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Clonal and horizontal transmission of carbapenem-resistant *Enterobacterales* strains and genes via flies

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Abstract

Background Antimicrobial resistance (AMR) is one of the most pressing global public health challenges; in particular, the rapid dissemination of carbapenem-resistant *Enterobacterales* (CRE) is emerging as a significant concern worldwide. Flies, serving as carriers of pathogens, pose a potential threat in the transmission of antibiotic-resistant bacteria (ARB) between animals and humans. The aim of this study was to evaluate and reveal the potential risk of AMR spread by flies.

Methods A total of 450 flies were collected from four farms, four rural areas, and four urban areas in Dengfeng, Henan, China. To select CRE strains on the surface of flies, three flies sampled from the same geographical location were arbitrarily selected and placed into one tube of brain heart infusion broth (BHI), and the supernatant was screened using CHROMagar™ mSuperCARBA culture medium. Different colors and shapes of colonies were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and 16S rRNA sequencing. Antimicrobial susceptibility testing for CRE strains was performed using broth microdilution. All CRE strains were whole-genome sequenced. Short-read sequencing was performed using MGISEQ-2000 and long-read sequencing was conducted using GridION.

Results Totally, 150 BHI tubes were screened for CRE strains, and 33 strains were identified as CRE positive. In 24 mSuperCARBA plates, only one species of CRE strain was isolated from each plate. In three plates, two different species of CRE strains were identified in each plate. In one plate, three different species of CRE strains were simultaneously isolated. Carbapenem resistance genes were detected in 81.8% of CRE strains, and bla_{NDM-1} was predominant (66.7%). No significant correlations between carbapenem-resistant phenotypes and carbapenem resistance genes were observed. The complete genomes of all 33 strains were obtained. Genome analysis revealed

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that clonal transmission events may have occurred among different farms and rural areas. Phylogenetic analysis revealed that bla_{NDM-1} IncFII plasmids could break bacterial species barrier for cross-host transmission in diverse areas.

Conclusions To understand and control the transmission of AMR from the perspective of One Health, it is imperative to enhance surveillance of ARB, antibiotic resistance genes, and antibiotic-resistant plasmids in flies.

Keywords Antimicrobial resistance, Carbapenem-resistant *Enterobacterales*, Plasmid, Fly

Background

Antimicrobial resistance (AMR) presents a significant global health challenge [1], as highlighted by the trends over the past few decades reported by the World Health Organization [2, 3]. AMR contributes to deaths, health complications, and healthcare expenditures in all countries, regardless of socioeconomic status [4]. Recent estimates indicate that more than 1.2 million deaths are caused by AMR annually, with projections indicating the potential escalation to nearly 10 million deaths by 2050 [5].

β-Lactams have been the mainstay of treatment for serious infections, and the most active β-lactams are carbapenems [6]. Carbapenems have been the gold standard for treating multidrug-resistant (MDR) bacterial infections or serious infections with gram-negative bacteria [7, 8]. Over the past 10 years, reports of carbapenemase-producing *Enterobacterales* have increased [9]. As a result of the widespread use of antibiotics, carbapenem-resistant strains often cause severe public health issues worldwide [10]. Carbapenem-resistant *Enterobacterales* (CRE) spread rapidly, with high lethality and few treatment options [11]. As such, CRE strains have been described as "nightmare bacteria," posing a substantial health burden [12].

The high prevalence of AMR can be partially attributed to the horizontal transfer of antibiotic resistance genes (ARGs), which is notably mediated by plasmids [13]. Plasmids are important vehicles for the spread of genetic information among bacteria [14, 15]. Plasmids are transmitted both vertically by cell division and horizontally to other bacteria [15–17]. They play a critical role in facilitating the transmission of AMR across bacterial species, and the plasmid-mediated transfer of ARGs among bacteria is a challenge to the "One Health" concept [13, 18].

Antibiotic-resistant bacteria (ARB) and ARGs can be transmitted among animals, environments, food, and humans [11, 19]. Flies have a remarkable ability to move freely between habitats and travel long distances (5–7 km) [1, 20]. They feed on trash, human and animal feces, and decomposing fruits and meat [21]; therefore, they are exposed to a rich bacterial environment. The number of bacteria carried by flies from contaminated to clean surfaces has been found to range from 50 to 50,000 CFU/mL [22]. Gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, are more frequently isolated from flies than gram-positive bacteria, indicating that the risk of *Enterobacterales* transmission via flies is high [23]. Enteric pathogens can survive on flies for up to 10 days [24]. Flies can spread bacteria to human food and utensils, threatening food safety and human health [25]. A study found that the odds of *E. coli* contamination on rice were 5.4 times ($P<0.001$, 95% CI: 2.5–11.7) greater if flies landed on the rice than if no flies landed on the rice [26]. In a longitudinal study of 160 urban and 80 rural families in India, the fly concentrations were identified as a predictor of infectious diarrhea. Fly density at the 75th percentile was linked to adjusted relative risks of diarrheal episodes and duration of diarrhea of 1.18 (95% CI: 1.03–1.34) and 1.15 (95% CI: 1.02–1.29), respectively [27]. Thus, the dissemination of ARB and ARGs by flies is a major issue.

In this study, CRE was characterized in flies collected from Dengfeng in Henan Province, China. By combining short- and long-read sequencing, the whole genomes of CRE isolates were obtained. Through analyses of strains and plasmid sequences, the clonal and horizontal transmission of ARB and ARGs in fly samples were evaluated and revealed the potential risk of AMR spread by flies.

Materials and methods

Sample collection and strain identification

Dengfeng, Henan, China covers an area of approximately 1220 km^2 and has a permanent population exceeding 700,000. Dengfeng is an agricultural city that primarily cultivates rice, wheat, corn, beans, and other crops. In total, 450 flies were collected from four farms (165 flies), four rural areas (150 flies), and four urban areas (135 flies) in Dengfeng in 2018. Geographic information was recorded using a global positioning system unit. Three flies collected from the same geographical location were arbitrarily selected, and placed into one tube of brain heart infusion broth (BHI) to select CRE strains on the surface bacteria of flies. The supernatant was screened for CRE strains using CHROMagar™ mSuperCARBA (bioMérieux, Paris, France) culture medium. Phenotypically different colonies were selected from each plate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Zybio, Chongqing, China) was used to identify each clone to the species level. A reliable species-level identification was achieved when the score was

2.00 or higher. Isolates with a score of < 2.00 were evaluated using 16S rRNA sequencing.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for CRE strains was performed using the reference broth microdilution method and the BD Phoenix M50 instrument (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with the following 28 antimicrobial agents: amikacin, gentamicin, tobramycin, amoxicillin-clavulanate, ampicillin-sulbactam, piperacillin-tazobactam, aztreonam, cefoperazone-sulbactam, ceftazidime, ceftriaxone, cefazolin, cefepime, cefoxitin, cefuroxime, chloramphenicol, ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, colistin, ertapenem, imipenem, meropenem, minocycline, tetracycline, tigecycline, nitrofurantoin, and trimethoprim-sulfamethoxazole. The Clinical and Laboratory Standards Institute M100-2022 (V32) breakpoints were used to assess the results in addition to tigecycline. Additionally, FDA breakpoints were used to interpret the tigecycline result.

