

Genomic Characterization of ESBL/AmpC-Producing *Escherichia coli* in Stray Dogs Sheltered in Yangzhou, China

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Purpose: Limited data are available on the prevalence and antimicrobial resistance of extended spectrum β -lactamase- (ESBL) and AmpC β -lactamase-producing *Escherichia coli* in stray dogs. We aimed to investigate the genomic characteristics of ESBL/AmpC-producing *E. coli* isolated from stray dogs sheltered in Yangzhou, China.

Methods: We collected 156 samples including 115 fecal swabs, 35 kennel floor swabs, two breeder hand and shoe sole swabs, and four feed samples. The isolates were tested for resistance by antimicrobial susceptibility testing and further analyzed for cefotaxime-resistant *E. coli* isolates by whole genome sequencing.

Results: We identified 80 cefotaxime-resistant *E. coli* isolates (51.3%), 59 isolates (73.8%) from feces and 21 (26.2%) from the environment. Whole-genome sequencing analysis showed that *bla*_{CTX-M-15} (n=30) and *bla*_{CTX-M-55} (n=29) were the most prevalent genotypes. Two isolates only carried the AmpC β -lactamase gene *bla*_{CMY-2}; one isolate had a combination of AmpC β -lactamase gene *bla*_{DHA-1} and ESBL β -lactamase gene *bla*_{CTX-M-14}. Other important resistance genes such as *bla*_{OXA-10}, *bla*_{TEM-1B}, *bla*_{TEM-135}, *bla*_{TEM-106}, *tet(A)*, *qnrS1*, *qnrB4*, and *oqxAB* were also detected. The serotype combination was highly abundant, with O10:H25 predominating (n=12). Most cefotaxime-resistant *E. coli* isolates belonged to phylogroup A (62.5%, n=50), followed by phylogroup B1 (26.3%, n=21). Thirty different sequence types (STs) and 27 distinct plasmid replicons were identified, among which ST2325 (n=12) and IncFII (n=38) was the most frequent ST and plasmid, respectively. ESBL/AmpC-producing isolates were divided into four major clades; clade IV was the primary lineage containing 37 isolates from feces and 13 from the environment. Three high-risk *E. coli* clone ST23 strains and one ST10 strain belonged to clades III and IV, respectively.

Conclusion: Our study provides a comprehensive overview of resistance profiles and genomic characteristics in ESBL/AmpC-producing *E. coli* and highlights the possible role of stray dogs as an antibiotic resistance gene reservoir.

Keywords: *Escherichia coli*, ESBL/AmpC, stray dogs, antimicrobial resistance, whole genome sequencing

Plain Language Summary

1. High prevalence (51.3%) of ESBL/AmpC-producing *E. coli* was found among stray dogs and in their surrounding environment.
2. *bla*_{CTX-M-15} and *bla*_{CTX-M-55} were the most prevalent genotypes.
3. Stray dogs could be an antibiotic resistance gene reservoir, representing a significant risk to public health.

Introduction

Antimicrobial resistance (AMR) has been highlighted as a serious threat to public health worldwide. Owing to their high efficacy and low toxicity, cephalosporins are widely used in human clinical therapy and veterinary medicine.¹ However, the intensive use of cephalosporins has led to the widespread dissemination of cephalosporins resistance in bacteria. An

important mechanism of resistance to cephalosporins in *Escherichia coli* is the production of extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases (AmpCs).²

ESBLs mainly contain *sulphydryl variable* (SHV), temoniera (TEM), oxacillinase (OXA), and cefotaximases (CTX-M). Currently, CTX-M type ESBL has become the most common ESBL worldwide, and the most common host is *E. coli*.³ Among the different CTX-M subtypes, CTX-M-1 and CTX-M-9 groups are predominant worldwide.^{4,5} High levels of AmpC β -lactamases via overexpression of the chromosomally encoded AmpC gene (c-AmpC) and/or presence of plasmid-mediated *ampC* gene (p-AmpC) are also important mechanisms mediating resistance to cephalosporins.⁶

