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ENVIRONMENTAL MICROBIOLOGY

# Implications of Foraging and Interspecies Interactions of Birds for Carriage of *Escherichia coli* Strains Resistant to Critically Important Antimicrobials

**Shewli Mukerji,[a,b] Samantha Gunasekera,[b] James Nicholas Dunlop,[c] Marc Stegger,[b,d] David Jordan,[e] Tanya Laird,[b] Rebecca Jane Abraham,[b] Mary Barton,[f] Mark O'Dea,[b] Sam Abraham[b]**

[a]School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, South Australia, Australia
[b]Antimicrobial Resistance and Infectious Diseases Laboratory, College of Science, Health, Engineering and Education, Murdoch University, Murdoch, Western Australia, Australia
[c]Conservation Council WA and Citizen Science Program, Perth, Western Australia, Australia
[d]Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen, Denmark
[e]NSW Department of Primary Industries, Wollongbar, New South Wales, Australia
[f]Clinical and Health Sciences, University of South Australia, Adelaide, South Australia, Australia

Mark O'Dea and Sam Abraham contributed equally. Author order was determined both alphabetically by first name and in order of increasing seniority in the field.

**ABSTRACT** Globally, gulls have been associated with carriage of high levels of *Escherichia coli* strains resistant to critically important antimicrobials (CIAs), a major concern, as these antimicrobials are the sole alternative or one among only a few alternatives available to treat severe life-threatening infections in humans. Previous studies of Australian silver gulls demonstrated high levels of resistance to CIAs, particularly fluoroquinolone and extended-spectrum cephalosporins, among *E. coli* strains (carriage at 24% and 22%, respectively). This study aimed to identify and characterize strains from four distinct bird species inhabiting a common coastal environment, determine the frequency of carriage of CIA-resistant *E. coli* strains, and examine if these resistant clones and their resistance-encoding mobile genetic elements (MGEs) could be transmitted between species. CIA-resistant *E. coli* was detected in silver gulls (53%), little penguins (11%), and feral pigeons (10%), but not in bridled terns. In total, 37 different sequence types (STs) were identified, including clinically significant human-associated lineages, such as ST131, ST95, ST648, ST69, ST540, ST93, ST450, and ST10. Five main mobile genetic elements associated with $bla_{CTX\text{-}M}$-positive *E. coli* strains isolated from three bird species were detected. Examination of clonal lineages and MGEs provided indirect evidence of transfer of resistance between bird species. The carriage of CIA-resistant *E. coli* by gulls and pigeons with proximity to humans, and in some instances food-producing animals, increases the likelihood of further bidirectional dissemination.

**IMPORTANCE** It has been shown that 20% of Australian silver gulls carry drug-resistant *Escherichia coli* strains of anthropogenic origin associated with severe diseases, such as sepsis and urinary tract infections, in humans. To further characterize the dynamics of drug-resistant *E. coli* in wildlife populations, we investigated the carriage of critically important antimicrobial (CIA) drug-resistant *E. coli* in four bird species in a common environment. Our results indicated that gulls, pigeons, and penguins carried drug-resistant *E. coli* strains, and analysis of mobile genetic elements associated with resistance genes indicated interspecies resistance transfer. Terns, representing a bird species that forages on natural food sources at sea and distant from humans, did not test positive for drug-resistant *E. coli*. This study demonstrates carriage of CIA-resistant bacteria in multiple bird species living in areas commonly

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Address correspondence to Mark O'Dea, m.o'dea@murdoch.edu.au, or Sam Abraham, s.abraham@murdoch.edu.au.

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inhabited by humans and provides further evidence for a leapfrog effect of resistance in wildlife, facilitated by feeding habits.

