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RESEARCH ARTICLE

Fecal carriage of third-generation cephalosporin-resistant *Escherichia coli* harboring antimicrobial resistance and virulence-associated genes in healthy companion dogs in Thailand

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ABSTRACT

Background and Aim: Third-generation cephalosporin (3GC)-resistant *Escherichia coli* in companion animals represents an important One Health concern because apparently healthy dogs may carry antimicrobial-resistant bacteria with virulence-associated characteristics. This study aimed to determine the prevalence of fecal carriage of 3GC-resistant *E. coli* in clinically healthy companion dogs in Khon Kaen, Thailand, and to characterize their antimicrobial resistance (AMR) profiles, extended-spectrum β-lactamase (ESBL) production, bla_{CTX-M} variants, phylogenetic groups, and extraintestinal virulence-associated genes.

Materials and Methods: A cross-sectional study was conducted from June to September 2025 using rectal swabs from 129 clinically healthy dogs attending five veterinary clinics using convenience sampling. 3GC-resistant isolates were identified using selective culture and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. ESBL production was assessed using combination disk testing, and antimicrobial susceptibility was determined using disk diffusion. Polymerase chain reaction and Sanger sequencing were used to characterize bla_{CTX-M} genes. Phylogenetic grouping and virulence-associated gene screening were performed to identify molecular profiles consistent with extraintestinal pathogenic *E. coli* (ExPEC) and uropathogenic *E. coli* (UPEC).

Results: 3GC-resistant *E. coli* was detected in 31/129 dogs (24.0%; 95% confidence interval: 17.4%–32.2%), yielding 32 isolates, including two phenotypically distinct isolates from one dog. Among these, 27/32 (84.4%) exhibited an ESBL-producing phenotype, and 22/27 (81.5%) ESBL-producing isolates carried bla_{CTX-M} genes. The identified variants included $bla_{CTX-M-55}$ (7/22), $bla_{CTX-M-15}$ (6/22), $bla_{CTX-M-27}$ (6/22), and $bla_{CTX-M-14}$ (3/22). Multidrug resistance (MDR) was observed in 25/32 isolates (78.1%). Phylogroup B2 predominated (15/32, 46.9%) and exhibited substantial virulence-gene carriage. One isolate (3.1%) met molecular criteria for UPEC only, five (15.6%) for ExPEC only, and seven (21.9%) for both pathotypes.

Conclusion: Clinically healthy companion dogs carried 3GC-resistant *E. coli* exhibiting MDR and virulence-associated molecular characteristics. These findings highlight the importance of companion animal AMR surveillance and responsible antimicrobial use within the One Health framework. However, the reported prevalence is limited to the sampled dogs and molecular pathotype classification does not independently establish clinical pathogenicity or zoonotic transmission.

Keywords: antimicrobial resistance, companion dogs, *Escherichia coli*, extended-spectrum β-lactamase, extraintestinal pathogenic *Escherichia coli*, multidrug resistance, third-generation cephalosporins, virulence-associated genes.

INTRODUCTION

Escherichia coli is a common inhabitant of the intestinal tract of humans and animals, where it generally exists as part of the normal microbiota. However, certain strains possess virulence-associated characteristics that enable them to cause extraintestinal infections when they enter susceptible anatomical sites [1–3]. Intestinal

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colonization by antimicrobial-resistant *E. coli* with virulence-associated determinants is of particular clinical and public health concern because asymptomatic carriers may harbor strains capable of causing subsequent opportunistic infections or carrying transferable antimicrobial resistance (AMR) determinants [4]. The emergence and dissemination of third-generation cephalosporin (3GC)-resistant Enterobacterales represent a major global AMR challenge because these organisms can compromise the effectiveness of clinically important antimicrobial agents [5]. In *E. coli*, resistance to 3GCs is commonly mediated by the production of extended-spectrum β-lactamases (ESBLs) and AmpC cephalosporinases [6]. Among these mechanisms, CTX-M-type ESBLs have become widely distributed across bacterial populations worldwide. Genes encoding CTX-M enzymes, particularly members of the $bla_{\text{CTX-M}}$ family, are frequently associated with mobile genetic elements (MGEs), which may facilitate horizontal transfer and dissemination of resistance determinants among bacterial populations [7–9].

Companion dogs are closely integrated into human households and frequently share living environments with their owners. This close association creates opportunities for exposure to common environmental sources of antimicrobial-resistant bacteria and potentially facilitates bacterial exchange between animals and humans. Importantly, clinically healthy dogs may asymptomatically carry antimicrobial-resistant *E. coli* in their intestinal tract, thereby contributing to the environmental circulation of resistance-associated bacteria and genetic determinants [7, 10–13]. The detection of antimicrobial-resistant *E. coli* in companion animals is therefore relevant to the One Health framework, which recognizes the interconnected nature of human, animal, and environmental health.

In Thailand, previous investigations have documented the occurrence of 3GC-resistant and $bla_{\text{CTX-M}}$-positive *E. coli* in companion animals. Such organisms have been identified in clinically healthy dogs in Chiang Mai and in clinical specimens obtained from companion animals in other regions of the country [9, 14–16]. These findings demonstrate that antimicrobial-resistant *E. coli* can occur in both apparently healthy and clinically affected companion animals. However, the epidemiological significance of intestinal carriage depends not only on the prevalence of resistant isolates but also on their antimicrobial susceptibility profiles, resistance-associated genetic determinants, phylogenetic backgrounds, and virulence-associated characteristics.

Certain *E. coli* lineages harbor virulence-associated genes linked to extraintestinal pathogenic *E. coli* (ExPEC) or uropathogenic *E. coli* (UPEC). The identification of these molecular characteristics among intestinal isolates may provide information about their potential to cause extraintestinal disease. Nevertheless, the presence of virulence-associated genes or meeting molecular pathotype criteria does not, by itself, establish clinical pathogenicity. Similarly, detection of antimicrobial-resistant *E. coli* in companion dogs does not demonstrate zoonotic transmission. Establishing transmission between animals and humans requires appropriately designed epidemiological investigations and higher-resolution molecular comparisons of isolates obtained from epidemiologically linked hosts.

Despite increasing recognition of antimicrobial-resistant *E. coli* in companion animals, integrated information on the fecal carriage of 3GC-resistant *E. coli* among clinically healthy companion dogs in northeastern Thailand remains limited. Previous investigations have provided evidence of resistant organisms in companion animals from several Thai regions [9, 14–16]; however, the simultaneous characterization of phenotypic AMR, ESBL production, $bla_{\text{CTX-M}}$ variants, phylogenetic groups, and ExPEC- and UPEC-associated virulence genes has not been adequately documented in healthy companion dogs from Khon Kaen Province.

This knowledge gap matters because asymptomatic intestinal carriage may go undetected during routine veterinary care. Moreover, assessing AMR alone may not adequately characterize the diversity of circulating *E. coli* populations. Information on the coexistence of resistance-associated determinants and virulence-associated genes, along with their distribution across phylogenetic groups, is needed to more fully describe the recovered isolates. Such integrated characterization may also help identify molecular profiles warranting further investigation, without assuming that the detected isolates necessarily cause disease or undergo transmission between animal and human hosts.

Khon Kaen Province is an important population and veterinary-service center in northeastern Thailand. However, region-specific evidence describing the carriage prevalence and combined phenotypic and molecular characteristics of 3GC-resistant *E. coli* in clinically healthy companion dogs attending veterinary clinics remains insufficient. Addressing this gap would provide baseline information for companion animal AMR surveillance and help establish priorities for future investigations of resistant bacterial populations at the human–animal interface.