ESBL/AmpC-encoding *E. coli* isolates have been widely reported worldwide, and with the assistance of mobile elements, have become one of the fastest-transmitting resistant pathogens in humans, animals, and the environment.⁷

Human-animal transmission of ESBL/AmpC-producing bacteria was previously described, non-human sources are important hosts involved in further spread of bacterial infections. Unlike wild animals or food animals, which have received widespread attention, the role of animals with a sheltered or stray background as important carriers of drug-resistant bacteria has been underestimated.^{8–10} The close contact of these animals with humans in some cases provides a suitable bridge for cross-species transmission of multidrug-resistant (MDR) bacteria. In fact, AMR surveillance of stray animals (mainly dogs) is critical because they may play a role in the transmission of MDR bacteria to humans via direct or indirect contact when sharing the same environment.¹¹ Potential risk factors for ESBL/AmpC carriage in stray dogs are free movement across large areas, scavenging of food remains and human/animal waste, lack of sanitation, and exposure to the environmental resistome.¹²

Currently, studies on ESBL/AmpC-carrying *E. coli* in stray dogs are scarce, and the potential transmission of ESBL/AmpC-carrying *E. coli* from stray dogs to the surrounding environment or humans has not been investigated in detail. Therefore, we aimed to investigate the prevalence and characterization of ESBL/AmpC-producing *E. coli* isolates from stray dogs and related environments and to elucidate their genetic diversity and dissemination mechanisms.

Materials and Methods

Sample Collection and Bacterial Isolation

A total of 156 samples were collected from Yangzhou Canine Inspection and Quarantine Institute in July 2021, including 115 fecal swabs, 35 kennel floor swabs, two breeder hand and shoe sole swabs, and four feed samples. The samples were pre-enriched in 2 mL of Luria-Bertani broth at 37 °C, 180 rpm for 24 hours. Ten-microliter aliquots of each enriched samples were inoculated on MacConkey agar plates supplemented with 2 mg/L cefotaxime and incubated at 37 °C overnight. One *E. coli* isolate per plate was selected for further purification and identified using Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using agar dilution or broth microdilution methods (limited to colistin and tigecycline). The tested antibiotics included ampicillin, cefazolin, cefotaxime, meropenem, gentamicin, amikacin, streptomycin, tetracycline, tigecycline, nalidixic acid, ciprofloxacin, colistin, trimethoprim-sulfamethoxazole, chloramphenicol, florfenicol, and fosfomycin. Results were interpreted according to the 2020 Clinical and Laboratory Standards Institute guidelines, except the breakpoints of streptomycin, tigecycline, and florfenicol were interpreted using European Committee on Antimicrobial Susceptibility Testing guidelines (<https://www.eucast.org/>). *E. coli* ATCC 25922 was used as the quality control strain.

Whole-Genome Sequencing and Analysis

All cefotaxime-resistant *E. coli* isolates were sequenced using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA), and at least 100-fold coverage of raw reads were collected. The raw sequence data were assembled into contigs using SPAdes 3.10.0.¹³ Species identification was also performed based on the genomic data (<https://pathogen.watch/>). Sequence types (STs) were identified using the *E. coli* Multilocus Sequence Typing (MLST) database (<https://bigsd.b.pasteur.fr/ecoli/>). Antimicrobial resistance genes and plasmid replicons were analyzed via the Center for Genomic Epidemiology pipeline

(<https://cge.cbs.dtu.dk/>). *In silico* *E. coli* phylogroup stratification was performed using ClermonTyping (<http://clermontyping.iame-research.center/>).¹⁴ The phylogenetic tree based on core genome single nucleotide polymorphisms (SNPs) was generated using ParSNP v. 1.2 (<https://github.com/marbl/parsnp>).¹⁵ ChiPlot (<http://chiplot.online/#>) was used to visualize the phylogenetic tree.