**KEYWORDS** Antimicrobial resistance, *Escherichia coli*, ST131, CTX-M, gulls, bird, mobile genetic elements, penguins, pigeons

Wildlife carriage of pathogenic bacteria resistant to critically important antimicrobials (CIAs), such as extended-spectrum cephalosporins (ESCs), fluoroquinolones (FQs), carbapenems, and colistin, is of concern for public health and animal health due to the potential to facilitate transmission to humans, other wildlife, and livestock (1–3). Despite insignificant levels of treatment with clinically relevant antimicrobials compared to humans, companion animals, and livestock, antimicrobial resistance (AMR) in wild animals and birds has increasingly been reported worldwide (4–7). Globally, gulls have been implicated in high levels of carriage of CIA-resistant bacteria that are of clinical significance to human health (8–10). Avian populations that adapt to the urban environment may potentially encounter, host, transmit, and even amplify human microorganisms, including potential pathogens. This may result in transmission of these organisms to species outside the urban environment at places where birds congregate, and particularly where colonial breeding or roosting occurs on coastal islands or on wetlands comprising a shared habitat (11).

Coastal islands adjacent to cities often support a range of colonially breeding species. They include gulls, which routinely scavenge in urban areas, and seabirds that forage for aquatic prey in a variety of marine habitats, from coastal shallows to open ocean (12). Coastal breeding islands in an urban context may provide useful One Health observatories to investigate and monitor the transmission of human microorganisms to and between wild bird populations (13). Of particular significance are the potential reservoirs and transmission pathways of bacteria of human origin that are resistant to antimicrobials.

A recent comprehensive study of Australian silver gulls (*Chroicocephalus novaehollandiae*), reported high levels of carriage of FQ- and ESC-resistant *Escherichia coli* strains nationally (24% and 22%, respectively) (14). Genomic characterization revealed that the majority of these CIA-resistant *E. coli* strains originated from humans and belonged to globally disseminated pandemic, multidrug-resistant, extraintestinal, and pathogenic clonal lineages. They included *E. coli* sequence type 131 (ST131), responsible for causing human sepsis and urinary tract infections in Australia and worldwide (15); a rapidly emerging FQ-resistant extraintestinal pathogenic *E. coli* (ExPEC) clone, ST1193 (16); and other clinically relevant STs, including ST10, ST69, and ST38. The study concluded that Australian silver gulls may act as ecological sponges, accumulating and transmitting CIA-resistant pathogenic *E. coli*.

A key unresolved issue is the role of other wild birds in the ecology of *E. coli* strains that are resistant to CIAs, particularly birds that have some form of ecological interaction with silver gulls. To explore this, we studied silver gulls and three other bird species cohabiting on a nearshore breeding island in an urbanized coastal environment. All four species forage from a central location (Penguin Island; 32°17′S, 115°41′E) but occupy different foraging niches. Silver gulls are opportunistic coastal foragers that also scavenge on human food waste and garbage. Feral pigeons (*Columba livia*) in the area forage on grain spillage from the East Rockingham industrial zone and on the seeds of agricultural weeds. In contrast to the gulls and pigeons, little penguins (*Eudyptula minor*) are an aquatic species foraging on schooling baitfish in waters close to the coast. A fourth species, bridled terns (*Onychoprion anaethetus*), are pelagic seabirds that forage on small fish and crustaceans found on the mid- to outer continental shelf but share their breeding habitat on Penguin Island with nesting silver gulls. All four study species nest and/or roost colonially on Penguin Island, a 12.5-ha aeolianite limestone island 600 m off the coast of suburban Rockingham, approximately 50 km south of Perth, Western Australia. The island supports approximately 3,000 pairs of breeding

silver gulls, 500 pairs of little penguins, up to 900 feral pigeons, and 4,000 pairs of bridled terns (12).

The aim of the study was to identify and characterize the CIA-resistant *E. coli* strains isolated from the four different bird species, including determining the phylogenetic relatedness of the isolates and the mobile genetic elements (MGEs) conferring resistance to CIAs. This would allow investigation into AMR mobility through colonizing bacteria in each of the four bird species in an environment with a limited influx of genes from nonavian species. It was hypothesized that discrete MGEs would be associated with resistance genes and that characterization of these would provide indications of gene movement throughout populations. Although it is widely acknowledged that the directionality of movement of AMR is ambiguous and that carriage in wildlife may originate from humans, the study aimed to facilitate better understanding of the routes of further amplification and dissemination of these CIA-resistant *E. coli* strains within the ecological system.