DNA extraction and whole-genome sequencing

Genomic DNA was extracted using the Wizard Genomic DNA Extraction Kit (Promega, Madison, WI, USA). Short-read sequencing was performed using MGISEQ-2000 (MGI, Shenzhen, China). Long-read sequencing was conducted using the Native Barcoding Kit 24 V14 (SQK-NBD114.24, ONT), R10.4.1 flow cells (FLO-MIN114, ONT), and a GridION device (ONT, Oxford, UK) with MinKNOW v23.07.5 and super-accuracy base-calling mode.

Bioinformatics and statistical analyses

Complete genome sequences were obtained using both short and long reads via Unicycler v0.4.8. CheckM2 v1.0.2 was used to assess the level of contamination of assembled genomes, and all genomes revealed < 5% contamination. To detect ARGs, plasmid replicon types, and insertion sequences, ResFinder v4.4.2, PlasmidFinder v2.1, and ISfinder v1.0.3 were used, respectively. Pyani v0.2.9 was used to analyze the average nucleotide identity (ANI).

Statistical analyses were performed using IBM SPSS Statistics 27 (IBM SPSS, Turkey). Spearman correlation coefficients were calculated to assess the correlations between antibiotic resistance phenotypes and their related ARGs. Regions of recombination were identified and excluded using Gubbins v3.3.3. IQ-TREE v2.1.2 was used to construct phylogenetic trees based on recombination-free single nucleotide polymorphisms (SNPs) with the maximum likelihood method. Plasmid genome comparisons were visualized using R v4.3.3 and BRIG v0.95.

Results

CRE isolated from fly samples

Among 150 fly samples collected from 12 locations in Dengfeng, 28 samples collected from nine locations were identified as CRE positive: there were 20 CRE-positive samples from the four farms, five from two rural areas, and three from three urban areas (Fig. 1). The CRE-positive rate in the samples collected from farms was higher than that in the samples collected from rural areas and urban areas. For 24 samples, only one CRE strain was isolated. For three samples, two different species of CRE strains were identified in each. In one sample, three different species of CRE strains were isolated simultaneously. In total, 33 CRE strains were isolated: 15 *Escherichia coli* (45.5%), six *Citrobacter freundii* (18.2%), two *Raoultella ornithinolytica* (6.1%), two *Moellerella wisconsensis* (6.1%), two *Proteus mirabilis* (6.1%), one *Klebsiella oxytoca* (3.0%), one *Providencia alcalifaciens* (3.0%), one *Providencia rettgeri* (3.0%), one *Proteus terrae* (3.0%), one *Morganella morganii* (3.0%), and one *Enterobacter cloacae* (3.0%).

Antimicrobial susceptibility of 33 CRE strains

The 33 CRE strains identified in this study exhibited resistance to first-line clinical antibiotics, including third-generation cephalosporins/carbapenems/colistin. The resistance rate to third-generation cephalosporins was notably high, exceeding 80%. Specifically, the resistance rate was highest for ceftriaxone (93.9%), followed by cefoperazone-sulbactam (84.8%) and ceftazidime (81.8%). The resistance rates to carbapenems, except for meropenem (24.2%), all exceeded 60% [ertapenem (72.7%), imipenem (63.6%)]. Furthermore, 3.0% of CRE isolates were resistant to colistin, and resistance to tigecycline was not detected. For these first-line clinical drugs, 93.9% of CRE isolates were resistant to both third-generation cephalosporins and carbapenems, making clinical treatment difficult. For 15 carbapenem-resistant *E. coli* strains, 14 strains (93.3%) were also resistant to third-generation cephalosporins and carbapenems. However, all isolates were sensitive to colistin or tigecycline.

The resistance rates to other classes of antibiotics, except for minocycline (18.2%), and nitrofurantoin (18.2%), all exceeded 20%. Some isolates exhibited resistance to aminoglycosides, including gentamicin (69.7%), tobramycin (54.5%), and amikacin (45.5%). Additionally, 93.9% of isolates were resistant to ampicillin-sulbactam; 87.9% of isolates were resistant to cefazolin, cefuroxime, and amoxicillin-clavulanate; 81.8% of isolates were resistant to cefoxitin and tetracycline; 75.8% of isolates were resistant to piperacillin-tazobactam; 72.7% of isolates were resistant to trimethoprim-sulfamethoxazole; 69.7% of isolates were resistant to chloramphenicol; and 60.6% of isolates were resistant to moxifloxacin. In addition, the