This study aimed to determine the fecal carriage prevalence of 3GC-resistant *E. coli* among clinically healthy

companion dogs attending veterinary clinics in Khon Kaen Province, Thailand, and to characterize the phenotypic and molecular properties of the recovered isolates.

Specifically, the study sought to evaluate antimicrobial resistance patterns and multidrug resistance (MDR), identify phenotypic ESBL production, detect and characterize *bla*$_{CTX-M}$ gene variants, determine the phylogenetic distribution of the isolates, and investigate virulence-associated genes linked to ExPEC and UPEC molecular pathotypes. The study also aimed to describe the occurrence of these characteristics among isolates recovered from asymptomatic dogs, providing region-specific baseline evidence relevant to AMR surveillance. The study further aimed to explore potential associations between selected dog-level characteristics and fecal carriage of 3GC-resistant *E. coli*.

By integrating phenotypic antimicrobial susceptibility testing with molecular characterization of resistance-associated and virulence-associated determinants, this investigation was intended to improve understanding of the diversity of 3GC-resistant *E. coli* carried by clinically healthy companion dogs. The findings may inform future surveillance programs, support responsible antimicrobial use in veterinary practice, and provide a foundation for subsequent One Health investigations involving epidemiologically linked animal, human, and environmental samples.

MATERIALS AND METHODS

Ethical approval

The study protocol, sample size, and procedures for collecting fecal samples from clinically healthy companion dogs were approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Approval No. IACUC-KKU-24/68). Written informed consent was obtained from dog owners before collecting animal-related information and fecal samples. All rectal swabs were collected by licensed veterinarians.

Study period and location

The study was conducted from June to September 2025 at five veterinary clinics in Mueang Khon Kaen District, Khon Kaen Province, northeastern Thailand. The five participating veterinary clinics were purposively selected based on convenience. The participating clinics provided routine veterinary services, including annual health examinations, vaccination, parasite control, and elective surgical procedures.

Study design

A cross-sectional, clinic-based study was conducted to investigate the fecal carriage of 3GC-resistant *E. coli* in clinically healthy companion dogs. Dogs were recruited through convenience sampling, and multiple eligible dogs from the same household could participate. Dogs from the same household were assigned a common household identifier for statistical analysis. Fecal samples were collected using rectal swabs and examined for 3GC-resistant *E. coli*. Recovered isolates were subsequently characterized for ESBL production, antimicrobial susceptibility, *bla*$_{CTX-M}$ gene variants, phylogenetic groups, and virulence-associated genes. Associations between dog-level factors and carriage were evaluated using statistical methods accounting for within-household clustering.

Sample size determination and animal selection

The sample size was calculated using the WinEpi program (http://www.winepi.net/uk/index.htm). The calculation was based on an estimated population of 130,680 owned dogs in Khon Kaen Province, obtained from the Rabies One Data platform (https://rabiesonedata.online/; accessed February 2, 2025). An expected prevalence of 3GC-resistant *E. coli* of 9.2% was assumed based on a previous study [11], with a 95% confidence level and an absolute precision of approximately 5%. No design-effect adjustment was incorporated to account for potential clustering by veterinary clinic or household.

A total of 129 clinically healthy dogs older than 1 year were enrolled from the five participating veterinary clinics. Clinical health status was determined from medical histories and physical examinations performed by licensed veterinarians at enrollment. Eligible dogs were presented for routine veterinary services, including health examinations, vaccination, parasite control, castration, or ovariohysterectomy. Dogs showing clinical evidence of illness or presented for evaluation, treatment, or follow-up of an ongoing illness were excluded. Recent gastrointestinal disturbances, diarrhea, and hospitalization were not systematically assessed as separate exclusion criteria. Multiple dogs from the same household were eligible for inclusion.

Information on previous illnesses and antimicrobial use during the preceding 6 months was obtained directly

from dog owners and was based on owner recall. Only the antimicrobial agents used and their indications were recorded. Treatment dates, duration, dosage, and routes of administration were not collected, and owner-reported antimicrobial exposure was not verified against veterinary medical records.

Fecal sample collection

One fecal sample was collected from each of the 129 enrolled dogs using a rectal swab administered by a licensed veterinarian. Each swab was aseptically placed into a tube containing Luria–Bertani (LB) broth (HiMedia, Maharashtra, India), maintain at 4°C, and processed for selective isolation and identification of 3GC-resistant *E. coli* within 12 hr of collection.

Screening and identification of 3GC-resistant *E. coli*

Upon arrival at the laboratory, fecal samples in LB broth were vortexed to ensure thorough mixing without prior enrichment. Subsequently, 10 µL of each suspension was spread onto eosin methylene blue agar (HiMedia) supplemented with 2 µg/mL cefotaxime and incubated overnight at 37°C [17]. The cefotaxime concentration of 2 µg/mL was selected based on the previously described selective culture method [17] to facilitate the recovery of cefotaxime-tolerant *E. coli*. This screening strategy was not intended to comprehensively detect all clinically relevant mechanisms of resistance to 3GCs.

Three presumptive *E. coli* colonies exhibiting a characteristic green metallic sheen on eosin methylene blue agar were randomly selected as representative colonies and subcultured onto 5% sheep blood agar. When colonies from the same sample exhibited distinct morphologies on blood agar, each morphotype was independently selected for further characterization. When colonies showed identical morphological characteristics, only one representative colony was retained for subsequent analysis.

Bacterial species identification was performed using the Bruker MALDI Biotyper system (Bruker Daltonics, Bremen, Germany), based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. A MALDI Biotyper identification score ≥2.0 was considered acceptable for species-level identification. Instrument calibration and quality control were performed using the Bruker Bacterial Test Standard according to the manufacturer's recommendations.

Phenotypic detection of ESBL production

Susceptibility to cefotaxime and ceftazidime, as representative 3GCs, was assessed using the disk diffusion method. All isolates exhibited resistance to cefotaxime and/or ceftazidime. The isolates were subsequently evaluated for ESBL production using the combination disk test according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [17]. *E. coli* American Type Culture Collection (ATCC) 25922 was used as the ESBL-negative quality control strain. An ESBL-positive control strain was not included in the test.

The antimicrobial disks included ceftazidime (30 µg), cefotaxime (30 µg), ceftazidime/clavulanic acid (30/10 µg), and cefotaxime/clavulanic acid (30/10 µg) (Oxoid, Cheshire, United Kingdom). An increase of ≥5 mm in the inhibition zone diameter around a disk containing clavulanic acid compared with the corresponding antimicrobial disk without clavulanic acid was interpreted as phenotypic confirmation of ESBL production [17].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller–Hinton agar (HiMedia), with *E. coli* ATCC 25922 serving as the quality control strain [18]. Bacterial suspensions were adjusted to a 0.5 McFarland standard before inoculation. Inoculated plates were incubated at 37°C for 16–18 h under aerobic conditions.

The following antimicrobial agents (Oxoid) were evaluated: ampicillin (10 µg), amoxicillin/clavulanic acid (30/10 µg), cephalexin (30 µg), imipenem (10 µg), enrofloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg), sulfamethoxazole/trimethoprim (25 µg), doxycycline (30 µg), and chloramphenicol (30 µg).

