 24 spotted deer (*Axis axis*), ten chickens, six rabbits, and five geese. The adjacent cage holds Rusa deer (*Rusa timorensis*), covering an area of 22 m x 12 m. Furthermore, a separate enclosure for a hog deer (*Axis porcinus*) is situated next to the Rusa deer cage. All the mentioned animals reside in open-air mesh cages, with a portion of the area covered by a roof and a dirt floor. In addition to the animals as mentioned above, there is a small open-air mesh cage measuring 12.5 m^2, covered with a roof and featuring a cement floor, where five guinea pigs are housed. This cage is located near the spotted deer enclosure. Sample collections were conducted three times during the study period: October 2021 (n = 14), January 2022 (n = 36), and April 2022 (n = 16). Initially, pooled fecal samples were collected from healthy animals in October 2021. Subsequently, in November 2021, some of the deer exhibited symptoms of FMD. Following the incidence of FMD, an unapproved antimicrobial product was subsequently administered to the deer by the keeper. The product was added to the drinking water without proper and precise dose estimation. The label of the unapproved antimicrobial product showed two types of antimicrobials (streptomycin and oxytetracycline), calcium gluconate and vitamins. The pooled fecal samples were again collected in January 2022 and April 2022.

Samples from captive animals: A total of 66 pooled fecal samples were collected from various animals within the Mini Zoo throughout the study period. Among these samples, 33 were obtained from the spotted deer, 20 from Rusa deer, six from geese, three from chickens, three from rabbits, and one from guinea pigs (Table 1). Unfortunately, collecting fecal samples directly from the deer and other animals was impossible due to their skittish nature. Thus, the dropped fresh fecal samples were collected. The collection of fresh pooled fecal samples from the deer and other animals was conducted during the early morning hours, specifically between 6 and 7 AM. A significant amount of fresh feces was observed at the deer feeding area. The defecating area was divided into sections of approximately one square meter to collect the pooled fecal samples. All fresh feces found (approximately 2-3 droppings per sample) were pooled together for the other animal species. However, in certain collection times, collecting fecal samples from some animals was impossible due to their anxious behavior. The exact number of samples collected during each collection time and from each animal species is presented in Table 1.

Bacterial culture and identification: The fecal samples were subjected to inoculation onto MacConkey agar (Oxoid, Ltd, Hampshire, UK) and MacConkey agar supplemented with 1 µg/ml cefotaxime (CTX) (Sigma-Aldrich, USA; MAC-CTX). These culture plates were then incubated aerobically at 37°C overnight. Potential AMR bacteria were isolated on MAC-CTX from the fecal samples by selecting 3-5 colonies that exhibited characteristics suggestive of *Escherichia coli*. To confirm the identity of the isolates at the species level, polymerase chain reaction (PCR) was conducted using previously reported primers (Wang *et al*., 1996).

Antimicrobial susceptibility testing: CTX-resistant *E. coli* isolates were subjected to antimicrobial phenotypic analysis using the disk diffusion method (CLSI, 2013). Eight antimicrobial agents (Oxoid, Ltd, Hampshire, UK) were used, namely: CTX (30 µg/disk), chloramphenicol (CHL, 30 µg/disk), ciprofloxacin (CIP, 5 µg/disk), gentamicin (GEN, 10 µg/disk), imipenem (IMI, 10 µg/disk), nalidixic acid (NAL, 30 µg/disk), sulfamethoxazole/trimethoprim (SXT, 23.75/1.25 µg/disk), and tetracycline (TET, 30 µg/disk). The resistance profiles for all antimicrobial agents were determined based on the guidelines set by the Clinical and Laboratory Standards Institute (CLSI, 2020). Confirmation of ESBL production was performed using the double-disk synergy test with clavulanate (10 µg/disk)/amoxicillin (20 µg/disk), CTX (30 µg/disk), ceftazidime (30 µg/disk), and cefpodoxime sodium (10 µg/disk) (Oxoid, Ltd, Hampshire, UK), as previously described (Luzzaro *et al*., 2001).