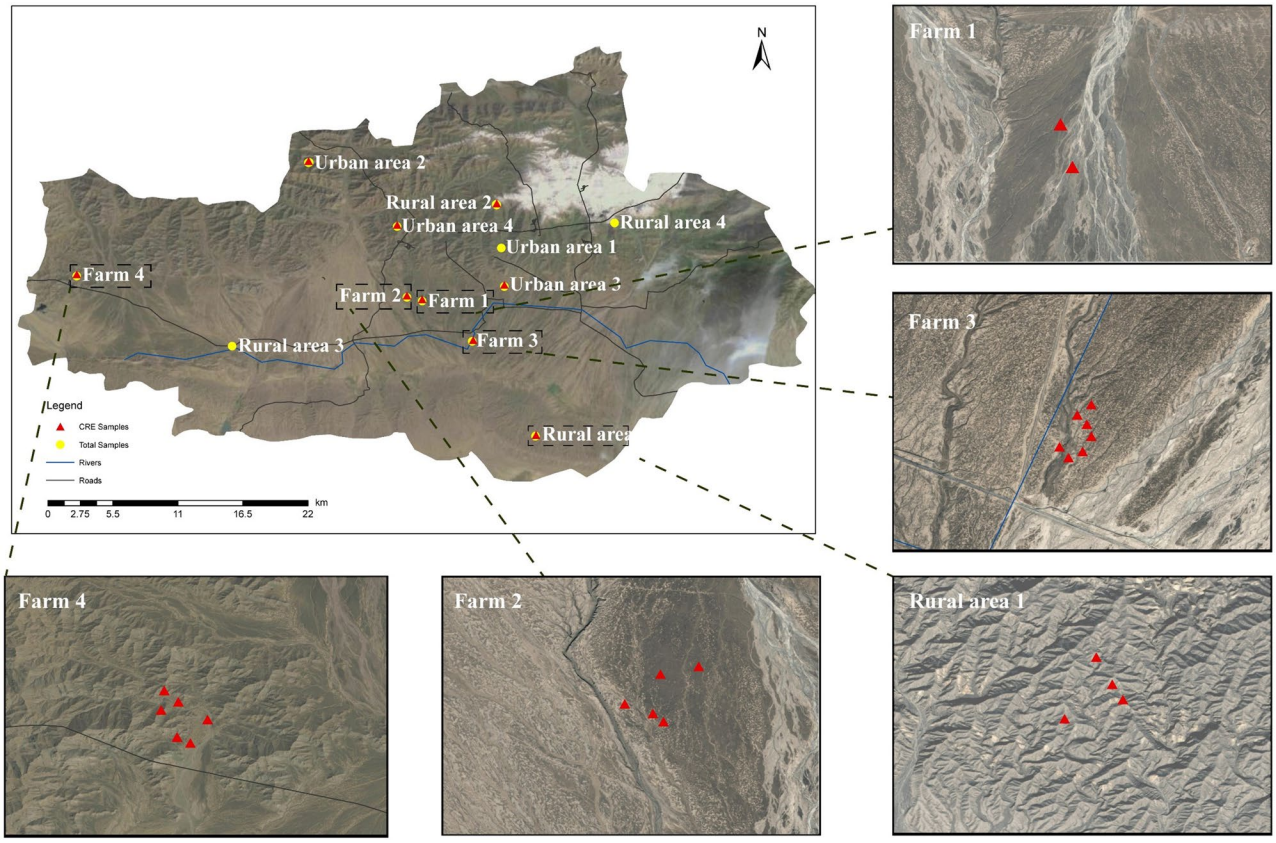

Fig. 1 Geographical locations of samples and CRE-positive samples. Yellow circles indicate the locations of all collected samples, and red triangles represent the locations of CRE-positive samples

cefepime and ciprofloxacin resistance rates were nearly 50%. All isolates were MDR, and one strain was resistant to 10 classes of drugs (Fig. 2).

Genome sequencing of 33 CRE strains

We sequenced all 33 CRE strains, and 63 total plasmid sequences were obtained, which included 53 complete plasmid sequences and 10 nearly complete plasmid sequence fragments. For eight strains, only one plasmid sequence was obtained for each; for 12 strains, two plasmid sequences were obtained simultaneously; for nine strains, three plasmid sequences were obtained simultaneously; and for one strain, four plasmid sequences were obtained simultaneously.

We obtained 34 chromosome sequence fragments, which included 21 complete chromosome sequences and 13 nearly complete chromosome sequence fragments (Supplementary Table S1). Sixteen types of plasmids were identified, and the highest prediction rate was IncFII (22.2%), followed by Col3M (11.1%), IncFIA/IncFIB (9.5%), IncFIB (7.9%), IncX1/IncY (4.8%), IncX4 (4.8%), IncFIA (3.2%), IncHI2/IncHI2A (3.2%), Incl1-l (3.2%), IncFIA/IncFIB/IncFIII (1.6%), IncHI2A (1.6%), IncX1 (1.6%), IncY (1.6%), IncX3 (1.6%), IncQ1 (1.6%), and p0111 (1.6%). Twenty (60.6%) isolates carried more than one plasmid type. Among these plasmid types, the species range of host bacteria carrying IncFII was narrow, including *E. coli* and *C. freundii*, and they were mainly isolated from farm 4. In contrast, the species range of host bacteria carrying Col3M was broad, including *P. mirabilis*, *P. rettgeri*, *M. wisconsensis*, *P. alcalifaciens*, and *P. terrae* (Fig. 3).

There was an average of 16.5 ARGs per isolate, and all isolates carried two or more ARGs. The overall prediction rate for β-lactam resistance genes was 90.1%, among which 66.7% carried $bla_{\mathrm{NDM-1}}$. The predominant phenicol ARG was *floR* (75.8%). The prediction rate of tetracycline resistance genes was 84.8%, and the predominant genotype was *tet(A)* (63.6%). The fosfomycin ARG prediction rate was 27.3%. The prediction rate of quinolone resistance genes was 66.7%, and the most prevalent genes were *oqxA* and *oqxB* (30.3%). The sulfonamide resistance gene prediction rate was 94.0%, including *sul1* (69.7%), *sul2* (63.6%), and *sul3* (39.4%). The predominant macrolide-resistant gene was *mph(A)* (45.5%). The trimethoprim resistance gene prediction rate was 70.0%, and the predominant genotype was *dfrA1* (30.3%). The main aminoglycoside resistance genes were *aph(3")-Ib* (57.6%)

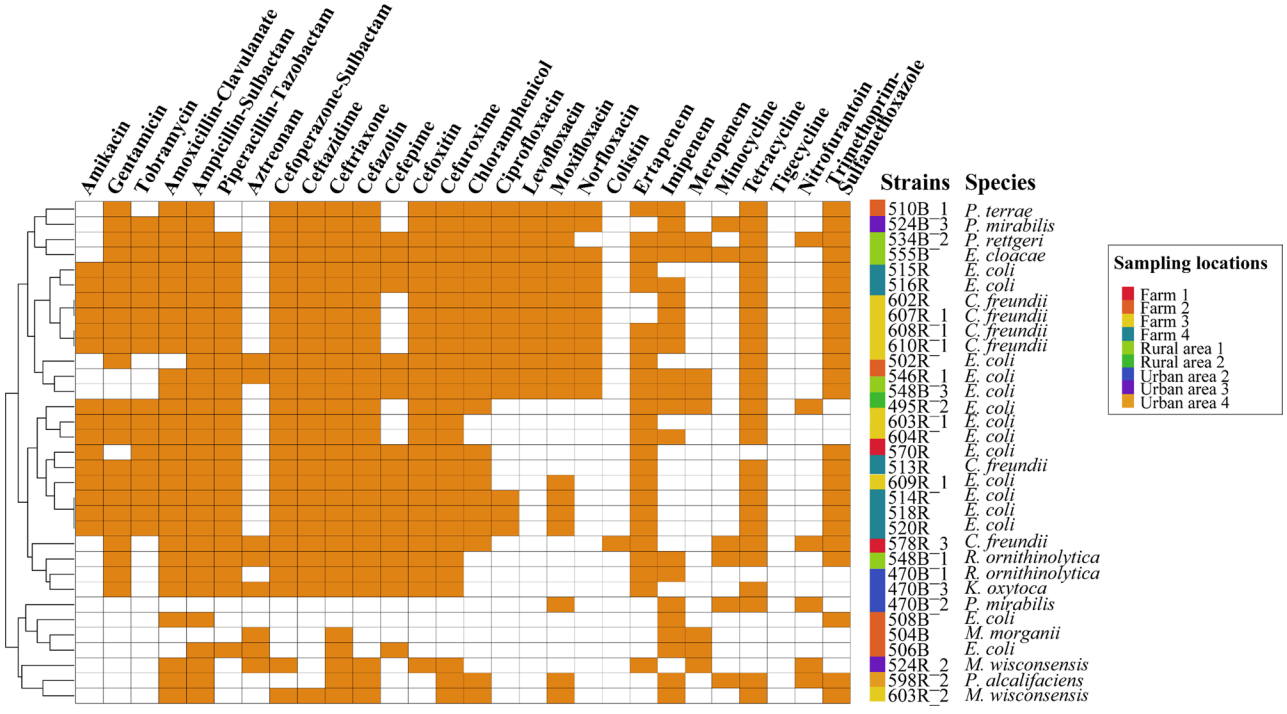

Fig. 2 Antibiotic resistance phenotypes of 33 CRE strains. Orange indicates antibiotic resistance