Results

Prevalence of Cefotaxime-Resistant *E. coli* Strains and Antimicrobial Susceptibility

Of total 156 samples, 80 cefotaxime-resistant *E. coli* isolates (51.3%) were identified, including 59 isolates (73.8%) from feces and 21 isolates (26.2%) from the environment. The environmental isolates included 20 isolates from floor swabs and one from breeder shoe sole swabs.

Eighty cefotaxime-resistant *E. coli* isolates showed differential resistance to 16 antibiotics (Table 1). All isolates were resistant to ampicillin and cefotaxime. High resistance rates (>60%) were observed for tetracycline and trimethoprim-sulfamethoxazole. However, all the isolates were susceptible to meropenem, amikacin, colistin, and tigecycline. Most cefotaxime-resistant *E. coli* isolates, including those of feces origin (57/59, 96.6%) and environmental origin (20/21, 95.2%), exhibited MDR phenotypes (≥ 3 antimicrobial classes) (Figure 1). The isolates from feces had more complex drug resistance phenotypes than those from the environment, and two isolates from feces showed resistance to 12 agents, with a resistance spectrum of ampicillin, cefazolin, cefotaxime, gentamicin, streptomycin, tetracycline, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, florfenicol, and fosfomycin.

Distribution of Antimicrobial Resistance Genes in Cefotaxime-Resistant *E. coli*

In total, 43 different resistance determinants were identified from 80 cefotaxime-resistant *E. coli* isolates, which was consistent with their MDR phenotypes. Eight *bla*_{CTX-M} variants were identified among 78 isolates (97.5%), namely, *bla*_{CTX-M-15} (n=30), *bla*_{CTX-M-55} (n=29), *bla*_{CTX-M-14} (n=9), *bla*_{CTX-M-132} (n=4), *bla*_{CTX-M-27} (n=2), *bla*_{CTX-M-65} (n=2), *bla*_{CTX-M-3} (n=1), and *bla*_{CTX-M-102} (n=1) (Figure 2). Additionally, two isolates only carried the AmpC β -lactamase gene *bla*_{CMY-2}. One isolate had a combination of AmpC β -lactamase gene *bla*_{DHA-1} and ESBL β -lactamase gene *bla*_{CTX-M-14}. Other β -lactamase genes were also detected, such as *bla*_{OXA-10} (n=2) and TEM-type genes (30.0%, 24/80), including *bla*_{TEM-1B} (n=16), *bla*_{TEM-135} (n=4), and *bla*_{TEM-106} (n=3).

Table 1 Antimicrobial Resistance Profiles of 80 Cefotaxime-Resistant *Escherichia coli* Isolates

Antibiotics	MIC Breakpoint $\mu\text{g/mL}$	Resistance % (No. of Resistant Isolates)		
		Feces (n = 59)	Environment (n = 21)	Total (n = 80)
Ampicillin	≥ 32	100.0 (59)	100.0 (21)	100.0 (80)
Cefazolin	≥ 8	25.4 (15)	23.8 (5)	25.0 (20)
Cefotaxime	≥ 4	100.0 (59)	100.0 (21)	100.0 (80)
Meropenem	≥ 4	0 (0)	0 (0)	0 (0)
Gentamicin	≥ 16	25.4 (15)	9.5 (2)	21.3 (17)
Amikacin	≥ 64	0 (0)	0 (0)	0 (0)
Streptomycin	≥ 64	25.4 (15)	23.8 (5)	25.0 (20)
Tetracycline	≥ 16	81.4 (48)	85.7 (18)	82.5 (66)
Tigecycline	≥ 1	0 (0)	0 (0)	0 (0)
Nalidixic acid	≥ 32	32.2 (19)	28.6 (6)	31.3 (25)
Ciprofloxacin	≥ 1	28.8 (17)	19.0 (4)	26.3 (21)
Colistin	≥ 4	0 (0)	0 (0)	0 (0)
Trimethoprim-sulfamethoxazole	$\geq 4/76$	61.0 (36)	71.4 (15)	63.8 (51)
Chloramphenicol	≥ 32	32.2 (19)	47.6 (10)	36.3 (29)
Florfenicol	≥ 32	32.2 (19)	42.9 (9)	35.0 (28)
Fosfomycin	≥ 256	28.8 (17)	4.8 (1)	22.5 (18)

Abbreviation: MIC, minimum inhibitory concentration.