Beta-lactamase gene identification: To identify the presence of ESBL genes, multiplex PCR was conducted following the protocol described by Dallenne *et al.* (2010). For each animal type and collection time, an isolate with the highest number of occurrences in the antimicrobial susceptibility profile was selected for further analysis. Subtyping of CTX-M-group beta-lactamases, specifically CTX-M-1 and CTX-M-9 groups, was performed by sequencing. The sequencing utilized previously reported primer pairs (Mena *et al*., 2006; Kojima *et al*., 2005), and both strands of the amplified DNA fragments were sequenced by Solgent Analysis Services, Korea. The obtained nucleotide sequences were translated into encoded amino acid sequences, which were then analyzed using BLAST (National Center for Biotechnology Information [NCBI], Bethesda, MD, USA) to determine their similarity to known sequences in the database.

Data analysis: Data analysis was performed using R version 4.2.0 (R Core Term, 2020). The diameter of inhibition zone data was analyzed using ANOVA and Turkey's *post hoc* test at a 5% significance level. The diameter of the inhibition zone of each antimicrobial and collection times were dependent and independent variables for ANOVA, respectively. The difference in resistance of ESBL-producing *Escherichia coli* (ESBL-Ec) to the number of antimicrobial classes over different collection times was compared by the chi-square test, while Pearson's correlation analyzed the relationship of AMR among the sampling. The threshold for statistical significance was $P < 0.05$.

Ethics statement: All animal procedures were approved by the Ethics Committee for Animal Research and Welfare of Masarakham University, Thailand (protocol code: IACUC-MSU-22/2021).

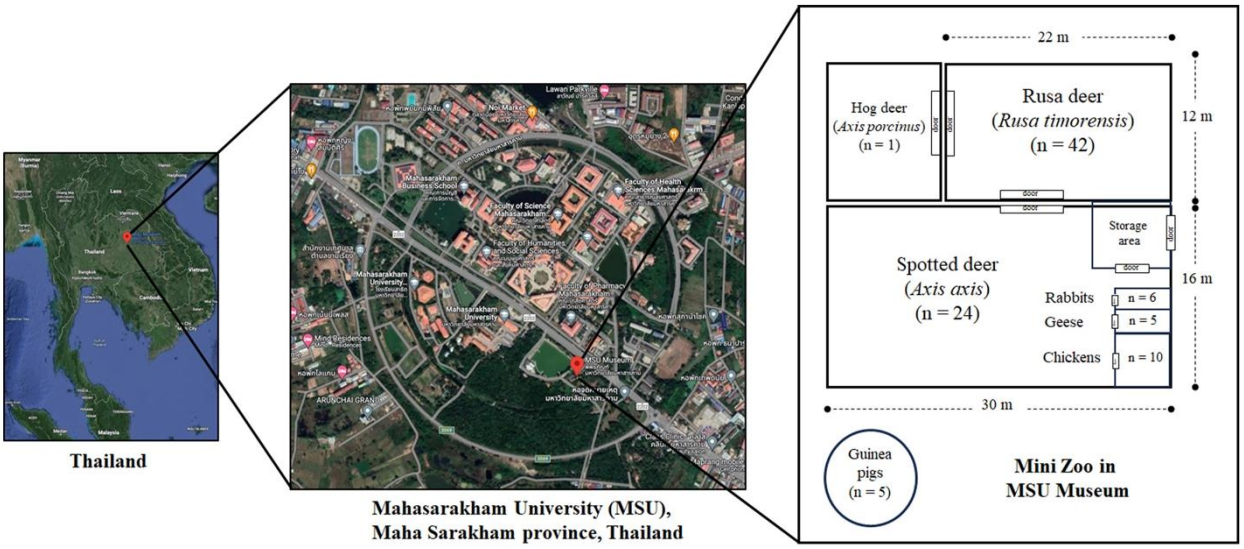

Figure 1 Mini Zoo in MSU Museum located on the Kham Riang campus of Mahasarakham University (MSU), Maha Sarakham province, Thailand. n, number of animals

Table 1 The percentage of ESBL-producing *Escherichia coli* infection rate in animals reared in a Mini Zoo on University campus.