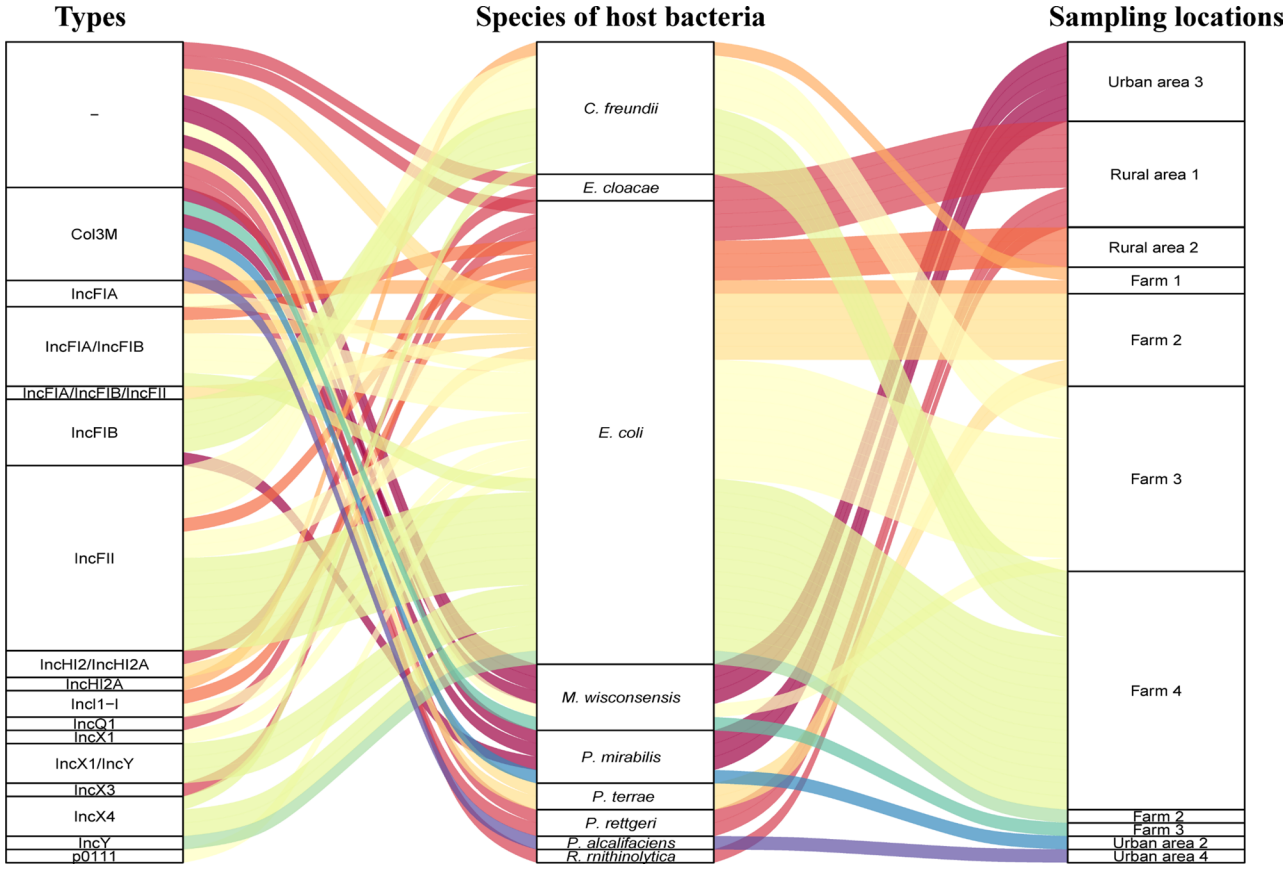

Fig. 3 Relationships among plasmid types, species of host bacteria, and sampling locations for 63 plasmids

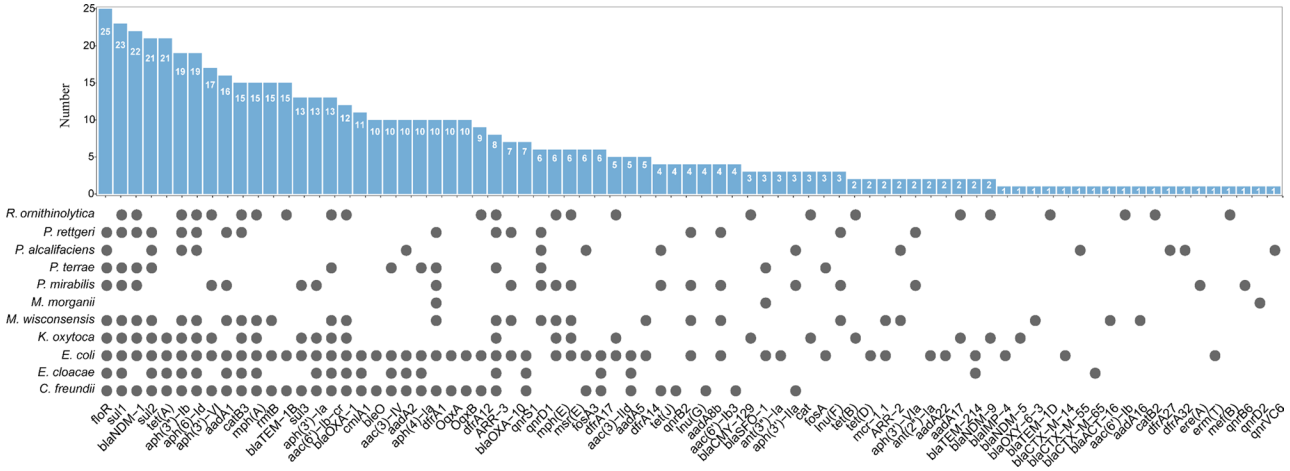

Fig. 4 Distribution of ARGs in 33 CRE strains. Blue bars indicate the number of ARGs, gray circles indicate the presence of ARGs in various species