## RESULTS

**Carriage of CIA-resistant *E. coli*.** Among the four bird species, the highest level of carriage of CIA-resistant *E. coli* was detected in silver gulls. Of the 100 gull swabs that were collected, 53 (53%) of the samples carried resistant forms of *E. coli* that demonstrated visible growth on selective agars (MacConkey agar supplemented with ciprofloxacin and Brilliance ESBL agar [ThermoFisher Scientific]). Resistant *E. coli* strains were isolated from 10 pigeon samples (10%) and 6 penguin samples (11%). No bridled terns tested positive for CIA-resistant *E. coli*. These rates of detection of CIA-resistant *E. coli* in swabs from the four different bird species were statistically significant ($P < 0.0001$). Figure 1 presents the frequency of growth of resistant *E. coli* observed for each bird species on selective medium.

**Phenotypic and genotypic characteristics of CIA-resistant *E. coli*.** Disc diffusion susceptibility testing of all *E. coli* isolates from the gulls that were identified as resistant on the basis of growth on selective media ($n = 94$) demonstrated high levels of resistance to ampicillin (86%), ciprofloxacin (49%), and ceftriaxone (55%) and low levels of resistance to cefoxitin (15%) and amoxicillin-clavulanate (19%). Resistance to ampicillin, ciprofloxacin, and ceftriaxone was common among *E. coli* strains recovered from antimicrobial-supplemented selective agar from feral pigeons ($n = 16$) and little penguins ($n = 9$) (Table 1). However, none of the *E. coli* isolates demonstrated resistance to the carbapenems (imipenem and meropenem). The overall frequencies of resistance based on disc diffusion testing in *E. coli* from selective plating in each of the three bird species are presented in Table 1.

**Molecular characterization of sequence types and antimicrobial resistance genes.** Among *E. coli* strains isolated from silver gulls ($n = 94$), feral pigeons ($n = 16$), and little penguins ($n = 10$), 37 STs were identified in total. In *E. coli* strains isolated from silver gulls, 30 different STs were detected, while 4 different STs were found in feral pigeons and 3 STs in little penguins. Among the 94 *E. coli* strains from silver gulls, the predominant STs included the following: ST131, 9.6% (9/94); ST69, 6.4% (6/94); ST10, 3.2% (3/94); and ST648, 3.2% (3/94). In feral pigeons, the most predominant were ST131, which was present in 25% (4/16) of samples, and ST 69, ST450, and ST695, which were each present in 12.5% (2/16) of samples. In *E. coli* strains isolated from little penguins, ST1598 and ST95 were the most frequent types and were each present in 20% (2/10) of samples. Dissemination of STs, based on the core genome single-nucleotide polymorphism (SNP) phylogeny of a subset of silver gull isolates and all feral pigeon and little penguin isolates, is demonstrated in Fig. 2.

**Examination of antimicrobial resistance genes and MGEs.** A range of resistance genes were present, likely representing the diverse origins of the isolates (see Fig. S2 in the supplemental material). Most isolates contained a combination of SNPs within the quinolone resistance-determining regions (QRDRs), accounting for the phenotypic resistance to ciprofloxacin noted in all three bird species. In addition, a small subset of seagull isolates carried the plasmid-mediated quinolone resistance genes *qnrS* ($n = 4$)