Animal species (Total of animals)	Number of ESBL-Ec-positive pooled samples in each collection period of each animal species (%Infection rate, positive/total of samples)			
	October 2021	January 2022	April 2022	Total
Spotted deer (24)	8 (100%, 8/8)	19 (100%, 19/19)	6 (100%, 6/6)	100%, 33/33
Rusa deer (42)	1 (100%, 1/1)	13 (100%, 13/13)	6 (83.3%, 5/6)	95%, 19/20
Geese (5)	3 (33.3%, 1/3)	3 (66.7%, 2/3)	none	50%, 3/6
Chicken (10)	1 (0%, 0/1)	none	2 (50%, 1/2)	33.3%, 1/3
Guinea pigs (5)	1 (0%, 0/1)	none	none	0%, 0/1
Rabbit (6)	None	1 (0%, 0/1)	2 (50%, 1/2)	33.3%, 1/3
Total	14 (71.4%, 10/14)	36 (94.4%, 34/36)	16 (81.3%, 13/16)	86.4%, 57/66

none, no sample to collect.

Results

Prevalence of ESBL-Ec in captive deer and other animals: The prevalence of ESBL-Ec in captive wild animals, including spotted deer, Rusa deer, geese, chickens, rabbits, and guinea pigs, was assessed (Table 1). A total of 66 samples were collected at three different sampling times, and ESBL-Ec was isolated from 57 of these samples (86.4%). The highest prevalence of ESBL-Ec was observed in the spotted deer (33 of 33 samples), followed by Rusa deer (19 of 20 samples), geese (3 of 6 samples), chickens (1 of 3 samples), and rabbit (1 of 3 samples). Interestingly, no CTX-resistant *E. coli* was isolated from the two samples of guinea pigs. Furthermore, the study found variations in the detection rates of ESBL-Ec among the different sampling periods. The second sampling exhibited the highest prevalence, with 94.4% (34/36) of samples tested positive for ESBL-Ec. This was followed by the third sampling with 81.3% (13/16) and the first with 71.4% (10/14) prevalence.

Antimicrobial resistance of ESBL-Ec in three collection times: The percentage of resistance to various antimicrobial agents in ESBL-Ec isolates obtained from captive wild animals across three collection times was assessed. We recorded a notable increase in the percentage of resistance to TET and SXT during the second sampling, which was maintained at a high level during the third sampling. In contrast, lower percentages of resistance to CHL, CIP, GEN, and NAL were recorded in the second and third sampling compared to the first sampling period (Fig. 2 and Table 2). It is worth noting that all ESBL-Ec isolates remained susceptible to IMI across all three collection times.

Table 2 presents the difference in bacterial inhibition zone (mean ± SE) of each antimicrobial among three collection times. The results of the bacterial inhibition zone of TET and SXT indicated statistically significant differences between the bacterial collection times for each antimicrobial agent ($P < 0.05$). Our findings revealed that TET and SXT inhibition zones against ESBL-Ec were significantly lower in the second and/or third sampling compared to the first sampling ($P < 0.05$). Conversely, the mean diameter of the inhibition zones for CHL, GEN, and NAL was significantly wider in the second and/or third sampling compared to the first sampling ($P < 0.05$). However, no significant differences were observed among the three collection times for CIP and IMI (Table 2).

Multidrug resistance (MDR) was observed across all collection times in the ESBL-Ec isolates. Our analysis revealed a significant association ($P < 0.0001$) between the number of antimicrobial classes and the collection times (Table 3). The Chi-square analysis showed significance at $P < 0.0001$ with chi-square statistics at 37.79 and moderate correlation at 0.47 as Phi coefficient. In the first sampling, the highest percentage of bacteria resistance was observed for one antimicrobial class, followed by the percentage of resistance to ≥ 4 classes of antimicrobials. After a month of the deer being exposed to oxytetracycline and streptomycin, there was a notable increase in the percentages of bacteria that resisted two and three classes of antimicrobials. In comparison, the percentages of resistance to one class and ≥ 4 classes of antimicrobials decreased. In the last sampling, the highest percentage of resistance was still observed for three classes of antimicrobials, and there was an increased percentage of resistance to one class and ≥ four classes of antimicrobials (Table 3).