Table 1 Carriage rate of carbapenem resistance genes in 33 CRE strains

Species (N)	Carriage rate of carbapenem resistance genes, n (%)			
	bla_{NDM-1}	bla_{NDM-5}	bla_{NDM-9}	bla_{NDM-1}+ bla_{IMP-4}
E. coli (N = 15)	10 (30.3)	1 (3.0)	1 (3.0)	0 (0.0)
C. freundii (N = 6)	6 (18.2)	0 (0.0)	0 (0.0)	0 (0.0)
R. ornithinolytica (N = 2)	1 (3.0)	0 (0.0)	0 (0.0)	1 (3.0)
P. terrae (N = 1)	1 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)
P. mirabilis (N = 2)	1 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)
K. oxytoca (N = 1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)
P. rettgeri (N = 1)	1 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)
E. cloacae (N = 1)	0 (0.0)	0 (0.0)	1 (3.0)	0 (0.0)
M. wisconsensis (N = 2)	2 (6.1)	0 (0.0)	0 (0.0)	0 (0.0)
P. alcalifaciens (N = 1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
M. morganii (N = 1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total (N = 33)	22 (66.7)	1 (3.0)	2 (6.1)	2 (6.1)

and *aph(6)-Id* (57.6%). The *mcr-1.1* gene (6.1%) was also detected (Fig. 4). Among the 33 isolates, the prediction rate of carbapenem resistance genes was 81.8% (27/33); these genes were isolated from 23 samples, including bla_{NDM-1} (66.7%) from 20 samples, bla_{NDM-5} (3.0%) from one sample, bla_{NDM-9} (6.1%) from two samples, and bla_{NDM-1}+bla_{IMP-4} (6.1%) from one sample (Table 1). Carbapenem resistance genes were mostly located on plasmids (74.1%), including two bla_{NDM-9} genes and 18 (63.6%) bla_{NDM-1} genes. In addition, bla_{NDM-1}, bla_{IMP-4}, and bla_{NDM-5} were located on chromosomes in six, two, and one strain, respectively.

Spearman correlation coefficients were measured to assess the correlations between every antibiotic resistance phenotype and their related ARGs. As a result, a strong correlation was observed between antibiotic resistance phenotypes (gentamicin, amoxicillin-clavulanate, ampicillin-sulbactam, cefepime, cefoxitin, chloramphenicol, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole) and their related ARGs (Fig. 5). However, no significant correlations between carbapenem-resistant phenotypes and carbapenem-related resistance genes were observed, which may be explained by the complex drug resistance mechanism.

bla_{NDM-1} was the predominant carbapenem resistance gene in our study. In total, 22 strains carrying bla_{NDM-1} were all resistant to carbapenems, but not every strain was entirely resistant to three types of carbapenem antibiotics (ertapenem, meropenem, and imipenem). Among them, 20 strains carrying bla_{NDM-1} were resistant to ertapenem, 13 strains were resistant to imipenem, and three strains were resistant to meropenem.

Furthermore, two metrics (recall and precision) were used to assess the prediction ability of bla_{NDM-1} for carbapenem-resistant phenotypes. The precision values of bla_{NDM-1} to ertapenem, imipenem, and meropenem resistance phenotypes were 83.3%, 54.2%, and 12.5%, respectively. The recall values of bla_{NDM-1} to these antibiotics were 83.3%, 61.9%, and 37.5%, respectively. Eight strains were resistant to meropenem in our study, of which three strains carried bla_{NDM-1}; 24 strains were resistant to ertapenem, of which 20 strains carried bla_{NDM-1}; and 21 strains were resistant to imipenem, of which 13 strains carried bla_{NDM-1}. bla_{NDM-1} was highly correlated with ertapenem resistance and not highly correlated with meropenem resistance. These results demonstrated the bla_{NDM-1} was highly correlated with ertapenem resistance and not highly correlated with resistance to meropenem.

Clonal transmissions of CRE strains and horizontal transmissions of bla_{NDM-1}

Phylogenetic trees based on SNPs were constructed for 15 *E. coli* isolates and six *C. freundii* isolates to identify the clonal clusters of strains. ANI analysis of the plasmids was used to determine the plasmid similarity to assess

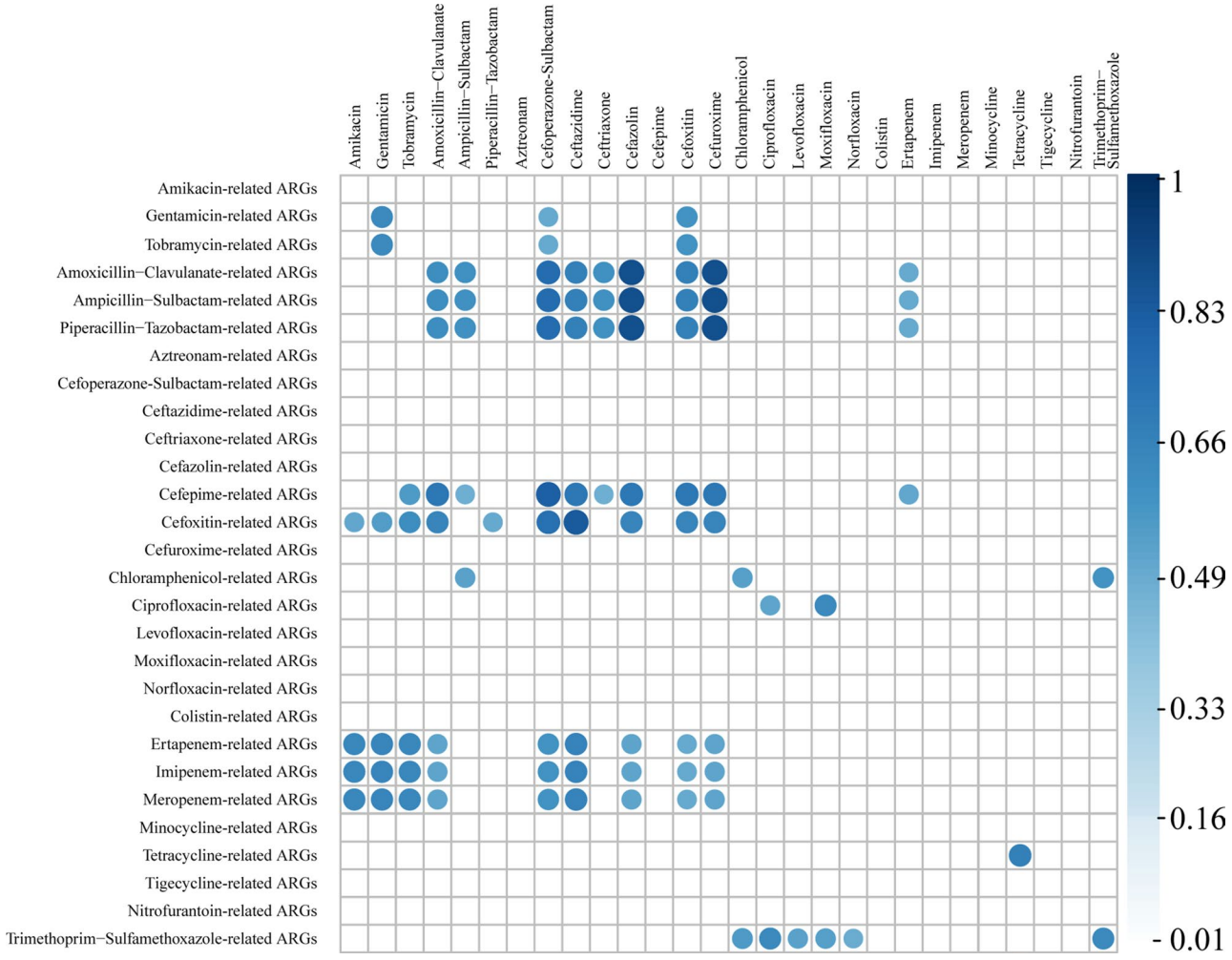

Fig. 5 Correlation between antibiotic resistance phenotypes and their related ARGs. The horizontal coordinates indicate antibiotic resistance phenotypes, and the vertical coordinates indicate phenotype-related ARGs. Blue circles represent significant positive correlations. Darker colors represent stronger correlations

the clonal proliferation of strains and the horizontal transmissions of ARGs and plasmids. When two strains have similar chromosomes and plasmids, they are both from the same clone. When two strains have comparable plasmids but distinct chromosomes, this is thought to be caused by horizontal transmission of plasmids between strains.