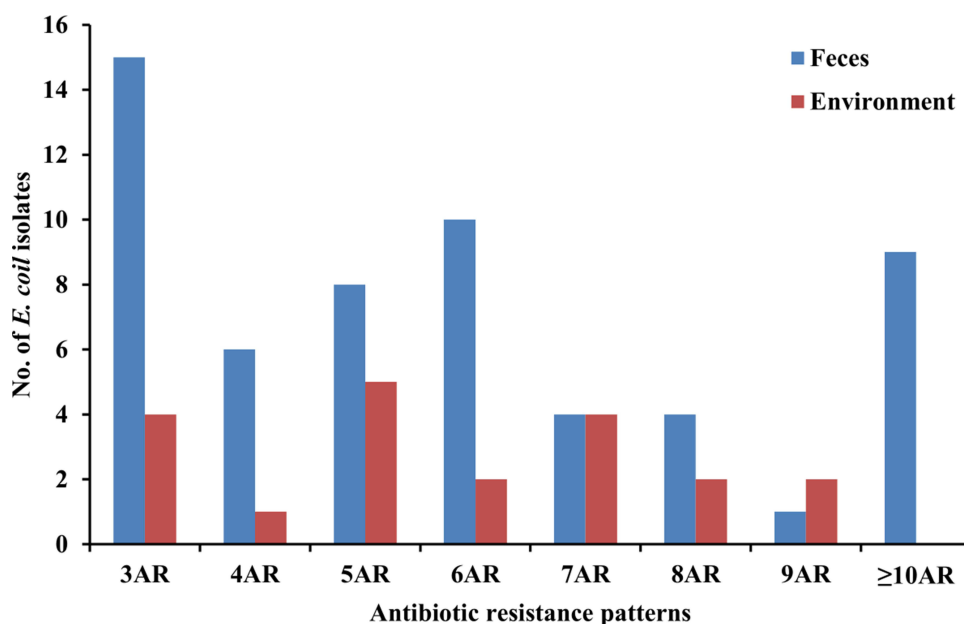


Figure 1 Antimicrobial resistance (AR) patterns of cefotaxime-resistant *E. coli* isolates from stray dog feces and the environment.