Antimicrobial resistance profiles and associated resistance genes of ESBL-Ec: One hundred and sixty-seven ESBL-Ec isolates, derived from 57 samples, exhibited diverse distributions across distinct animal species. In Table 4, we showed that the highest numbers of ESBL-Ec isolates were found in the spotted deer (96 isolates), followed by Rusa deer (57 isolates), geese (8 isolates), rabbits (3 isolates), and chickens (3 isolates). The AMR profiles of the ESBL-Ec isolates from animals in the Mini Zoo exhibited a wide range of resistance patterns. The highest number of CTX-resistant isolates was observed in the spotted deer and geese in the first sampling. Additionally, all ESBL-Ec isolates from Rusa deer exhibited resistance to 4-7 antimicrobial classes. During the second sampling, ESBL producers from the spotted deer and geese demonstrated the highest resistance rate to CTX-TET-SXT, while Rusa deer had the highest number of ESBL-Ec isolates resistant to CTX-TET. In the last sampling, the spotted and Rusa deer exhibited the highest number of ESBL-Ec isolates resistant to CTX-TET-SXT. Notably, ESBL-Ec isolates resistant to CTX were observed in the last sampling of rabbits, while ESBL-Ec isolates resistant to MDR were found in chickens.

We further determined the resistance genes underlying the ESBL-Ec identified. Our study revealed the CTX-M group-producing *E. coli* distribution and identified specific CTX-M subtypes within the ESBL-Ec isolates. All 165 ESBL-Ec isolates obtained from samples of the spotted deer, Rusa deer, geese, and

rabbits were found to be CTX-M-1 group producers. In contrast, the 3 ESBL-Ec isolates from chickens belonged to the CTX-M-9 group. Further analysis of the ESBL-Ec isolates involved selecting 10 isolates based on the highest occurrence of AMR profiles per animal species per collection time. Subtyping these isolates depicted the presence of specific CTX-M subtypes. Among the selected isolates, six were identified as $bla_{\text{CTX-M-15}}$, two as $bla_{\text{CTX-M-79}}$, one as $bla_{\text{CTX-M-55}}$, and one as $bla_{\text{CTX-M-65}}$. During the first sampling, CTX-M-15, CTX-M-79, and CTX-M-55 genes were observed in ESBL-Ec isolates from spotted deer, Rusa deer, and geese, respectively. In the second sampling period, ESBL-Ec carrying the CTX-M-15 gene was detected in isolates from the animal mentioned above species. Also, in the last sampling, the CTX-M-15 gene was found in spotted deer and rabbits isolates, while the CTX-M-79 and CTX-M-65 genes were observed in isolates from Rusa deer and chickens, respectively (Table 4).

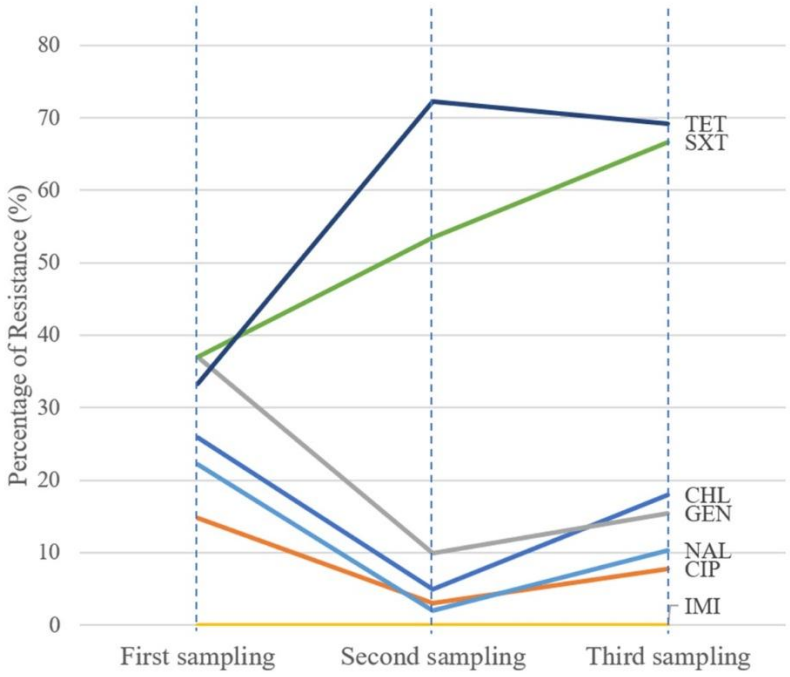

Figure 2 The percentage of resistance of ESBL-producing *Escherichia coli* from 3 collection times. CHL, Chloramphenicol; CIP, Ciprofloxacin; GEN, Gentamycin; IMI, Imipenem; NAL, Nalidixic acid; SXT, Sulfamethoxazole/trimethoprim; TET, Tetracycline

Table 2 The average of inhibition zones (mean ± SE) of antimicrobials and percentage of resistance of 167 ESBL-producing *Escherichia coli* from 3 collection times.