For the phylogenetic tree of *E. coli* isolates, two strains in clade I were isolated from different samples in farm 2 with nine SNPs. Because they had similar genomes, they may have come from the same clone. In clade II, five strains with the same carbapenem-encoding gene ($bla_{\mathrm{NDM-1}}$) that were isolated from five different samples in the same geographical location belonged to two clusters (516R, 514R and 520R, 518R, 515R). Isolates within the two clusters exhibited 10 and 26 SNPs, respectively, which indicated that two clonal transmission events may have occurred. Strains in clade III were widely distributed, including in rural area 1, rural area 2, farm 1, and farm 3. Three strains (495R_2, 603R_1, and 604R) with the same carbapenem-encoding gene ($bla_{\mathrm{NDM-1}}$) in a cluster with 31 SNPs were isolated from three different samples in two different locations (straight-line distance > 10 km), indicating that there may have been a clonal transmission event (Fig. 6a).

The phylogenetic tree of *C. freundii* isolates revealed two clades (Fig. 6b). In clade II, four *C. freundii* strains (607R_1, 610R_1, 608R, and 602R) from farm 3 formed a cluster with only seven SNPs. This indicated that these four strains spread clonally within farm 3.

To further analyze the horizontal transmission of plasmids, we constructed a phylogenetic tree based on ANI analysis for 53 complete plasmid sequences and 10 nearly complete plasmid sequence fragments (Fig. 7a). The plasmids could be divided into two clades, exhibiting significant differences in the carriage rate of $bla_{\mathrm{NDM-1}}$, *tet(A)*, *aph(3')-VI*, *qnrD1*, and *rmtB* between these clades ($P < 0.05$). In clade II, a cluster included 12 complete

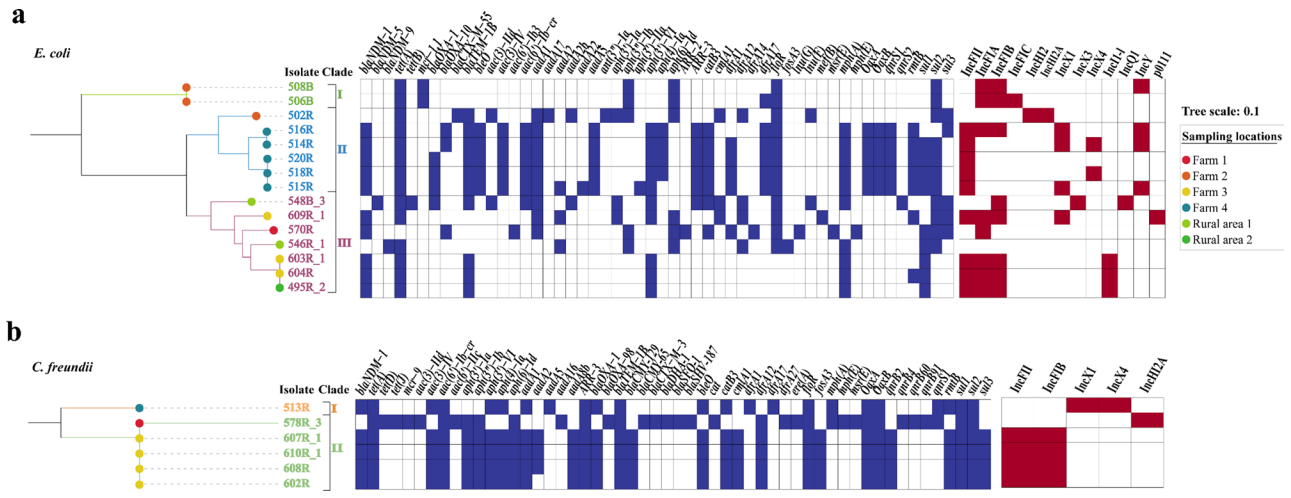

Fig. 6 Phylogenetic trees of 15 *E. coli* strains (**a**) and six *C. freundii* strains (**b**). *Note* The phylogenetic tree is shown on the left, a heatmap of ARG carriage is shown in the middle (blue indicates the presence of ARGs), and the distribution of plasmid types is shown on the right (red indicates the presence of plasmid types)