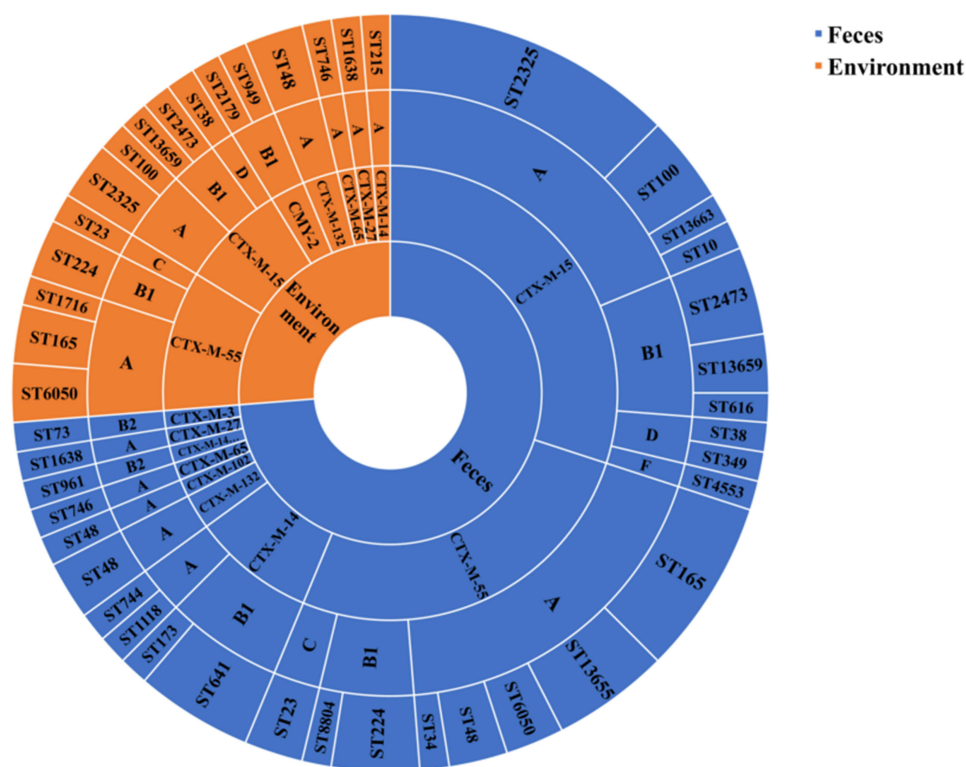


Figure 2 Distribution of ESBL/AmpC β-lactamase genes, sequence types (STs), and phylogroups in *E. coli* isolates.

In addition to β-lactamase, genes accounting for resistance to aminoglycosides, trimethoprim, sulfonamides, tetracyclines, macrolides, phenicols, rifampicin, Fosfomycin, and fluoroquinolones were also identified (Figure 3). The *tet(A)* gene, which confers resistance to tetracycline, was detected in 57 isolates (71.3%). Plasmid-mediated quinolone resistance gene *qnrS1* was detected in 57 isolates (71.3%); *qnrB4* and *oqxAB* were also found in one isolate (1.2%) and two isolates (2.5%), respectively. The phenicol resistance gene *floR* (32.5%, 26/80), the sulfonamide resistance gene