The results were categorized as susceptible, intermediate, or resistant according to the applicable interpretive criteria and inhibition zone diameter breakpoints. Canine-specific veterinary breakpoints from the Clinical and Laboratory Standards Institute (CLSI) VET01S, 7th edition (2024), were applied to ampicillin, amoxicillin/clavulanic acid, gentamicin, and amikacin [18]. For imipenem, sulfamethoxazole/ trimethoprim, doxycycline, and chloramphenicol, human CLSI interpretive criteria were applied because canine-specific veterinary breakpoints were unavailable [18]. Human EUCAST criteria (version 16.0, 2026) were used for cephalexin [19], whereas enrofloxacin was interpreted according to CLSI VET01S, 4th edition (2018), because disk diffusion breakpoints were unavailable in the current edition [20].

Isolates resistant to at least one antimicrobial agent in three or more antimicrobial classes were classified as MDR [21]. For MDR classification, the ten antimicrobial agents tested were assigned to eight antimicrobial categories, including penicillins/β-lactamase inhibitor combinations (ampicillin and amoxicillin/clavulanic acid), first-generation cephalosporins (cephalexin), carbapenems (imipenem), fluoroquinolones (enrofloxacin), aminoglycosides (gentamicin and amikacin), folate pathway inhibitors (sulfamethoxazole/trimethoprim), tetracyclines (doxycycline), and phenicols (chloramphenicol). MDR classification was based exclusively on the ten antimicrobial agents tested in this study. Intermediate susceptibility results were not considered resistant for MDR classification.

Detection and characterization of *bla*$_{CTX-M}$ genes

Genomic DNA was extracted from all 3GC-resistant *E. coli* isolates using the boiling method and used as the template for subsequent polymerase chain reaction (PCR) assays [22].

Only isolates exhibiting a phenotypically confirmed ESBL-producing profile were screened for common *bla*$_{CTX-M}$ gene groups, including groups 1, 2, 9, and 8/25, using simplex and multiplex PCR assays as previously described [23]. The primers, annealing temperatures, and expected amplicon sizes are presented in Table 1.

E. coli ATCC 25922 was used as the negative control. The previously characterized *E. coli* strains CUVET16-707 (GenBank accession Nos. CP115356–CP115357) and CUVET20-PYO1 (GenBank accession Nos. CP115390–CP 115391) served as positive controls for *bla*$_{CTX-M}$ groups 1 and 9, respectively [9]. Positive-control strains for *bla*$_{CTX-M}$ groups 2 and 8/25 were unavailable.

PCR amplification was performed in a total reaction volume of 25 µL using 5× FIREPol Master Mix (Solis Biodyne, Tartu, Estonia). Full-length *bla*$_{CTX-M}$ genes were amplified using the corresponding group-specific primers and subjected to bidirectional Sanger sequencing [24]. Forward and reverse sequence reads were quality-checked, trimmed, and assembled to generate consensus sequences. The resulting sequences were compared with reference sequences available in the National Center for Biotechnology Information GenBank database using the Basic Local Alignment Search Tool (BLAST) to identify *bla*$_{CTX-M}$ variants.

The nucleotide sequences generated in this study were deposited in GenBank under accession Nos. QB016904–QB016925.

Phylogenetic grouping and detection of virulence-associated genes

Phylogenetic grouping of *E. coli* isolates was performed using quadruplex and simplex PCR assays to assign isolates to phylogroups A, B1, B2, C, D, E, and F, following the method described by Clermont *et al.* [25]. Isolates yielding unclassifiable phylogenetic profiles were retested to confirm the results.

Virulence-associated genes linked to ExPEC were detected using previously described multiplex PCR protocols [26, 27]. The investigated genes included those associated with adhesion (*afa*, *crl*, *iha*, *papC*, *sfa/foc*, *yfcV*, and *tsh*), cytotoxicity (*hlyA*, *cnf*, *sat*, and *vat*), and iron acquisition (*iroN*, *fyuA*, *chuA*, and *iucD*). Two additional simplex PCR assays were performed to detect *kpsII*, associated with group II capsule formation, and *iutA*, associated with iron acquisition. The primers, amplification conditions, and expected amplicon sizes are provided in Table 1 [23, 24, 26–32].

PCR assays were conducted in a total reaction volume of 25 µL using 5× FIREPol Master Mix (Solis Biodyne). The previously characterized *E. coli* strains CUVET16-707 (GenBank accession Nos. CP115356–CP115357), CUVET20-PYO1 (CP115390–CP115391), CUVET20-PYO4 (CP115376–CP115380), and CUVET21-37 (CP115351–CP115355) were used as positive controls. Collectively, these strains provided positive-control coverage for all investigated virulence-associated genes [9]. DNase-free water was used as a no-template control in this PCR assays. No separate bacterial DNA negative control was included.

Isolates harboring at least two of the five virulence-associated genes (*papC*, *sfa/foc*, *afa*, *iutA*, and *kpsII*) were classified as ExPEC according to the molecular criteria described previously [33]. Isolates carrying at least three of the four genes (*yfcV*, *vat*, *fyuA*, and *chuA*) were classified as UPEC according to the established molecular criteria [29]. These classifications were based on gene detection profiles rather than confirmed clinical pathogenicity.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 29.0.2.0 (20) (IBM Corp., Armonk, NY, USA). Categorical variables were summarized as frequencies and percentages, whereas continuous variables were expressed as medians and interquartile ranges (IQRs). Exploratory descriptive analyses and cross-tabulations were performed to assess data distributions, missing values, and sparse observations across predictor categories.

Table 1: Primers and PCR conditions for detecting *bla*$_{CTX-M}$ genes and ExPEC associated virulence genes in *Escherichia coli* isolates recovered from clinically healthy companion dogs.