Antimicrobials	Collection times	Average of inhibition zones: Mean ± SE (mm)*	Percentage of antimicrobial resistance (%)
Chloramphenicol	First	$20.48^{a} \pm 1.80$	25.93
	Second	$25.47^{b} \pm 0.58$	4.95
	Third	$22.41^{ab} \pm 1.29$	17.95
Ciprofloxacin	First	$24.03^{a} \pm 1.80$	14.81
	Second	$25.75^{a} \pm 0.43$	2.97
	Third	$25.46^{a} \pm 0.81$	7.70
Gentamycin	First	$10.74^{a} \pm 1.62$	37.04
	Second	$15.51^{b} \pm 0.54$	9.90
	Third	$15.41^{b} \pm 1.08$	15.38
Imipenem	First	$32.00^{a} \pm 0.45$	0
	Second	$31.13^{a} \pm 0.23$	0
	Third	$31.15^{a} \pm 0.39$	0
Nalidixic acid	First	$17.26^{a} \pm 1.93$	22.22
	Second	$21.66^{b} \pm 0.37$	1.98
	Third	$20.95^{b} \pm 1.19$	10.26
Sulfamethoxazole/trimethoprim	First	$17.37^{a} \pm 2.64$	37.04
	Second	$11.64^{ab} \pm 1.25$	53.47
	Third	$9.03^{b} \pm 2.08$	66.67
Tetracycline	First	$19.59^{a} \pm 2.35$	33.33
	Second	$10.27^{b} \pm 0.92$	72.28
	Third	$13.15^{b} \pm 1.53$	69.23

*The values with different superscript letters in a column of each antimicrobial are significantly different ($P < 0.05$).

Table 3 The percentage of multidrug-resistant ESBL-producing *Escherichia coli* in three collection times.

Collection times	No. of isolates	Percentage of antimicrobial resistance in the number of antimicrobial class(es) (n/N)				Chi-Square	Phi Coefficient
		1 class	Two classes	Three classes	≥4 classes		
First	27	44.44% (12/27)	7.40% (2/27)	18.52% (5/27)	29.63% (8/27)		
Second	101	3.70% (14/101)	41.58% (42/101)	36.63% (37/101)	7.92% (8/101)		
Third	39	25.64% (10/39)	7.69% (3/39)	46.15% (18/39)	20.51% (8/39)		
Total	167	21.30% (36/167)	27.81% (47/167)	35.50% (60/167)	14.37% (24/167)	37.79	0.47

n is the number of ESBL-Ec isolates; N is the total number of ESBL-Ec isolates in each sampling.

Table 4 Antimicrobial resistance profiles of 167 ESBL-producing *Escherichia coli* and their ESBL genes isolated from the spotted deer, Ruza deer, and other animal species in Mini Zoo were collected between October 2021 and April 2022.