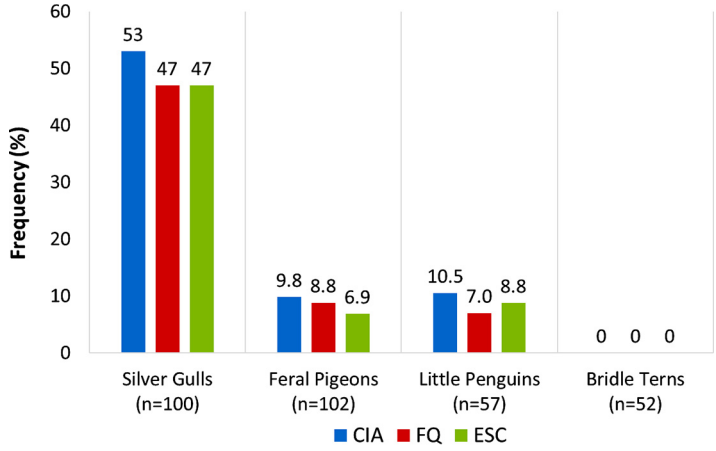


**FIG 1** Frequency (percent) of carriage of resistant *E. coli* strains in fecal swabs from the four bird species. CIA, resistant to fluoroquinolones and/or extended-spectrum cephalosporins; FQ, fluoroquinolone resistant; ESC, extended-spectrum cephalosporin resistant. Resistance was measured on selective medium containing each drug and confirmed by antimicrobial susceptibility testing using disc diffusion.

and *qnrB* ($n = 1$). The *qnrS* gene was detected in ST457, ST10, and ST58 isolates, while an ST617 isolate harbored *qnrB*. However, there was no clear pattern in the carriage of resistance genes across sequence types. The most frequently occurring ESC resistance gene identified in *E. coli* isolates across the three bird species was $bla_{\text{CTX-M-15}}$, found in 7.4% (7/94) of silver gull origin isolates, 25% (4/16) of feral pigeon origin isolates, and 50% (5/10) of little penguin origin isolates. The $bla_{\text{CTX-M-15}}$ gene was found across three different STs in feral pigeons (ST69, ST93, and ST450), three in little penguins (ST95, ST648, and ST1598), and six in silver gulls (ST10, ST127, ST457, ST617, ST648, and ST1598) (Fig. 2).

In order to further characterize the degree of similarity between resistance elements, and thus the indication that horizontal gene transmission was potentially occurring across species, detailed analysis of the CTX-M-type $\beta$-lactamase-encoding genes was undertaken, which revealed that there were two main mobile genetic elements associated with $bla_{\text{CTX-M-15}}$-positive *E. coli* isolates from all three bird species. A 2,971-bp transposition unit (TU) containing an IS*Ecp1* insertion sequence 48 bp upstream from $bla_{\text{CTX-M-15}}$ and a partial *ORF477*Δ downstream (MGE 1) was present in four silver gull origin *E. coli* isolates and three feral pigeon origin *E. coli* isolates, located chromo-

**TABLE 1** Resistance profiles of isolates obtained from media selecting for extended-spectrum cephalosporin and fluoroquinolone resistance determined by disc diffusion assay[a]

| Antimicrobial | Frequency of resistance (% of isolates) | | | |
|---|---|---|---|---|
| | Silver gull ($n = 94$) | Feral pigeon ($n = 16$) | Little penguin ($n = 9$) | *P* value |
| Amoxicillin-clavulanate | 20.2 | 0 | 11.1 | 0.117 |
| Ampicillin | 86.2 | 87.5 | 100 | 0.771 |
| Cefoxitin | 16.0 | 0 | 0 | 0.154 |
| Ceftriaxone | 55.3 | 62.5 | 66.7 | 0.792 |
| Chloramphenicol | 21.3 | 18.7 | 22.2 | 1.000 |
| Ciprofloxacin | 47.9 | 62.5 | 66.7 | 0.371 |
| Gentamicin | 10.6 | 6.2 | 22.2 | 0.475 |
| Imipenem | 0 | 0 | 0 | NA |
| Meropenem | 0 | 0 | 0 | NA |
| Streptomycin | 45.7 | 56.3 | 22.2 | 0.274 |
| Tetracycline | 52.1 | 56.2 | 55.6 | 0.945 |
| Trimethoprim-sulfamethoxazole | 50.0 | 56.2 | 44.4 | 0.892 |

[a]Data are grouped according to bird species of origin, with associated interspecies carriage *P* value. No resistant isolates from bridled terns were detected.