Target gene/ Group	Primer name	Sequence (5'–3')	Annealing (°C)	Amplicon (bp)	Reference
bla$_{CTX-M}$ group 1	MultiCTXMGp1_for	TTAGGAARTGTGCCGCTGYA	60	688	[23]
	MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT			
bla$_{CTX-M}$ group 2	MultiCTXMGp2_for	CGTTAACGGCACGATGAC	60	404	[23]
	MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT			
bla$_{CTX-M}$ group 9	MultiCTXMGp9_for	TCAAGCCTGCCGATCTGGT	60	561	[23]
	MultiCTXMGp9_rev	TGATTCTCGCCGCTGAAG			
bla$_{CTX-M-8}$, -25, -26, and -39 to -41	CTX-Mg8/25_for	AACRCRCAGACGCTCTAC	60	800	[23]
	CTX-Mg8/25_rev	TCGAGCCGGAASGTGTYAT			
bla$_{CTX-M}$ group 1	CTX-M-1full_for	CTTCCAGAATAAGGAATCCC	50	949	[24]
	CTX-M-1full_rev	CGTCTAAGGCGATAAACAAA			
bla$_{CTX-M}$ group 9	CTX-M-9full_for	TGACCGTATTGGGAGTTTG	50	902	[24]
	CTX-M-9full_rev	ACCAGTTACAGCCCTTCG			
hlyA	hlyA-f	GTCCATTGCCGATAAGTTT	58	352	[26]
	hlyA-r	AAGTAATTTTTGCCGTGTTTT			
Iha	iha-f	TAGTGCGTTGGGTTATCGCTC	58	609	[26]
	iha-r	AAGCCAGAGTGGTTATTCGC			
afa	afa-f	TAAGGAAGTGAAGGAGCGTG	58	810	[26]
	afa-r	CCAGTAACTGTCCGTGACA			
sfa/foc	sfa-f	GTCCTGACTCATCTGAAACTGCA	58	1242	[26]
	sfa-r	CGGAGAACTGGGTGCATCTTA			
crl	crl-f	TTTCGATTGTCTGGCTGTATG	58	250	[26]
	crl-r	CTTCAGATTCAGCGTCGTC			
cnf	cnf-f	TCGTTATAAAATCAAACAGTG	58	446	[26]
	cnf-r	CTTTACAATATTGACATGCTG			
Sat	sat-f	TGCTGGCTCTGGAGGAAC	58	667	[26]
	sat-r	TTGAACATTCAGAGTACCGGG			
iroN	iroN-f	ATCCTCTGGTCGCTAACTG	58	847	[26]
	iroN-r	CTGCACTGGAAGAACTGTTCT			
papC	papC-f	TGATATCACGCAGTCAGTAGC	58	501	[27]
	papC-r	CCGGCCATATTCACATAAC			
iucD	iucD-f	ACAAAAAGTTCTATCGCTTCC	58	714	[27]
	iucD-r	CCTGATCCAGATGATGCTC			
tsh	tsh-f	ACTATTCTCTGCAGGAAGTC	58	824	[26]
	tsh-r	CTTCCGATGTTCTGAACGT			
vat	vat_for	TCAGGACACGTTCAGGCATTCAGT	63	1100	[28]
	vat_rev	GGCCAGAACATTTGCTCCCTTGTT			
fyuA	fyuA_for	GTAAACAATCTTCCCGCTCGGCAT	63	850	[28]
	fyuA_rev	TGACGATTAACGAACCGGAAGGGA			
chuA	chuA_for	CTGAAACCATGACCGTTACG	63	652	[29]
	chuA_rev	TTGTAGTAACGCACTAAACC			
yfcV	yfcV_for	ACATGGAGACCACGTTCACC	63	292	[30]
	yfcV_rev	GTAATCTGGAATGTGGTCAGG			
iutA	iutA_for	GGCTGGACATCATGGGAACTGG	63	302	[31]
	iutA_rev	CGTCGGGAACGGGTAGAATCG			
kpsII	kpsII-F	GCGCATTTGCTGATACTGTTG	58	577	[32]
	K2-R	AGGTAGTTCAGACTCACACCT			

PCR = Polymerase chain reaction; bp = Base pairs; ExPEC = Extraintestinal pathogenic *Escherichia coli*; CTX-M = CTX-M-type β-lactamase; *bla*$_{CTX-M}$ = Genes encoding CTX-M β-lactamases. Degenerate nucleotide codes: R = A or G; Y = C or T; S = G or C. Primer names and oligonucleotide sequences are presented in roman type. Blank cells indicate values shared with the preceding primer of the same pair.

Associations between dog-level factors and fecal carriage of 3GC-resistant *E. coli* were assessed using univariable generalized estimating equations (GEE) with a binomial distribution and logit link function. An exchangeable working correlation structure and robust covariance estimator were applied, with household specified as the clustering unit to account for potential within-household correlation. Sensitivity analyses were performed by repeating the univariable GEE models using an independent working correlation structure. The resulting odds ratios (ORs), 95% confidence intervals (CIs), and statistical significance were compared with those obtained from the primary exchangeable correlation models to assess the sensitivity of the findings to the assumed working correlation structure.

The results were expressed as ORs with 95% CIs. Because of sparse observations within individual breed categories, breed-specific ORs were not estimated. Instead, the overall association between breed and carriage status was assessed using a household-clustered permutation test with 100,000 permutations to account for within-household correlation.

Individual antimicrobial agents previously administered to exposed dogs were summarized descriptively and were not evaluated as separate predictors because of the small number of exposed animals. A two-sided $p < 0.05$ was considered statistically significant. Unknown, unrecalled, and missing information was excluded from the corresponding analyses.

RESULTS

Demographic and clinical characteristics of healthy dogs

A total of 129 clinically healthy companion dogs were recruited from five participating veterinary clinics, which contributed 62, 53, 4, 8, and 2 dogs, respectively. Of these, 48 dogs (37.2%) belonged to 19 households from which more than one dog was sampled.

The demographic characteristics and clinical histories of the enrolled dogs are presented in Table 2. The median age was 3.0 years (IQR: 2.0–6.0 years; range: 1–16 years) among dogs with available age information. Chihuahuas and mixed breed dogs were the most frequently represented breeds, each accounting for 16.1% of dogs with available breed information. Routine or preventive veterinary care was the most frequently reported reason for clinic attendance (55.0%), followed by grooming or boarding (35.0%) and elective surgery (10.0%). Among the 109 dogs with available antimicrobial use histories, seven (6.4%) had received antimicrobial treatment during the preceding 6 months. Amoxicillin/clavulanic acid was the most frequently reported antimicrobial agent, accounting for six of the seven exposed dogs (85.7%).

Univariable GEE analyses accounting for household clustering identified no statistically significant associations between the evaluated dog-level characteristics and fecal carriage of 3GC-resistant *E. coli* (Table 2). Age showed an inverse association with carriage that approached, but did not reach, statistical significance (OR = 0.89 per 1-year increase; 95% CI: 0.80–1.00; $p = 0.056$). No multivariable analysis was performed because of the limited number of positive dogs and sparse observations in several predictor categories.

Prevalence of fecal carriage of 3GC-resistant *E. coli*

Of the 129 fecal samples examined, 31 were positive for 3GC-resistant *E. coli*, corresponding to a carriage prevalence of 24.0% (31/129; 95% CI: 17.4%–32.2%). Among positive dogs with available age information, the median age was 3.5 years (IQR: 2.0–6.0 years; range: 1–8 years). Only one positive dog with an available antimicrobial use history had received antimicrobial treatment during the preceding 6 months (Table 2).

A total of 32 isolates were recovered from the 31 positive samples. One dog yielded two isolates with distinct colony morphologies, including a typical colony and a hemolytic colony, which were retained separately for subsequent phenotypic and molecular characterization.

Table 2: Demographic and clinical characteristics of healthy dogs and dog-level factors associated with carriage of 3GC-resistant *Escherichia coli*.

Characteristic	Total dogs, n (%)	Positive dogs, n/N (%)	OR (95% CI)	p-value
Veterinary clinic (n = 129)				0.159
A	62 (48.1)	10/62 (16.1)	Reference	—
B	53 (41.1)	18/53 (34.0)	2.47 (0.97–6.27)	0.057
C–E	14 (10.9)	3/14 (21.4)	1.35 (0.32–5.74)	0.683
Age, years (n = 107)	Median 3.0 (IQR: 2.0–6.0); range: 1–16	Median 3.5 (IQR: 2.0–6.0); range: 1–8 (n = 28)	0.89 (0.80–1.00) per year	0.056
Sex (n = 114)				
Female	61 (53.5)	17/61 (27.9)	Reference	—
Male	53 (46.5)	12/53 (22.6)	0.85 (0.38–1.90)	0.686
Breed (n = 112)			Not estimated	0.613
Chihuahua	18 (16.1)	7/18 (38.9)	—	—
Mixed breed	18 (16.1)	7/18 (38.9)	—	—
Pomeranian	17 (15.2)	4/17 (23.5)	—	—
Pembroke Welsh Corgi	10 (8.9)	1/10 (10.0)	—	—