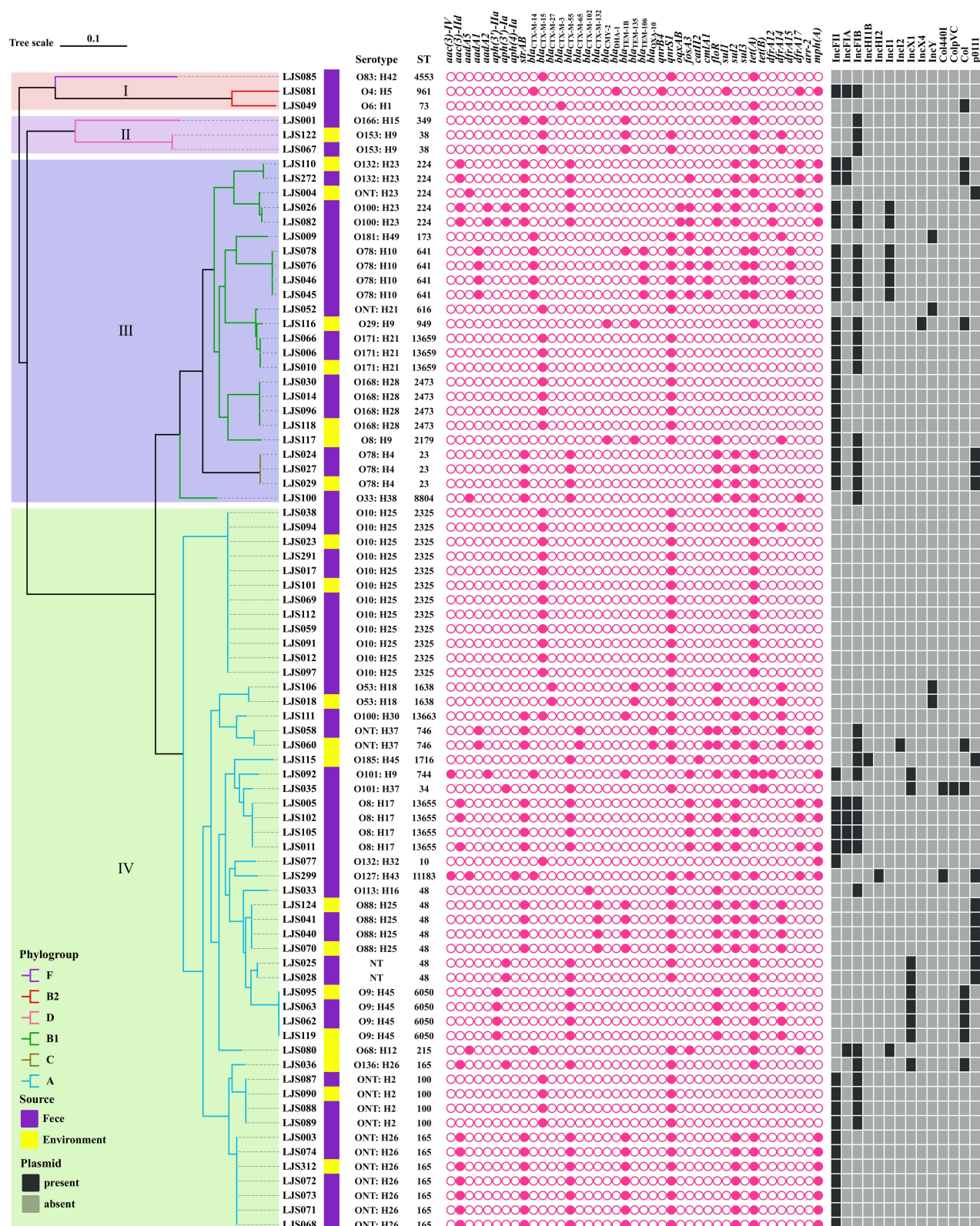


Figure 3 Phylogenetic relationships between the 80 cefotaxime-resistant *E. coli* strains. The sequence type, serotype, source, resistance genes, and plasmid replicons for each strain are indicated on the right side of the tree.

sul2 (37.5%, 30/80), the aminoglycoside resistance gene *strAB* (35.0%, 28/80), the trimethoprim resistance gene *dhfrA14* (21.3%, 17/80), and the macrolide resistance gene *mph(A)* (22.5%, 18/80) were also identified.

Phylogroup, Multilocus Sequence Typing, and Serotyping of Cefotaxime-Resistant *E. coli*

Most cefotaxime-resistant *E. coli* isolates belonged to phylogroup A (62.5%, n=50), followed by phylogroup B1 (26.3%, n=21) (Figure 2). The remaining strains belonged to phylogroup C (3.7%, n=3), D (3.7%, n=3), B2 (2.5%, n=2), and F (1.3%, n=1). Most of the isolates from stray dog feces (46.3%, n=37) and the environment (16.3%, n=13) belonged to phylogroup A.

In silico multilocus sequence typing revealed 30 different STs among 80 cefotaxime-resistant *E. coli* isolates (Figure 2). Among them, the most frequent was ST2325 (n=12), followed by ST165 (n=8), ST48 (n=7), ST224 (n=5), ST100 (n=4), ST641 (n=4), ST2473 (n=4), ST6050 (n=4), ST13655 (n=4), ST23 (n=3), ST13659 (n=3), ST38 (n=2), ST746 (n=2), and ST1638 (n=2). The remaining isolates belonged to a variety of ST types (Figure 2). Additionally, two isolates belonged to phylogenetic group B2, isolated from stray dog feces, with MLST types ST73 and ST961, respectively. Three isolates of phylogenetic group D were derived from the environment (ST38) and feces (ST38 and ST349), respectively (Figure 2). The *bla*_{CTX-M-15}-positive isolates were distributed across 10 different ST types, including ST38 and ST349. Twelve ST types were shared between fecal and environmental isolates and mainly encoded CTX-M-15 and CTX-M-55.

In serotype analysis, a total of 29 different serotypes were identified in 80 isolates. The serotype combination was highly abundant, with O10:H25 being predominant (n=12). O-serotypes are more abundant than flagellar serotypes (Figure 3).