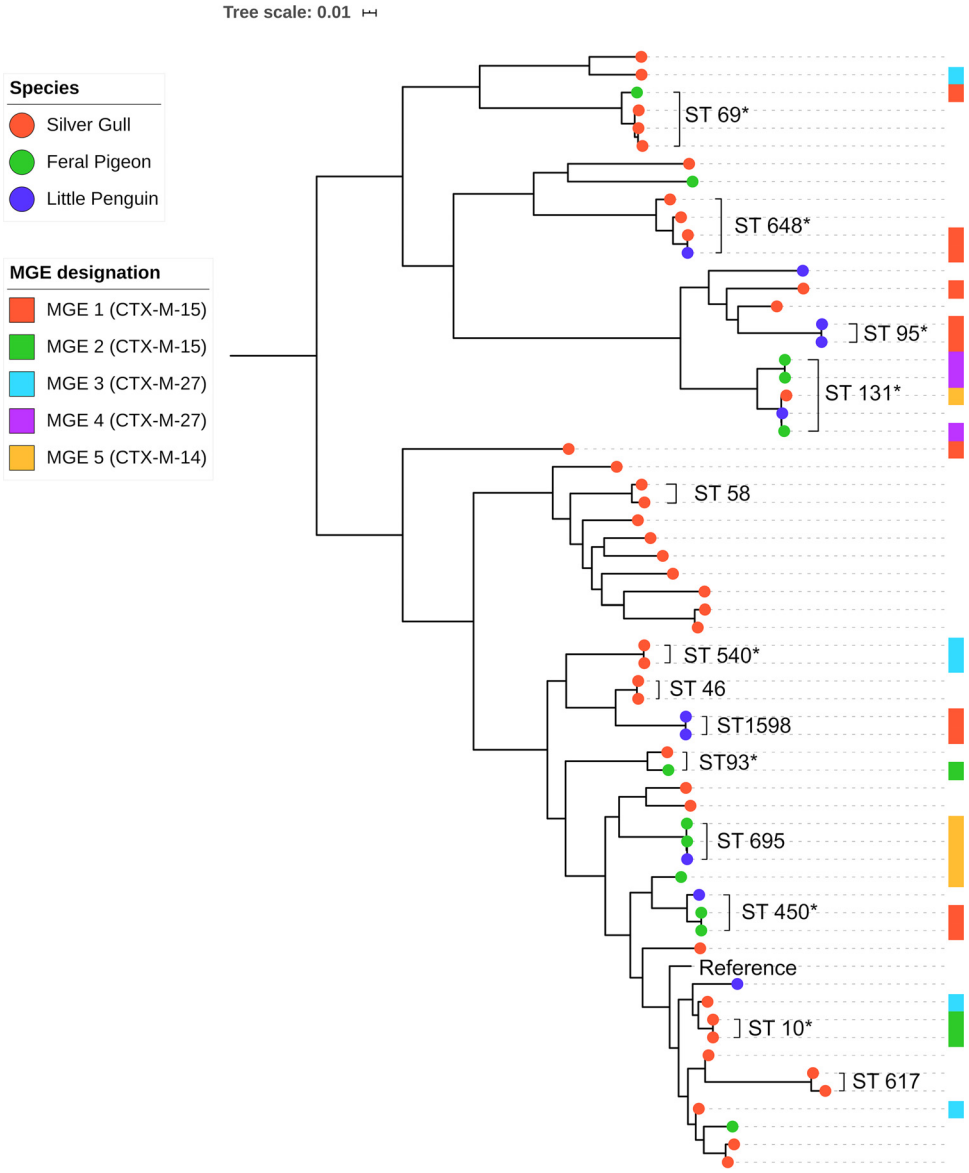


**FIG 2** Maximum-likelihood midpoint-rooted phylogenetic tree representing clonal diversity among 61 *E. coli* isolates from silver gulls, feral pigeons, and little penguins on Penguin Island, Western Australia. The colored circles represent different bird species the *E. coli* strain was isolated from. The scale bar corresponds to ~2,650 SNPs. The asterisks mark clinically significant human-associated CIA-resistant *E. coli* lineages. Mobile genetic element designations (1 to 5) are as follows: IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15}$-*ORF477*Δ (2,971-bp TU), $bla_{CTX\text{-}M\text{-}15}$-Tn*2*, IS*26*-$bla_{CTX\text{-}M\text{-}27}$-IS*903C*, IS*26*-$bla_{CTX\text{-}M\text{-}27}$-IS*903B*, and IS*Ecp1*-$bla_{CTX\text{-}M\text{-}14}$/IS*Ecp1*-$bla_{CTX\text{-}M\text{-}14}$-IS*903C*.

somally in all cases, based on MLplasmids analysis (see below). Five little penguin origin *E. coli* isolates carried the transposition unit, which was chromosomally integrated in three isolates and plasmid mediated in the remaining two (Fig. 3).

Searching for related mobile genetic elements using NCBI nucleotide BLAST revealed high levels of homology (single SNP differences only) between the 2,971-bp transposition unit seen in silver gulls, feral pigeons, and little penguins on Penguin Island and human clinical $bla_{CTX\text{-}M\text{-}15}$-positive *E. coli* isolates observed internationally, including a uropathogenic human ST131 isolate from Australia (GenBank accession no. CP036245.1).

A Tn*2* transposon that carried the complete $bla_{CTX\text{-}M\text{-}15}$ gene, a truncated IS*Ecp1* (potentially artificially truncated during contig assembly), and a partial *ORF477*Δ (MGE 2; 4,570 bp long) was found in two silver gull origin *E. coli* isolates and one feral pigeon origin isolate. This mobile genetic element was plasmid mediated in all cases (Fig. 3).