Characteristic	Total dogs, n (%)	Positive dogs, n/N (%)	OR (95% CI)	p-value
Other	8 (7.1)	1/8 (12.5)	—	—
Poodle	7 (6.2)	1/7 (14.3)	—	—
Siberian Husky	6 (5.4)	2/6 (33.3)	—	—
Cavalier King Charles Spaniel	5 (4.5)	2/5 (40.0)	—	—
Bichon Frisé	4 (3.6)	0/4 (0.0)	—	—
Japanese Spitz	4 (3.6)	0/4 (0.0)	—	—
Thai Ridgeback	4 (3.6)	1/4 (25.0)	—	—
Dachshund	3 (2.7)	0/3 (0.0)	—	—
French Bulldog	2 (1.8)	1/2 (50.0)	—	—
Jack Russell Terrier	2 (1.8)	1/2 (50.0)	—	—
Shih Tzu	2 (1.8)	0/2 (0.0)	—	—
Yorkshire Terrier	2 (1.8)	0/2 (0.0)	—	—
Reason for veterinary visit (n = 120)				0.099
Routine/preventive care	66 (55.0)	16/66 (24.2)	Reference	—
Grooming/boarding	42 (35.0)	5/42 (11.9)	0.46 (0.15–1.41)	0.175
Elective surgery	12 (10.0)	5/12 (41.7)	2.33 (0.64–8.56)	0.202
Illness within preceding 6 months (n = 113)				
No	101 (89.4)	25/101 (24.8)	Reference	—
Yes	12 (10.6)	3/12 (25.0)	1.40 (0.36–5.46)	0.628
Antimicrobial use within preceding 6 months (n = 109)				
No	102 (93.6)	25/102 (24.5)	Reference	—
Yes	7 (6.4)	1/7 (14.3)	0.67 (0.15–3.02)	0.600
Antimicrobial agent among exposed dogs (n = 7)			Descriptive only	—
Amoxicillin/clavulanic acid	6/7 (85.7)	0/6 (0.0)	—	—
Cephalexin	1/7 (14.3)	1/1 (100.0)	—	—
Household structure (n = 129)				
Single-dog household	81 (62.8)	22/81 (27.2)	Reference	—
Multiple-dog household	48 (37.2)	9/48 (18.8)	0.47 (0.16–1.42)	0.182

3GC = Third-generation cephalosporin; OR = Odds ratio; CI = Confidence interval; IQR = Interquartile range; GEE = Generalized estimating equations. The total number of dogs varies among characteristics because of missing information. Percentages in the positive dog column represent the proportion of positive dogs within each category. ORs, 95% CIs, and p-values were estimated using univariable logistic GEE with household clustering. Clinics C–E were combined because of small sample sizes. Overall p-values for veterinary clinic and reason for visit were obtained using Wald tests. Breed was assessed using an exact permutation test with 100,000 permutations. Antimicrobial agents were summarized descriptively.

Phenotypic characteristics and AMR profiles

The combination disk test identified 27 of the 32 isolates (84.4%) as phenotypic ESBL producers. The remaining five isolates were ESBL-negative by this test and exhibited resistance profiles suggestive of alternative β-lactam resistance mechanisms, including possible AmpC production.

Antimicrobial susceptibility testing revealed that all 32 isolates were resistant to at least two antimicrobial agents, including ampicillin and cephalexin (Figure 1). None of the isolates exhibited resistance to imipenem or amikacin.

Fifteen distinct AMR profiles were identified among the 32 isolates (Table 3). The most frequently observed profile was resistance to ampicillin and cephalexin, detected in six isolates (18.8%). Overall, 25 isolates (78.1%) met the criteria for MDR, indicating that resistance to multiple antimicrobial classes was common among the recovered isolates.

Table 3: Isolate-level phenotypic and molecular characteristics of 3GC-resistant *Escherichia coli* isolates (n = 32) recovered from clinically healthy companion dogs in Khon Kaen, Thailand, according to phylogroup.

Phylogroup	Isolate ID	Dog ID	ESBL phenotype	*bla*$_{CTX-M}$ variant	Resistance profile	MDR	Molecular pathotype
A	CA07	CA07	Positive	*bla*$_{CTX-M-15}$	AMP–AMC–LEX–SXT	Yes	Neither
A	CA41	CA41	Positive	*bla*$_{CTX-M-14}$	AMP–LEX–ENR–SXT–CHL	Yes	Neither
A	CA62	CA62	Negative	—	AMP–AMC–LEX–ENR–SXT–DOX	Yes	Neither
A	CB18	CB18	Positive	*bla*$_{CTX-M-55}$	AMP–AMC–LEX–GEN–ENR–SXT–CHL	Yes	Neither
A	CB35	CB35	Positive	*bla*$_{CTX-M-55}$	AMP–LEX–GEN–ENR–SXT	Yes	Neither
A	CB54	CB54	Positive	—	AMP–AMC–LEX–ENR–SXT–DOX	Yes	Neither
A	CC03	CC03	Negative	—	AMP–AMC–LEX–ENR–SXT–DOX	Yes	Neither

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Phylogroup	Isolate ID	Dog ID	ESBL phenotype	*bla*$_{CTX-M}$ variant	Resistance profile	MDR	Molecular pathotype
B1	CA61	CA61	Positive	*bla*$_{CTX-M-55}$	AMP–LEX–GEN–DOX	Yes	Neither
B1	CB06	CB06	Positive	*bla*$_{CTX-M-15}$	AMP–LEX	No	Neither
B1	CB51	CB51	Positive	*bla*$_{CTX-M-15}$	AMP–LEX	No	Neither
B1	CB52	CB52	Negative	—	AMP–AMC–LEX–ENR–SXT–DOX	Yes	Neither
B2	CA01	CA01	Negative	—	AMP–AMC–LEX–ENR–SXT	Yes	UPEC
B2	CA15	CA15	Positive	*bla*$_{CTX-M-55}$	AMP–AMC–LEX–GEN–SXT–CHL	Yes	UPEC + ExPEC
B2	CA18	CA18	Positive	*bla*$_{CTX-M-14}$	AMP–LEX–GEN–SXT	Yes	UPEC + ExPEC
B2	CA21	CA21	Positive	—	AMP–AMC–LEX–ENR–SXT–CHL	Yes	ExPEC
B2	CA24	CA24	Positive	*bla*$_{CTX-M-27}$	AMP–LEX–GEN–SXT	Yes	UPEC + ExPEC
B2	CB12	CB12	Positive	*bla*$_{CTX-M-27}$	AMP–AMC–LEX–ENR–SXT–CHL	Yes	ExPEC
B2	CB13	CB13	Positive	*bla*$_{CTX-M-15}$	AMP–LEX	No	Neither
B2	CB30	CB30	Positive	*bla*$_{CTX-M-15}$	AMP–LEX	No	Neither
B2	CB40	CB40	Positive	*bla*$_{CTX-M-27}$	AMP–LEX–GEN–SXT	Yes	UPEC + ExPEC
B2	CB42	CB42	Positive	*bla*$_{CTX-M-27}$	AMP–LEX–GEN–SXT	Yes	UPEC + ExPEC
B2	CB43.H	CB43	Positive	*bla*$_{CTX-M-27}$	AMP–AMC–LEX–SXT	Yes	UPEC + ExPEC
B2	CB43.T	CB43	Positive	—	AMP–AMC–LEX–SXT–CHL	Yes	Neither
B2	CB50	CB50	Positive	*bla*$_{CTX-M-55}$	AMP–AMC–LEX–ENR–SXT–CHL	Yes	ExPEC
B2	CD04	CD04	Positive	*bla*$_{CTX-M-55}$	AMP–LEX	No	Neither
B2	CC02	CC02	Positive	—	AMP–AMC–LEX–SXT	Yes	Neither
D	CA25	CA25	Positive	*bla*$_{CTX-M-15}$	AMP–LEX	No	ExPEC
D	CB17	CB17	Negative	—	AMP–AMC–LEX	No	Neither
D	CB25	CB25	Positive	*bla*$_{CTX-M-55}$	AMP–LEX–GEN–ENR	Yes	ExPEC
D	CB36	CB36	Positive	*bla*$_{CTX-M-14}$	AMP–LEX–DOX	Yes	Neither
D	CB38	CB38	Positive	*bla*$_{CTX-M-27}$	AMP–LEX–GEN–SXT	Yes	UPEC + ExPEC
Unclassifiable	CB05	CB05	Positive	—	AMP–AMC–LEX–SXT	Yes	Neither