Plasmid Replicons in Cefotaxime-Resistant *E. coli*

Plasmid replicon analysis revealed that 27 distinct plasmid replicons were identified in 80 cefotaxime-resistant *E. coli* isolates. As shown in Figure 3, IncF was the most common replicon type, including IncFII (n=38), IncFIA (n=8), and IncFIB (n=34). Other replicons such as IncI, IncHI, IncN, IncX, IncY, and Col-like plasmid (for example, Col440I, ColpVC) were also detected. In total, 56.3% isolates (n=45) carried two or more different plasmid replicons, and 17.5% (n=14) did not harbor any detectable plasmid replicons.

Phylogenetic Analysis of Cefotaxime-Resistant *E. coli* Strains

To explore the relationship among the 80 cefotaxime-resistant *E. coli* isolates, we constructed a phylogenetic tree based on core genome SNPs. All ESBL-producing isolates were divided into four major clades, namely I (n=3, 3.75%), II (n=3, 3.75%), III (n=24, 30.00%), and IV (n=50, 62.50%) (Figure 3). Clade I contained three *E. coli* isolates from feces; the other clades contained both fecal and environmental isolates. Clade IV was the primary lineage, containing 37 isolates from feces and 13 isolates from the environment. ST2325, ST48, and ST165 were the predominant STs in this clade; one high-risk clone ST10 strain was designated as belonging to clade IV. Clade III was the second most prevalent lineage; ST224, ST641, and ST2473 were the predominant STs in this clade. Three high-risk clone ST23 strains belonged to clade III.

Discussion

Although the seriousness of antibiotic resistance has been recognized and certain interventions have been implemented to prevent the spread of antibiotic resistance, the problem is still growing. The relevance and role of AMR dissemination transcends the human context and is closely related to the human–animal–environment interface.^{16,17} As a bacterial host species that is closely related to the human living environment, stray dogs serve as a suitable bridge for the interspecies transmission of antimicrobial-resistant bacteria owing to their close contact with humans.

In the present study, high carriage (51.3%) of cefotaxime-resistant *E. coli* in stray dogs and shelters was reported. Apart from resistance to cefotaxime, the isolates showed resistance to ampicillin (100%), followed by high resistance to tetracycline and trimethoprim-sulfamethoxazole. This is consistent with the findings of many previous studies on

companion animals and livestock^{18,19} and may be because β -lactams and tetracyclines are commonly used antibiotics in human and veterinary settings. The findings also suggest that resistance is broadly distributed across a wide range of environmental scenarios. Additionally, various rates of resistance to antibiotics such as quinolones (nalidixic acid and ciprofloxacin), gentamicin, and streptomycin, were observed in our isolated strains. These drugs are first-line antibiotics used to treat common infections and are critical to humans. The current treatment of ESBL *Enterobacteriaceae* infections, based on their susceptibility to drugs, is still preferred to carbapenems, and when carbapenems are not effective, colistin and tigecycline may be considered, but the treatment of the infection itself is complex and requires careful evaluation.²⁰

Nearly all isolates (96.3%) exhibited multidrug-resistant phenotypes, with 11.3% of isolates showing resistance to more than 10 antibiotics. A previous study reported higher rates of drug resistance and multidrug resistance in *E. coli* strains from kennel dogs than those observed in strains from individually bred dogs.²¹ It is hypothesized that stray dogs themselves may carry multidrug-resistant bacteria. During shelter periods, dogs are exposed to poor sanitation and group housing, as well as other dogs and their bacteria-carrying feces, which are likely to contain multiple resistance genes that can be transmitted horizontally. Closely related extended-spectrum cephalosporin-resistant *E. coli* isolates have been found in dogs and humans sharing a shelter environment, suggesting clonal interspecies or dog-to-dog cross-transmission.²²