Collection period (n)	Animal species (N)	Antimicrobial resistance profiles	Percentage (%) of ESBL-Ec isolates (n/N)		Sub-families of CTX-Ms (CTX-M group)	Subtyping of CTX-Ms*
October 2021 (27)	Spotted deer (21)	CTX	33.33%	(9/27)	CTX-M-1	CTX-M-15
		CTX, GEN	3.70%	(1/27)	CTX-M-1	nd
		CTX, TET	3.70%	(1/27)	CTX-M-1	nd
		CTX, GEN, TET	7.4%	(2/27)	CTX-M-1	nd
		CTX, GEN, SXT	11.11%	(3/27)	CTX-M-1	nd
		CTX, TET, NAL, CHL	3.70%	(1/27)	CTX-M-1	nd
		CTX, NAL, CHL, SXT, CIP	3.70%	(1/27)	CTX-M-1	nd
		CTX, GEN, TET, CHL, SXT	3.70%	(1/27)	CTX-M-1	nd
		CTX, GEN, TET, NAL, SXT, CIP	3.70%	(1/27)	CTX-M-1	nd
		CTX, TET, NAL, CHL, SXT, CIP	3.70%	(1/27)	CTX-M-1	nd
		CTX, GEN, CHL, SXT	3.70%	(1/27)	CTX-M-1	nd
	Rusa deer (3)	CTX, TET, CHL, SXT, NAL	3.70%	(1/27)	CTX-M-1	nd
		CTX, TET, GEN, CHL, SXT, NAL, CIP	3.70%	(1/27)	CTX-M-1	CTX-M-79
	Geese (3)	CTX	11.11%	(3/27)	CTX-M-1	CTX-M-55
		Subtotal	100%	(27/27)		
January 2022 (101)	Spotted deer (57)	CTX	11.88%	(12/101)	CTX-M-1	nd
		CTX, GEN	0.99%	(1/101)	CTX-M-1	nd
		CTX, TET	7.92%	(8/101)	CTX-M-1	nd
		CTX, SXT	6.93%	(7/101)	CTX-M-1	nd
		CTX, TET, SXT	22.77%	(23/101)	CTX-M-1	CTX-M-15
		CTX, SXT, CIP	0.99%	(1/101)	CTX-M-1	nd
		CTX, TET, CHL, SXT	0.99%	(1/101)	CTX-M-1	nd
		CTX, GEN, TET, CHL, SXT	1.98%	(2/101)	CTX-M-1	nd
		CTX, GEN, TET, NAL, CHL, SXT	0.99%	(1/101)	CTX-M-1	nd
		CTX, TET, NAL, CHL, CIP, SXT	0.99%	(1/101)	CTX-M-1	nd
	Rusa deer (39)	CTX	1.98%	(2/101)	CTX-M-1	nd
		CTX, TET	20.79%	(21/101)	CTX-M-1	CTX-M-15
		CTX, SXT	4.95%	(5/101)	CTX-M-1	nd
		CTX, TET, SXT	4.95%	(5/101)	CTX-M-1	nd
		CTX, TET, GEN	2.97%	(3/101)	CTX-M-1	nd
		CTX, TET, GEN, SXT	2.97%	(3/101)	CTX-M-1	nd
	Geese (5)	CTX, TET, SXT	4.95%	(5/101)	CTX-M-1	CTX-M-15
		Subtotal	100%	(101/101)		
April 2022 (39)	Spotted deer (18)	CTX	10.26%	(4/39)	CTX-M-1	nd
		CTX, SXT	5.13%	(2/39)	CTX-M-1	nd
		CTX, TET, SXT	28.21%	(11/39)	CTX-M-1	CTX-M-15
		CTX, TET, CHL, SXT	2.56%	(1/39)	CTX-M-1	nd
	Rusa deer (15)	CTX	7.69%	(3/39)	CTX-M-1	nd
		CTX, TET	2.56%	(1/39)	CTX-M-1	nd
		CTX, TET, SXT	12.80%	(5/39)	CTX-M-1	CTX-M-79
		CTX, TET, GEN	5.13%	(2/39)	CTX-M-1	nd

	CTX, TET, GEN, SXT, CHL	7.69%	(3/39)	CTX-M-1	nd
	CTX, TET, GEN, SXT, NAL, CIP	2.56%	(1/39)	CTX-M-1	nd
Rabbits (3)	CTX	7.69%	(3/39)	CTX-M-1	CTX-M-15
Chickens (3)	CTX, TET, NAL, CHL, SXT	2.56%	(1/39)	CTX-M-9	nd
	CTX, TET, NAL, CHL, SXT, CIP	5.13%	(2/39)	CTX-M-9	CTX-M-65
	Subtotal	100%	(39/39)		

*, an isolate of the highest percentage of AMR profile in each animal species of each sampling was selected to identify subtypes of CTX-Ms-group beta-lactamases.; nd, not determined; n, number of ESBL-Ec isolates; N, total of ESBL-Ec isolates in each sampling; CTX, cefotaxime; CHL, Chloramphenicol; CIP, Ciprofloxacin; GEN, Gentamycin; NAL, Nalidixic acid; SXT, Sulfamethoxazole/trimethoprim; TET, Tetracycline