3GC = Third-generation cephalosporin; ESBL = Extended-spectrum β-lactamase; MDR = Multidrug resistance; ExPEC = Extraintestinal pathogenic *Escherichia coli*; UPEC = Uropathogenic *Escherichia coli*; AMP = Ampicillin; AMC = Amoxicillin/clavulanic acid; LEX = Cephalexin; GEN = Gentamicin; ENR = Enrofloxacin; SXT = Sulfamethoxazole/trimethoprim; DOX = Doxycycline; CHL = Chloramphenicol. — = No detected *bla*$_{CTX-M}$ variant. Molecular pathotypes were assigned using predefined virulence-gene criteria.

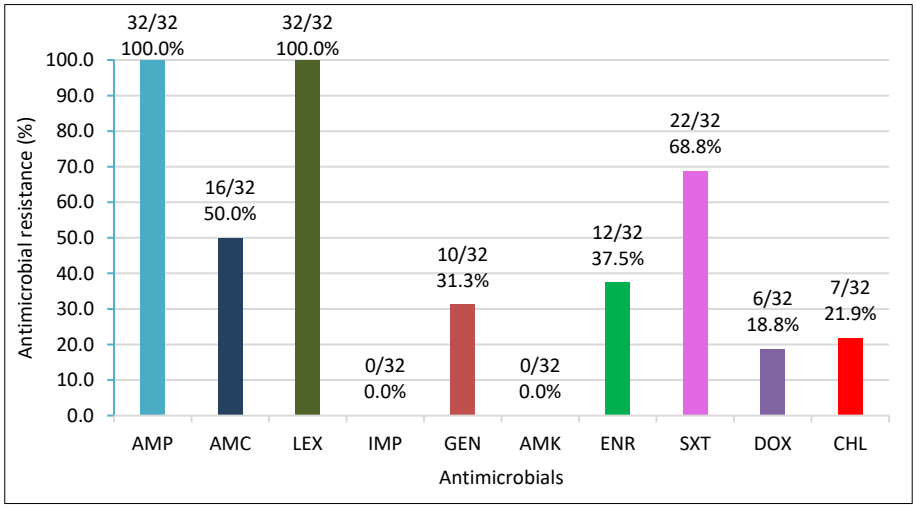


Figure 1: Number and frequency of antimicrobial resistance phenotypes among 3GC-resistant *Escherichia coli* isolates (n = 32) recovered from clinically healthy companion dogs in Khon Kaen, Thailand. Blue, dark blue, dark green, orange, green, pink, purple, and red bars represent aminopenicillins, β-lactam/β-lactamase inhibitor combinations, first-generation cephalosporins, aminoglycosides, fluoroquinolones, folate pathway inhibitors, tetracyclines, and phenicols, respectively. 3GC = Third-generation cephalosporin; AMP = Ampicillin; AMC = Amoxicillin/clavulanic acid; LEX = Cephalexin; IMP = Imipenem; GEN = Gentamicin; AMK = Amikacin; ENR = Enrofloxacin; SXT = Sulfamethoxazole/trimethoprim; DOX = Doxycycline; CHL = Chloramphenicol.

Distribution of *bla*$_{CTX-M}$ genes and variants

The *bla*$_{CTX-M}$ gene family was detected in 22 of the 27 phenotypically ESBL-positive isolates (81.5%; 95% CI: 63.3%–91.8%).

Among the 22 *bla*$_{CTX-M}$-positive isolates, group 1 was the most prevalent, accounting for 13 isolates (59.1%).

This group comprised *bla*$_{CTX-M-15}$ (6/22, 27.3%) and *bla*$_{CTX-M-55}$ (7/22, 31.8%). Group 9 was detected in nine isolates (40.9%), comprising *bla*$_{CTX-M-14}$ (3/22, 13.6%) and *bla*$_{CTX-M-27}$ (6/22, 27.3%).

No *bla*$_{CTX-M}$ genes belonging to groups 2 or 8/25 were detected. Furthermore, no isolate carried more than one detected *bla*$_{CTX-M}$ variant.

Phylogenetic distribution of 3GC-resistant *E. coli*

Four phylogroups were identified among the 32 isolates (Table 4). Phylogroup B2 was predominant, comprising 15 isolates (46.9%), followed by phylogroup A (7 isolates; 21.9%), phylogroup D (5 isolates; 15.6%), and phylogroup B1 (4 isolates; 12.5%). One isolate (3.1%) remained unclassifiable after repeat testing.

Table 4: Distribution of virulence-associated genes and molecular pathotypes among 3GC-resistant *Escherichia coli* isolates (n = 32) recovered from clinically healthy companion dogs in Khon Kaen, Thailand, according to phylogroup.

Phylogroup	Isolates, n (%)	Median number of virulence genes (range)	UPEC only, n (%)	ExPEC only, n (%)	UPEC and ExPEC, n (%)
A	7 (21.9)	2 (2–3)	0 (0.0)	0 (0.0)	0 (0.0)
B1	4 (12.5)	1 (1–3)	0 (0.0)	0 (0.0)	0 (0.0)
B2	15 (46.9)	6 (2–11)	1 (3.1)	3 (9.4)	6 (18.8)
D	5 (15.6)	5 (3–11)	0 (0.0)	2 (6.3)	1 (3.1)
Unclassifiable	1 (3.1)	1 (1)	0 (0.0)	0 (0.0)	0 (0.0)
Total	32 (100)	3 (1–11)	1 (3.1)	5 (15.6)	7 (21.9)

3GC = Third-generation cephalosporin; UPEC = Uropathogenic *Escherichia coli*; ExPEC = Extraintestinal pathogenic *Escherichia coli*. Percentages are calculated using all 32 isolates. Molecular pathotypes were assigned using predefined virulence-gene criteria.

Distribution of *bla*$_{CTX-M}$ variants by phylogroup and MDR status

Among the 22 *bla*$_{CTX-M}$-positive isolates, 11 (50.0%) belonged to phylogroup B2, four (18.2%) each to phylogroups A and D, and three (13.6%) to phylogroup B1 (Table 3). Overall, 17 of the 22 *bla*$_{CTX-M}$-positive isolates (77.3%) were classified as MDR.

Of the six isolates carrying *bla*$_{CTX-M-27}$, five (83.3%) belonged to phylogroup B2, and all six exhibited MDR. In contrast, *bla*$_{CTX-M-55}$ was distributed across phylogroups A, B1, B2, and D, with six of the seven isolates (85.7%) classified as MDR.

Distribution of virulence-associated genes and molecular pathotypes

The distribution of virulence-associated genes across phylogroups is presented in Table 4. All 32 isolates carried at least one of the investigated virulence-associated genes, with *crl*, associated with curli fimbriae, detected in every isolate.