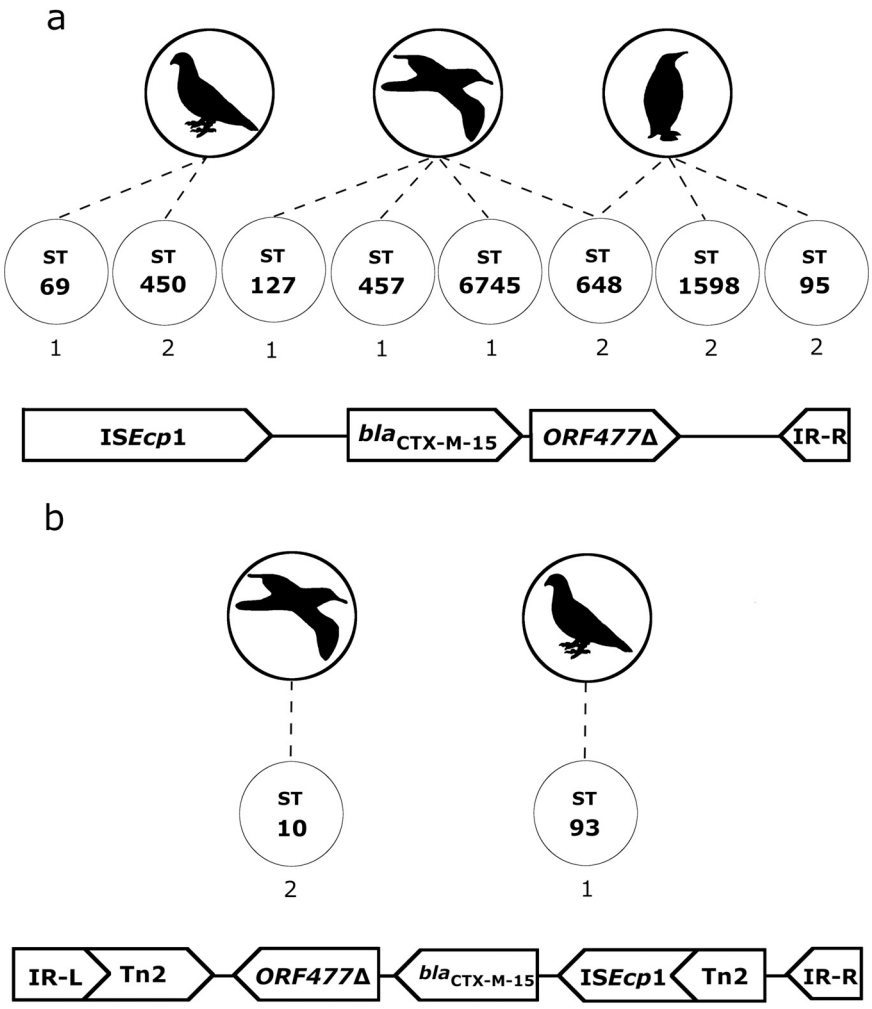


**FIG 3** Schematic diagram of commonly observed mobile genetic elements containing $bla_{CTX\text{-}M\text{-}15}$. (a) MGE 1. (b) MGE 2. The source animals, sequence types of origin, and numbers of isolates are indicated. IR-L, left inverted repeat; IR-R, right inverted repeat.

An NCBI nucleotide BLAST search revealed that two *E. coli* isolates from the United States and one *E. coli* isolate from India carried very similar mobile genetic elements (<5-bp difference); however, the majority of relevant hits were noticeably different (<93% query coverage).

The $bla_{CTX\text{-}M\text{-}27}$ gene was detected in 5% (5/94) of silver gull origin *E. coli* isolates and 19% (3/16) of feral pigeon origin *E. coli* isolates. All $bla_{CTX\text{-}M\text{-}27}$-positive feral pigeon origin *E. coli* isolates were ST131. All five silver gull origin *E. coli* isolates harboring $bla_{CTX\text{-}M\text{-}27}$ contained the ESC resistance gene within the same IS*26*-mediated composite transposon (MGE 3; 1,511 bp long), but this mobile genetic element was present across four STs (ST38, ST540, ST43, and ST3572). Based on analysis using MLplasmids, the $bla_{CTX\text{-}M\text{-}27}$-associated composite transposon was found to be chromosomally integrated in two $bla_{CTX\text{-}M\text{-}27}$-positive isolates and plasmid mediated in the remaining three $bla_{CTX\text{-}M\text{-}27}$-positive isolates. Human clinical and livestock-associated *E. coli* isolates from Japan (2 isolates), Vietnam (1 isolate), and Sweden (1 isolate) have been found to contain identical mobile genetic elements, although the majority of relevant hits had <95% query coverage.

Two out of three feral pigeon origin *E. coli* isolates harboring $bla_{CTX\text{-}M\text{-}27}$ contained the same IS*26*-mediated composite transposon (MGE 4; 1,513 bp long), which slightly differed from the composite transposon observed in silver gulls (Fig. 4). It was located chromosomally in one isolate and plasmid mediated in the other. The third isolate