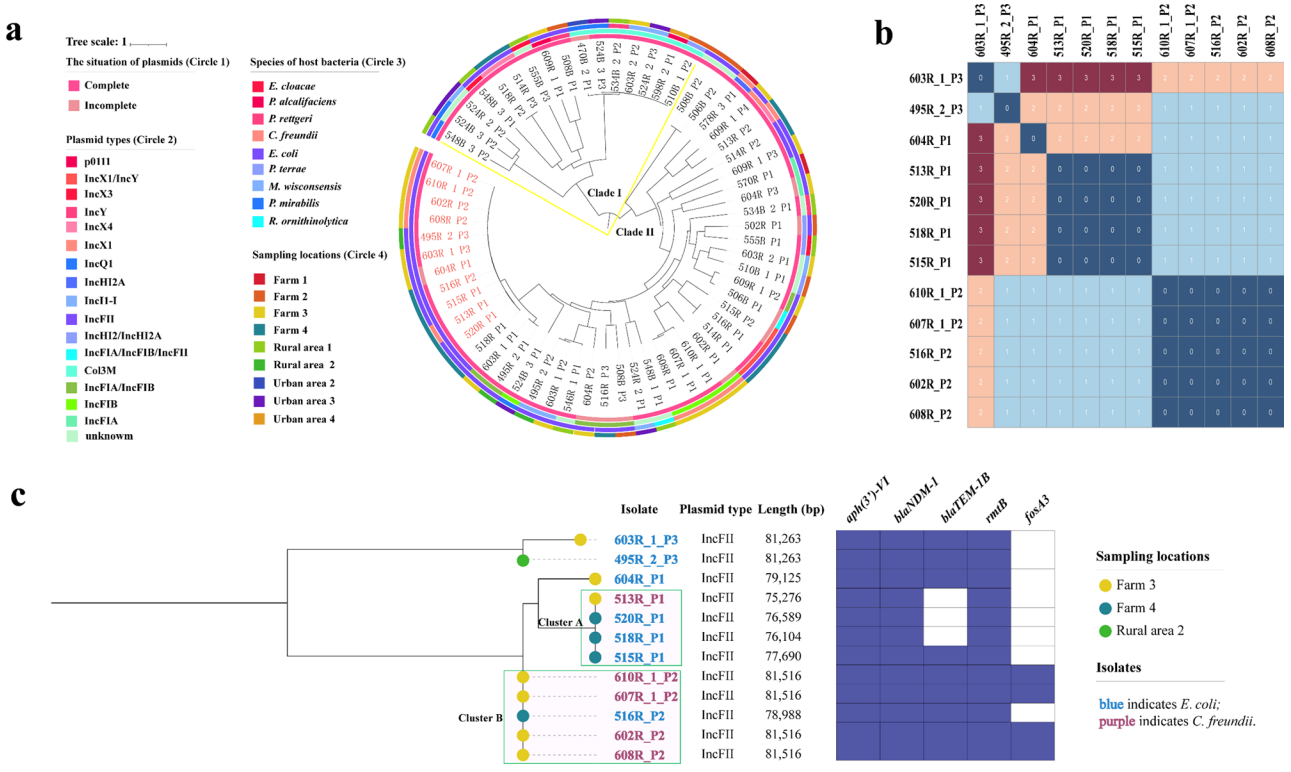

Fig. 7 Phylogenetic tree of 63 plasmids based on average nucleotide identity (**a**). Details of SNPs in recombination-free alignments for the red cluster in the phylogenetic trees (**b**). Phylogenetic tree based on SNPs in the red cluster (**c**). The phylogenetic tree is shown on the left, a heatmap of ARG carriage is shown on the right (blue indicates the presence of ARGs)

$bla_{\mathrm{NDM-1}}$ IncFII plasmids. Only three recombination-free SNPs were detected in these $bla_{\mathrm{NDM-1}}$ IncFII plasmids, indicating a horizontal transmission event of $bla_{\mathrm{NDM-1}}$ IncFII plasmids occurred among different host bacteria isolated from farm 3, farm 4, and rural area 2 (Fig. 7b).

To better clarify the relationships among these 12 plasmid sequences, we constructed a phylogenetic tree based on SNPs (Fig. 7c). Two clusters with no SNPs in recombination-free alignments were identified in these $bla_{\mathrm{NDM-1}}$ IncFII plasmids. In cluster A, four $bla_{\mathrm{NDM-1}}$ IncFII (515R_P1, 518R_P1, 520R_P1, and 513R_P1) plasmids with no SNPs in recombination-free alignments were isolated from four different samples from farm 4 and different host bacteria (*E. coli* and *C. freundii*); this provides

evidence for cross-host horizontal transmission of the bla_{NDM-1} IncFII plasmid (Supplementary Figure S1). A horizontal transmission event was also observed in cluster B, in which five bla_{NDM-1} IncFII plasmids (610R_1_P2, 607R_1_P2, 602R_P2, 608R_P2, and 516R_P2) with no SNPs in recombination-free alignments were isolated from different host bacteria (*E. coli* and *C. freundii*) in two different locations (straight-line distance > 28 km). All five bla_{NDM-1} IncFII plasmids in clade B harbored the ARGs bla_{NDM-1}, bla_{TEM-1B}, *aph(3')-IV*, and *rmtB*; however, one of them lacked the fosfomycin resistance gene *fosA*, indicating that a gene deletion might have occurred during cross-host transmission (Supplementary Figure S2).

Discussion

AMR is recognized as a One Health challenge owing to the rapid emergence and dissemination of ARB and ARGs in humans, wildlife, companion animals, agriculture, and the environment on a global scale. Antimicrobial-resistant ESKAPE (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) pathogens represent a global threat to human health [28]. Because of the limited treatment options for serious infections, ESKAPE pathogens are a serious issue in the post-antibiotic era. Furthermore, reports of CRE have increased worldwide [29]. CRE is usually associated with antibiotic use, prolonged hospitalization, and medical device use, accelerating the emergence of drug-resistant bacteria and increasing mortality rates; thus, CRE are listed as priority pathogens by the World Health Organization [30–32]. The Centers for Disease Control and Prevention showed that the mortality rate of CRE infections is 6.6% annually in the United States [33]. In China, the prevalence of CRE infections is high (4.0 per 10,000 discharges) [34]. In a hospital in Henan Province, surveillance of CRE infections from 2014 to 2016 revealed an increasing trend, which is alarming in this densely populated province [35].

Flies act as vectors and reservoirs for many microbes, including viruses, bacteria, and fungi [36]. The ability of flies to move freely makes them an important vector for pathogen transmission. A total of 11 species harbored CRE strains in this study, all of which are pathogenic, causing infectious diseases, such as bacteremia, sepsis, and pneumonia [37]. *Moellerella wisconsensis* is a rare clinical pathogenic bacterium associated with gastroenteritis, diarrhea, bacteremia, and urinary tract infections [38]. Our study represents the first report of carbapenem resistance in *M. wisconsensis*, indicating the need for increased attention and surveillance focused on this opportunistic pathogen.

In Thailand, no CRE strains were detected in 50 fly samples from five fresh food markets [39]; additionally, CRE strains were not detected in 163 flies from five sites in Berlin [40]. However, the prevalence of CRE in China differs significantly from those in other countries. In Shandong Province, 12 CRE strains were isolated from 180 flies (positive rate: 6.7%) [41], which demonstrated that the prevalence of CRE strains in flies in China was higher than those in other countries.

We found 12 previously reported sequence types (STs), with nine STs in *E. coli* (ST48, ST101, ST361, ST410, ST641, ST648, ST1397, ST2973, and ST6189), two STs in *C. freundii* (ST63 and ST219), and one ST in *E. cloacae* (ST993). Of the 12 previously reported STs, seven STs (ST48, ST101, ST361, ST410, ST641, ST648, and ST2973) in *E. coli* have been reported in clinical studies, with ST648, ST48, and ST410 being of great concern [42]. Additionally, we found 12 strains of different species that were represented by new STs. We have submitted the genome to PubMLST (https://pubmlst.org/) and got seven new STs (including ST251 and ST253 in *P. mirabilis*, ST1306 in *C. freundii*, ST252 in *P. terrae*, ST649 in *K. oxytoca*, ST48 in *P. rettgeri*, and ST49 in *P. alcalifacien*).

Escherichia coli is a common opportunistic pathogen in the intestinal tracts of animals and humans and has an increasing impact on available therapeutic options [43]. In this study, 93.3% of carbapenem-resistant *E. coli* strains were resistant to both third-generation cephalosporins and carbapenems. Tigecycline and polymyxin antibiotics, as the last line of defense against MDR gram-negative bacteria, play a crucial role in clinical treatment [44]. CRE strains detected in our study were exhibited resistance to third-generation cephalosporin/carbapenems/colistin. Furthermore, one *C. freundii* strain exhibited simultaneous resistance to third-generation cephalosporin, carbapenems, and colistin, posing significant challenges for the clinical treatment of CRE infections.