The number of virulence-associated genes detected per isolate ranged from 1 to 11. Phylogroups B2 and D exhibited the highest numbers of detected virulence-associated genes, with median counts of six (range: 2–11) and five (range: 3–11) genes per isolate, respectively. These genes represented several functional categories, including adhesion, cytotoxicity, iron acquisition, and capsule-associated functions.

In contrast, isolates belonging to phylogroups A and B1, together with the unclassifiable isolate, carried fewer virulence-associated genes and lacked the investigated genes associated with cytotoxicity and capsule formation.

Among the 32 isolates, one (3.1%) met the molecular criteria for UPEC only, five (15.6%) for ExPEC only, and seven (21.9%) for both UPEC and ExPEC. All isolates meeting these molecular pathotype criteria belonged to phylogroups B2 or D (Tables 3 and 4).

DISCUSSION

Fecal carriage of 3GC-resistant *E. coli* in healthy companion dogs

This study identified clinically important 3GC-resistant *E. coli* in fecal samples from clinically healthy companion dogs in Khon Kaen, Thailand, demonstrating that apparently healthy animals can asymptomatically carry antimicrobial-resistant bacteria with potential pathogenic significance. Given the importance of 3GCs in treating bacterial infections in human and veterinary medicine, the occurrence of resistant *E. coli* in companion dogs represents a relevant One Health concern.

The prevalence of fecal carriage of 3GC-resistant *E. coli* was 24.0%, exceeding the prevalence reported in Brazil (9.2%) and Japan (14.6%) but remaining lower than that reported in Romania (44.1%) [10–12]. Within Thailand, the observed prevalence was comparable to previous findings among healthy dogs (25.8%) and dogs

with periodontal disease (29.4%) in Chiang Mai [14, 34]. Differences in prevalence across geographical settings may reflect variations in environmental conditions, sanitation, animal population density, antimicrobial use practices, and exposure to resistant bacteria [7, 35]. However, differences in sampling strategies and study populations should also be considered when comparing these estimates.

The convenience sampling strategy, with most dogs recruited from two of the five participating clinics, limits the generalizability of the observed prevalence. Although dogs presented for elective surgery may have differed in previous veterinary healthcare exposure from those attending for routine or preventive care, the reason for veterinary attendance was not significantly associated with 3GC-resistant *E. coli* carriage. Furthermore, the inclusion of multiple dogs from some households warrants consideration when interpreting dog-level prevalence estimates and associated factors.

Potential sources and determinants of antimicrobial-resistant *E. coli* carriage

The capacity of *E. coli* to acquire and disseminate AMR genes through horizontal gene transfer, particularly via MGEs such as plasmids, contributes to the persistence and spread of AMR. Consequently, intestinal *E. coli* populations may acquire and maintain resistance determinants even in the absence of clinical infection or recent antimicrobial treatment [36].

Companion dogs may encounter resistant *E. coli* through contaminated food, environmental exposure, or contact with colonized animals or humans [36, 37]. Previous antimicrobial exposure may also contribute to the selection and persistence of resistant intestinal bacteria. These mechanisms offer possible explanations for the detection of resistant isolates among apparently healthy dogs without reported recent antimicrobial treatment. However, the present study did not investigate the sources of colonization or directly assess transmission.

Recent antimicrobial use was not significantly associated with carriage. Nevertheless, this finding should be interpreted cautiously because antimicrobial use histories relied on owner recall, information was unavailable for some dogs, and only one positive dog with an available history had received antimicrobial treatment during the preceding 6 months. The small number of exposed dogs substantially limited the statistical power to identify an association.

ESBL production and distribution of *bla*$_{\text{CTX-M}}$ variants

Phenotypic ESBL production was detected in 84.4% of the 3GC-resistant *E. coli* isolates, and 81.5% (22/27) of the ESBL-producing isolates carried *bla*$_{\text{CTX-M}}$ genes. These findings are consistent with previous studies identifying CTX-M-type enzymes as important contributors to 3GC resistance among *E. coli* isolates from humans and animals [6, 8].

The ESBL-producing isolates in which *bla*$_{\text{CTX-M}}$ genes were not detected may harbor other ESBL-associated genes, including *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, or *bla*$_{\text{CTX-M}}$ variants outside the investigated targets. Similarly, the five ESBL-negative isolates may possess alternative β-lactam resistance mechanisms, including AmpC β-lactamase production. Plasmid-mediated AmpC genes, particularly *bla*$_{\text{CMY-2}}$, have been reported in companion animals [23]. However, neither AmpC production nor the associated resistance genes were confirmed in this study. Therefore, the mechanisms responsible for 3GC resistance in these isolates remain unresolved.

The molecular epidemiology of ESBL-associated genes varies geographically and temporally [6, 8]. Although *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$ are widely reported variants [8], *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-27}}$ were the most frequently detected variants in the present study, each accounting for 31.8% and 27.3%, respectively, of the *bla*$_{\text{CTX-M}}$-positive isolates.

The relatively frequent detection of *bla*$_{\text{CTX-M-55}}$ is consistent with reports documenting its increasing occurrence among *E. coli* populations in Asia, particularly China [8, 38–40]. The CTX-M-55 enzyme differs from CTX-M-15 by an Ala77Val amino acid substitution, which has been associated with differences in enzymatic activity against certain cephalosporins [38]. The dissemination of *bla*$_{\text{CTX-M-55}}$ has also been linked to resistant *E. coli* populations in clinical settings and food-producing animals, frequently involving plasmid-mediated resistance [8, 38–40]. Nevertheless, the present study did not investigate the plasmid location or genetic context of the detected genes.

Epidemiological significance of *bla*$_{\text{CTX-M-27}}$

Among group 9 variants, *bla*$_{\text{CTX-M-27}}$ was detected more frequently than *bla*$_{\text{CTX-M-14}}$. The increasing occurrence of *bla*$_{\text{CTX-M-27}}$ has been documented in Japan, Thailand, and several European countries, highlighting its epidemiological importance in community-associated and healthcare-associated *E. coli* populations [15, 41–43].

The international expansion of the *E. coli* ST131 C1-M27 subclone, which belongs to phylogroup B2, has been associated with the dissemination of *bla*$_{CTX-M-27}$ [44, 45]. Previous studies have also reported characteristics that may contribute to the successful spread of this lineage [42, 44].

In the present study, five of the six *bla*$_{CTX-M-27}$-positive isolates belonged to phylogroup B2, and all six exhibited MDR. Although this distribution is compatible with the phylogenetic background associated with ST131 C1-M27, phylogroup assignment alone cannot establish sequence type or subclonal identity. Therefore, the presence of ST131 C1-M27 cannot be inferred without additional characterization using multilocus sequence typing or whole-genome sequencing.

AMR profiles and implications for treatment

All 32 isolates were resistant to ampicillin and cephalexin, and 78.1% were classified as MDR. The frequent occurrence of MDR phenotypes may reflect the coexistence of multiple resistance determinants on plasmids or other MGEs, as previously described [46]. However, the genetic organization and potential linkage of resistance determinants were not investigated in this study.

The detection of 3GC-resistant and MDR *E. coli* in healthy companion dogs raises concerns about potential limitations in antimicrobial treatment options if these organisms subsequently cause opportunistic infections. Nevertheless, resistance profiles obtained from fecal carriage isolates should not be interpreted as direct evidence of treatment failure in clinical infections.