Discussion

This study highlights the significant prevalence of ESBL-Ec in captive wild deer at the Mahasarakham University Mini Zoo (Table 1), located within the university campus (Fig. 1). The Mini Zoo is close (0.8 km) to small communities and paddy fields. Initially, the investigation focused on healthy animals in the Mini Zoo, with a particularly high occurrence of ESBL-Ec observed in wild deer during October 2021 (Table 1). However, there was an endemic outbreak of FMD in the region, affecting nearby cattle communities located approximately 0.8 km from the Mini Zoo within the same sub-district. It is important to note that FMD is a well-established endemic disease in the province. This FMD incidence subsequently spread to the captive wild deer in November 2021. Importantly, it is worth noting that the keeper could have contributed to the transmission of FMD to the deer population, as his cattle were infected with FMD before the deer contracted the disease. To manage the FMD outbreak, oxytetracycline and streptomycin were administered to the deer, leading to observations of a high prevalence of ESBL-Ec, accompanied by increased resistance rates to TET and SXT in the second and third sampling periods (Table 2). Viral and bacterial infections in captive deer might be attributed to potential shortcomings or failures in biosecurity measures. The breakdown in biosecurity measures has led to significant infectious disease outbreaks in large commercial food animal farms and zoos (Gray *et al*., 2018). Additionally, the presence of AMR bacteria is a result of antimicrobial use (Kimura *et al*., 2017), as has been observed in this study where there was an increase in TET resistance rate in ESBL-Ec after the animals were exposed to oxytetracycline (Table 2). The exact cause of the elevated SXT resistance rate remains uncertain, but it is possible that it resulted from the inclusion of SXT in the unapproved antimicrobial product, but it was not disclosed on the product's label. A study conducted in Poland demonstrated that administering trimethoprim and sulfamethoxazole to treat pigs led to a significant prevalence of SXT-resistant bacteria following exposure, primarily due to selection pressure (Mazurek *et al*., 2015). Additionally, although not investigated in the present study, the elevated SXT resistance could suggest the presence and potential transfer of resistance determinants such as *sul1* and *sul2*, which have been associated with high levels of SXT resistance (Ma *et al*., 2021). Despite being isolated and having no direct contact with domestic animals, our recent investigation unveiled a significant occurrence of ESBL-Ec among wild captive animals of various species. The selection pressure from antimicrobial use in captive animals likely contributed to the increased prevalence of MDR feature observed in ESBL-Ec, with a higher percentage of resistance specifically to TET due to the selective pressure exerted by oxytetracycline used (Fig. 2) (Græsbøll *et al*., 2019). In contrast, the low percentage of resistance to CHL, GEN, NAL, CIP, and IMI of ESBL-Ec in the animals was observed in the second and third sampling because the animals were not exposed to those antimicrobials (Fig. 2).

The ESBL-Ec rate was 86.4% in this current research, which was considered a high rate when compared to several previous studies in Thailand, including healthy dogs in Chiang Mai province (25.84%) (Thepmanee *et al*., 2018), calves and lactating cows in Lamphun province (80.56%) (Vannakovida *et al*., 2021). Our findings represent the first documentation of ESBL-Ec incidence among captive deer in Thailand. Interestingly, the incidence of ESBL-Ec carried by captive deer in the current study (86.4%) was higher than the reported prevalence of 1.56% for ESBL-Ec in 64 hunted roe deer from the central part of Rotkreuz, Switzerland (Stephan *et al*., 2012), 2% for ESBL-producing *E. coli* in wild boars from Poland (Literak *et al*., 2010)., 66.67% (2 of 3 animals) for ESBLs detected in wild deer from Portugal (Costa *et al*., 2006), and 59.01% (72 of 122 animals) of ESBL-Ec isolated from red deer's feces in Spain (Alonso *et al*., 2016). The prevalence recorded in this study was relatively high.

Notably, the prevalence of ESBL-Ec increased after the animals were exposed to non-beta-lactam drugs; however, intriguingly, it decreased by 13% within three months following this exposure. Kimura *et al.* (2017) show that the concentration of cefalexin-resistant *E. coli* isolated from fecal samples of healthy dogs decreased after 12 days post-exposure to cefalexin. Thus, discontinuing antimicrobial administration might have contributed to decreasing the isolation of AMR bacteria following antimicrobial exposure.