In this study, the most important ESBL enzyme was the CTX-M enzyme, and eight different CTX-M types were detected, indicating a high diversity of *bla*_{CTX-M} in *E. coli* isolates from stray dogs. These CTX-M variants were mainly clustered in the CTX-M-1 and CTX-M-9 groups, consistent with previous reports on the global prevalence of CTX-M genes.^{3,23} The most commonly detected genes in this study were *bla*_{CTX-M-15} and *bla*_{CTX-M-55}, which are currently the most common types detected in pets and other animals in China and many other countries.^{24,25} Several variants (eg, CTX-M-65, CTX-M-14, and CTX-M-3) are also very common in *E. coli* isolated from humans.^{26,27} Similarly, CMY-2, the most prevalent pAmpC β -lactamase identified in this study, has been previously reported in *E. coli* from companion animals and stray dogs.^{8,28} Additionally, *bla*_{DHA-1} and *bla*_{CTX-M-14} genes were identified in the same strain. DHA-1 pAmpC β -lactamase was initially very common in human isolates; this gradually became more prevalent in companion animals in Europe, Korea, and other locations.²⁹ Our study showed that the ESBL/AmpC-encoding genes in *E. coli* strains isolated from stray dogs (shelter/dog breeder) are becoming more diverse, posing a potential threat to humans.

Unlike previous reports, we did not find *E. coli* ST131 and ST410 clones with a high risk of international transmission.^{30,31} Results of molecular typing indicated that CTX-M-producing isolates were clonally diverse; other important *E. coli* genotypes identified in our study were ST10 group and ST38, isolated from both the environment and feces. Among them, the ST10 group (including ST10, ST48, and ST744) has been reported to be associated with urinary tract infections and sepsis in poultry and humans.³² Additionally, although most isolates were distributed in phylogenetic groups A and B1, a small number of isolates from phylogenetic group B2 and D were also identified. This is usually called virulence phylogeny, and most of these isolates contained more virulence factors.³³ ST38 (belonging to phylogenetic group D) is usually considered an enteroaggregative *E. coli* strain and is frequently associated with urinary tract infections.³⁴ In this study, two ST38 *E. coli* strains were isolated from fecal and environmental samples and showed a close phylogenetic relationship with the same resistance genes and IncFIB plasmids. Additionally, other ST types (such as ST48 and ST100) from different sources exist, but these contain the same resistance genes and plasmid replicons, indicating clonal transmission between different sources in the shelter. Simultaneously, highly diverse clones suggest the importance of horizontal transfer in the spread of resistance genes. Most *bla*_{CTX-M} genes are associated with some well-characterized plasmids. For example, *bla*_{CTX-M-15} in the internationally prevalent clone ST131, is usually associated with IncF plasmids, which are vectors for *bla*_{CTX-M-14} dissemination among *E. coli* in Spain.^{35–37}

The global prevalence of ESBL-*E. coli* is estimated to be relatively low in dogs compared to livestock or humans, but studies nonetheless reflect the existence of irrational antibiotic use.³⁸ Shelter animals are likely to be an unusual source of infection for shelter staff or potential adopters or even families, and when infected with disease can cause a variety of problems for humans, such as length of hospitalization, mortality, and medical costs.³⁹ Now that global data on the prevalence of ESBL-*E. coli* in stray dogs and cats are under reported, we still hope this study will serve as a wake-up call to improve public health.

We have to admit that there are some limitations in our study. The samples were small-scale, and only collected from Yangzhou City. Therefore more large-scale studies from different regions are needed.

Conclusion

In conclusion, our study demonstrated a high prevalence of ESBL- and pAmpC-producing *E. coli* among stray dogs in China. Owing to the complexity of the stray dog background, resulting in genetic diversity, further research is required to determine the spread of AMR genes in this population and their association with humans. It is critical to strengthen long-term monitoring, control the use of antibiotics, and reduce the risk of transmission of MDR bacteria among stray dogs.

Data Sharing Statement

The genome sequences in this study have been deposited into NCBI GenBank under BioProject PRJNA880799.

Ethical Statement

All the experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Yangzhou University, Yangzhou, China, in accordance with Chinese legislations on animal experimentation.

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Disclosure

The authors report no conflicts of interest in this work.

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