No resistance to imipenem or amikacin was detected among the investigated isolates. These findings describe *in vitro* susceptibility under the testing conditions and do not establish clinical treatment efficacy. Furthermore, the absence of detected resistance should not be interpreted as justification for empirical use of these agents. Appropriate antimicrobial stewardship, susceptibility-guided treatment, and avoidance of unnecessary exposure to critically important antimicrobials remain essential in companion animal practice.

Phylogenetic distribution and virulence-associated characteristics

In addition to AMR, a substantial proportion of the 3GC-resistant *E. coli* isolates carried virulence-associated genes. Overall, 13 of the 32 isolates (40.6%) met the molecular criteria for UPEC, ExPEC, or both. These isolates possessed combinations of genes previously associated with extraintestinal infections [29, 33]. However, molecular classification based on selected virulence determinants indicates potential pathogenic characteristics and does not demonstrate clinical pathogenicity or the ability to cause disease.

Phylogroups B2 and D collectively accounted for 62.5% of the isolates. Phylogroup B2, in particular, has frequently been associated with ExPEC lineages and a greater prevalence of virulence-associated determinants [9]. Consistent with this association, isolates belonging to phylogroups B2 and D exhibited higher numbers of detected virulence-associated genes than those belonging to phylogroups A and B1.

The predominance of phylogroups B2 and D among resistant fecal isolates suggests that clinically healthy companion dogs may carry *E. coli* populations possessing both AMR and virulence-associated characteristics. However, these findings do not establish that the isolates are capable of causing extraintestinal infection or that transmission between dogs and humans has occurred.

Phylogroups A and B1 were less frequently represented and are commonly associated with intestinal commensal *E. coli* populations [32]. Nevertheless, phylogroup membership alone is insufficient to determine whether an isolate is commensal or pathogenic, because resistance and virulence characteristics can vary within each phylogroup.

One isolate remained unclassifiable after repeated testing using the phylogrouping approach [25]. Although this PCR-based method is widely used, some isolates may yield ambiguous or unclassifiable profiles. Sequence-based characterization could provide greater phylogenetic resolution. In addition, the selective isolation of 3GC-resistant *E. coli* may have influenced the observed phylogroup distribution and the representation of associated virulence determinants [9, 47].

One Health implications and future surveillance

The occurrence of 3GC-resistant *E. coli* carrying virulence-associated genes in clinically healthy dogs has implications for One Health surveillance. Close contact between companion animals and their owners may create opportunities for the exchange of resistant bacteria, while shared environmental exposures may also contribute to colonization.

The present findings support the potential importance of healthy companion dogs as carriers of

antimicrobial-resistant *E. coli*. However, the study did not include paired human or environmental samples, and the direction, frequency, or occurrence of transmission could not be determined.

Integrated surveillance incorporating companion animals, humans, and shared environments would help clarify the distribution and potential movement of resistant *E. coli* populations. Combining longitudinal sampling with higher-resolution genomic characterization, including sequence typing, resistance-gene localization, and plasmid analysis, would further improve understanding of colonization persistence, bacterial relatedness, and possible transmission pathways.

Limitations

Several limitations should be considered when interpreting these findings. First, the clinic-based convenience sampling strategy, modest sample size, and unequal recruitment across clinics may limit the representativeness and generalizability of the results. The inclusion of multiple dogs from some households also introduced potential clustering. Although household clustering was considered in the association analyses, residual effects related to clinic-level sampling cannot be excluded.

Second, recent gastrointestinal disturbances, diarrhea, and hospitalization were not systematically assessed as separate exclusion criteria, although these factors may have influenced intestinal bacterial carriage. Furthermore, antimicrobial use histories depended on owner recall, and some demographic and clinical-history variables were incomplete. The small number of dogs with reported recent antimicrobial exposure limited the ability to assess antimicrobial use as a potential determinant of carriage.

Third, the restricted number of colonies selected for characterization may have underestimated within-host diversity. The molecular investigation was limited to selected resistance- and virulence-associated genes and did not include confirmatory AmpC testing, comprehensive resistance-gene detection, multilocus sequence typing, whole-genome sequencing, or plasmid characterization.

Fourth, the molecular criteria used to identify UPEC- and ExPEC-associated isolates indicate the presence of selected virulence-associated genes rather than confirmed pathogenicity.

Finally, the absence of paired human and environmental sampling, direct transmission analysis, longitudinal follow-up, and quantitative bacterial-load measurements prevented evaluation of transmission pathways, colonization duration, and bacterial carriage dynamics.

Despite these limitations, the study provides baseline evidence of 3GC-resistant *E. coli* carriage and associated resistance and virulence characteristics among clinically healthy companion dogs in Khon Kaen, Thailand, supporting further genomic and integrated One Health investigation.

CONCLUSION

This study demonstrated that clinically healthy companion dogs in Khon Kaen, Thailand, can carry 3GC-resistant *E. coli* in their intestinal tract, with a fecal carriage prevalence of 24.0%. Among the 32 recovered isolates, 84.4% exhibited an ESBL phenotype, 78.1% were classified as MDR, and 81.5% of the ESBL-producing isolates carried $bla_{\text{CTX-M}}$ genes. The predominant variants were $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-27}}$. Phylogroup B2 was the most prevalent, and 40.6% of the isolates met the molecular criteria for UPEC, ExPEC, or both. These findings demonstrate the coexistence of AMR and virulence-associated genetic characteristics in *E. coli* carried by apparently healthy dogs.

The detection of resistant *E. coli* in clinically healthy companion dogs highlights the importance of antimicrobial stewardship, appropriate infection prevention, and hygiene practices in veterinary clinics and households. Although no resistance to imipenem or amikacin was detected, these findings should not be interpreted as evidence of clinical treatment efficacy or justification for empirical antimicrobial use. The presence of resistant isolates carrying virulence-associated genes also supports the inclusion of healthy companion animals in AMR surveillance programs.

The study combined phenotypic antimicrobial susceptibility testing, ESBL detection, $bla_{\text{CTX-M}}$ gene characterization, phylogenetic grouping, and virulence-associated gene profiling. This integrated approach provided information on resistance phenotypes, selected genetic determinants, and molecular pathotype characteristics within the same collection of isolates. The inclusion of dogs attending multiple veterinary clinics also provided baseline data on asymptomatic carriage in a companion animal population in northeastern Thailand.

Clinically healthy companion dogs in Khon Kaen can asymptomatically carry 3GC-resistant *E. coli* exhibiting MDR phenotypes and virulence-associated genetic characteristics. The detection of $bla_{\text{CTX-M}}$ variants,

particularly *bla*$_{CTX-M-55}$ and *bla*$_{CTX-M-27}$, emphasizes the importance of continued molecular surveillance. Although zoonotic transmission and clinical pathogenicity were not demonstrated, these findings provide baseline evidence supporting responsible antimicrobial use, preventive hygiene measures, and integrated One Health surveillance to better understand the distribution and potential dissemination of resistant *E. coli* among companion animals, humans, and their shared environments.

DATA AVAILABILITY

The data supporting the findings of this study are included in the published article. Additional data are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

During the preparation of this work, the authors used ChatGPT in order to improve language clarity and grammatical structure. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

AUTHORS' CONTRIBUTIONS

PS: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, and writing – original draft preparation. CN, TK, PhS, and AP: Data curation, formal analysis, investigation, visualization, and writing – review and editing. PSi: Formal analysis, investigation, validation, and writing – review and editing. PN: Investigation and writing – review and editing. PP: Investigation, resources, supervision, and writing – review and editing. SJ: Conceptualization, methodology, project administration, resources, supervision, validation, and writing – review and editing. All authors have read and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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