Previous studies on captive wild animals also detected MDR bacteria (Oliveira de Araujo *et al.* 2020; Smoglica *et al.* 2023). The presence of MDR ESBL-Ec strains in captive wild animals (Table 3), with varying resistance profiles (Table 4) among different species and sampling periods, suggested the widespread of MDR ESBL-Ec in captive wild animals. The presence of AMR bacteria resulted from antimicrobial use (Kimura *et al*., 2017). After the animals were exposed to

oxytetracycline, this study showed an increase in the TET resistance rate of ESBL-Ec, indicating the increase of bacteria resistant to 3 classes of antimicrobials as shown in Table 2. The selection of MDR bacteria could be attributed to an inappropriate use of antimicrobial agents in animals (Brown *et al*., 2019). While the primary focus was on ESBL-Ec, the study found a significant and moderate association between the number of antimicrobial resistance classes and the collection times (Table 3). Thus, the results suggested that AMR bacteria may be resistant to a greater number of antimicrobial classes after antimicrobial use.

In this study, the predominant CTX-M subfamily identified in ESBL-Ec isolates from spotted deer, Rusa deer, geese, and rabbits was CTX-M-1, whereas in the chickens, it was CTX-M-9 (Table 4). This result suggested that CTX-M-1 was the predominant subfamily gene in AMR bacteria isolated from captive wild animals, followed by CTX-M-9 subfamily as the report of CTX-M-1 subfamily prevalence in ESBL producers isolated from dairy farms in Thailand (97.0%) (Saekhow and Sriphannam, 2021). In addition, this finding is consistent with some previous studies that have reported the prevalence of CTX-M-1 and CTX-M-9 subfamily-producing *E. coli* in various animal species, including wild and domesticated animals (Yossapol *et al*., 2021; Lay *et al*., 2021).

Our further analysis involving the selection of isolates based on AMR profiles revealed the presence of specific CTX-M subtypes (Table 4). The most frequently identified subtype was $bla_{\text{CTX-M-15}}$, found in ESBL-Ec isolates from multiple animal species and across different sampling periods. This is in line with other studies that have reported CTX-M-15 as one of the most prevalent subtypes in ESBL-Ec isolates from various sources, including animals, humans, and the environment (Chen *et al*., 2019; Valentin *et al*., 2014). Moreover, previous studies conducted in Thailand showed that the prevalence of CTX-M-15-producing bacteria was found to be highest in livestock (59.3%) (Lay *et al*., 2021) and the environment (44.7%) (Chotinantakul *et al*., 2022), followed by CTX-M-55-producing bacteria (26.3%) (Chotinantakul *et al*., 2022). Furthermore, the presence of other CTX-M subtypes, such as $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-65}}$, and $bla_{\text{CTX-M-79}}$, in specific animal species during certain sampling periods highlights the diversity of ESBL-Ec strains and their genetic variability. Although subtyping was done in some isolates of major AMR profiles of each animal species in each collection time, CTX-M-15-producing *E. coli* was detected in all animal species except chickens, where we found bacteria harboring the CTX-M-65 gene. These findings suggest the acquisition and dissemination of different CTX-M subtypes through horizontal gene transfer among the animal populations, which could be influenced by factors such as animal husbandry practices, exposures to antimicrobials, and interactions with the environment (Szmolka *et al*., 2013).

A plasmid analysis would have further highlighted the possibility of horizontal gene transfer in disseminating resistance genes (Yossapol *et al*., 2021); however, this analysis was not conducted in this study. Nonetheless, these findings highlighted the presence and dissemination of ESBL-producing bacteria in captive wild animal populations. Understanding the prevalence and distribution patterns of ESBL-Ec among different animal species and over time is essential for devising effective strategies to combat antimicrobial resistance in these environments. This research contributes to our understanding of ESBL emergence and transmission dynamics in captive wild animals, providing valuable insights for the management and conservation of these species.

In conclusion, this study indicates a high prevalence of ESBL-Ec in captive wild animals at the Mini Zoo, with the effect of antimicrobials used in animals having AMR bacteria harboring variations in resistance patterns and the presence of specific resistance genes. The findings highlight the complex dynamics of AMR in these animal populations and emphasize the importance of antimicrobial use in animals with AMR bacteria and continuous surveillance and management strategies to prevent the spread of MDR bacteria.

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