Aminoglycoside resistance genes [*aph(3")-Ib*, *aph(6)-Id*, *aac(3)-IV*, *aadA1*, *aadA5*, *aph(4)-Ia*] and the sulfonamide resistance genes (*sul1*and *sul2*) were commonly found in the chromosome. In two *E. coli* strains that were isolated from farm 2, the ARGs located in the chromosome were the same [*aph(3")-Ib*, *aph(6)-Id*, *floR*, *sul2*]. In two *E. coli* strains that were isolated from farm 4, the ARGs located in the chromosome were the same [*aac-IV*, *aac(6')-Ib-cr*, *aadA1*, *aadA5*, *aph(4)-Ia*, *ARR-3*, bla_{OXA-1}, bla_{TEM-1B}, *bleO*, *catB3*, *dfrA1*, *dfrA17*, *floR*, *mph(A)*, *OqxA*, *OqxB*, *qnrS1*, *sul1*, *sul2*, *tet(A)*]. In three *C. freundii* strains that were isolated from farm 3, the ARGs located in the chromosome were the same [$bla_{CMY-129}$ and *qnrB2*]. The most common carbapenem resistance gene identified in our study was bla_{NDM-1} (66.7%). Carbapenem resistance genes can be transmitted among species of bacteria via plasmids or transposons. The bla_{NDM} gene has become one of the most prevalent types of

carbapenemase-encoding genes worldwide [45]. Among strains collected from patients in China, bla_{NDM} (35.7%) also represents the most common carbapenemase-encoding gene [46]. In a hospital in Henan Province [47], the prevalence rate of bla_{NDM-1} (33.3%) was lower than that in our study.

Phenotypes depend upon multiple genetic factors, and mechanisms of CRE resistance include enzyme production, efflux pumps, porin mutations, penicillin-binding protein alteration, and biofilm production [48]. In our study, six strains remained carbapenemase free and were resistant to carbapenems. Among them, three strains harbored *marA*, which binds to the marbox at nucleotides 1349 to 1364 and autoactivates marRAB expression, thus conferring resistance to beta-lactam antibiotics [49, 50]. Another three strains harbored *TolC* and *H-NS*. TolC forms the outer membrane channel of most MDR efflux pumps found in *Enterobacterales*, and it was suggested as a potential target for efflux inhibitors [51]. H-NS endures envelope stress and could alleviate the stress induced by metallo-β-lactamases expression, which is the mechanism by which carbapenem resistance occurs [52]. In addition, we did not detect a significant correlation between carbapenem-resistant phenotypes and carbapenem-related resistance genes, which indicates the complexity of carbapenem resistance mechanisms.

Flies provide a suitable environment for ARB and facilitate the horizontal transfer of ARGs [53], suggesting that they could play an important role in the dissemination of ARB and ARGs [54, 55]. In the present study, the most prevalent CRE-positive plasmid was IncFII. Plasmid-mediated horizontal transfer of ARGs is one of the main mechanisms underlying the transmission of resistance genes, and the IncFII plasmid disseminates various ARGs among *Enterobacterales* [56]. In our study, 63.6% of bla_{NDM-1} were associated with the IncFII plasmid, which was consistent with previous results showing that bla_{NDM-1} is disseminated by IncF (IncFII and IncFIB) plasmids in Vietnam [57, 58].

The transferability of bla_{NDM-1} may increase the diversity of its bacterial hosts and pose a threat to human health, clinical care, and food safety. In our study, bla_{NDM-1} IncFII plasmids were highly similar in a variety of host bacteria separated by a distance of 28 km, indicating a potential risk for horizontal transmission between bacteria in various areas. The frequent transmission of antibiotic-resistant plasmids across various regions and hosts suggested the importance of surveillance of CRE and ARGs [59, 60]. Furthermore, a study demonstrated that NDM-5-positive plasmids have a broad range of hosts and can be transferred across various bacterial phyla, highlighting the potential challenge of horizontal gene transfer events within complex bacterial communities [61]. Clonal transmission of CRE strains from various samples was found in our study. Similarly, clonal transmission events of CRE strains have been detected in well water and animal feces in different counties in a province or in different provinces in Inner Mongolia and Shandong, China [59]. Therefore, within the One Health concept, it is necessary to enhance surveillance of antibiotic-resistant plasmids and to develop preventive measures to mitigate dissemination.

In our study, only fly samples were collected; therefore, we were unable to compare the relationship between strains carried by flies and clinical isolates. Additionally, three flies were considered one sample, which may have resulted in overestimation of the CRE-positive rate. Thus, to improve our understanding of the significance of monitoring CRE isolates, we will collect more types of strains at the same time and use one fly per sample in the future.

Conclusions

We investigated CRE strains isolated from flies, which act as reservoirs of pathogens. Flies have a wide distribution and play an important role in the transmission of AMR between animals and humans. The prevalence rate of carbapenem resistance genes in flies collected from Dengfeng, China is high. bla_{NDM-1} could break the host bacterial species barrier for cross-host transmission via the IncFII plasmid or clonal transmission in the same or different places. Within the One Health framework, actions should be implemented to minimize and mitigate the spread of ARB and ARGs. Therefore, in addition to monitoring pathogens in humans, it is imperative to enhance the monitoring of CRE isolates, carbapenem-resistant plasmids, and carbapenem resistance genes in vectors, with particular attention to flies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13099-024-00665-1.

Supplementary Material 1

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Author contributions

Formal analysis, Jiaqi Liu and Zhenpeng Li; Investigation, Jialiang Xu, Jiayong Zhao and Xin Lu; Methodology, Mengyu Wang, Yao Peng, and Yuan Zhang; Resources, Tian Tian and Gailing Yuan; Supervision, Zhenpeng Li and Xin Lu; Visualization, Zhenpeng Li and Zhe Li; Writing – original draft, Jiaqi Liu and Xin Lu; Writing – review & editing, Jialiang Xu, Biao Kan and Xin Lu. All authors have read and agreed to the published version of the manuscript.

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Data availability
Sequence data that support the findings of this study have been deposited in the GenBank with the accession code PRJNA1067853, PRJNA1098918, PRJNA1098920, PRJNA1098929, PRJNA1098930, PRJNA1098932, PRJNA1098933, PRJNA1098937, PRJNA1098941, PRJNA1098944, PRJNA1098947, PRJNA1098949, PRJNA1098951, PRJNA1098955, PRJNA1098958, PRJNA1098959, PRJNA1099412, PRJNA1098967, PRJNA1098968, PRJNA1098969, PRJNA1098970, PRJNA1098972, PRJNA1098974, PRJNA1098975, PRJNA1098977, PRJNA1099319, PRJNA1099321, PRJNA1099322, PRJNA1099323, PRJNA1099324, PRJNA1099326, PRJNA1099327, PRJNA1099328.

Declarations

Ethical approval
This study was conducted in compliance with the recommendations of the Declaration of Helsinki and the Ethics Committee of the Institute of Infectious Disease Prevention and Control, Henan Center for Disease Control and Prevention (ethical approval number: 2015-YM-006-02).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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