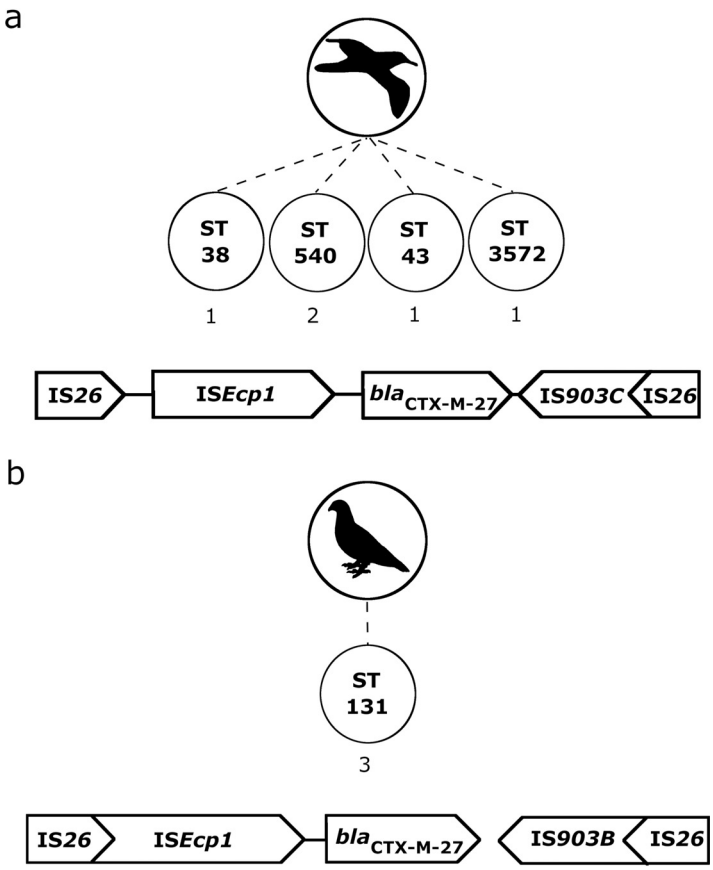


**FIG 4** Schematic diagrams of both mobile genetic elements carrying $bla_{CTX\text{-}M\text{-}27}$, including source animals, sequence types, and total numbers of isolates. (a) MGE 3. (b) MGE 4.

harbored a similar mobile genetic element with minor differences of three and one base deletions in the IS*Ecp1* and IS*903B* sequences, respectively, but was otherwise identical to the composite transposon observed in the other two feral pigeon origin *E. coli* isolates harboring $bla_{CTX\text{-}M\text{-}27}$. Searches for similar mobile genetic elements using NCBI nucleotide BLAST found 20 isolates across North America, South America, Europe, and Asia containing identical mobile genetic elements.

The $bla_{CTX\text{-}M\text{-}14}$ gene was present in 3.2% (3/94) of silver gull origin *E. coli* isolates and was chromosomally integrated in all cases. Two of these isolates contained a complete IS*Ecp1* sequence located 42 bp upstream from $bla_{CTX\text{-}M\text{-}14}$, while one isolate contained only 204 bp of the 3′ end of IS*Ecp1* (potentially due to contig assembly), also located 42 bp upstream from $bla_{CTX\text{-}M\text{-}14}$. The $bla_{CTX\text{-}M\text{-}14}$ gene was also present in 12.5% (2/16) of *E. coli* isolates from feral pigeons and 10% (1/10) of little penguin origin *E. coli* isolates. Both $bla_{CTX\text{-}M\text{-}14}$-positive feral pigeon origin *E. coli* isolates and the little penguin origin *E. coli* isolate were ST695 (Fig. 5). All three isolates harbored the same mobile genetic element conferring resistance to ESCs (MGE 5), with the mobile genetic element comprising the entire sequence of IS*Ecp1* at the 5′ end and then $bla_{CTX\text{-}M\text{-}14}$ 42 bp downstream. Searches for similar mobile genetic elements using NCBI BLAST found that over 25 isolates across Asia, Europe, North America, and Asia contained a highly conserved element (1-bp difference). Among the isolates included in this study, the number of interisolate SNPs observed in all five mobile genetic elements was low. SNPs were present only in MGE 3 and MGE 5 and consisted of a single nucleotide difference in each, indicating that shared MGEs were highly similar among both the different *E. coli* lineages and the bird species.

Less prominent ESC resistance genes found in silver gulls included $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CMY\text{-}2}$. One *E. coli* isolate from a silver gull harbored $bla_{CTX\text{-}M\text{-}3}$; however, the

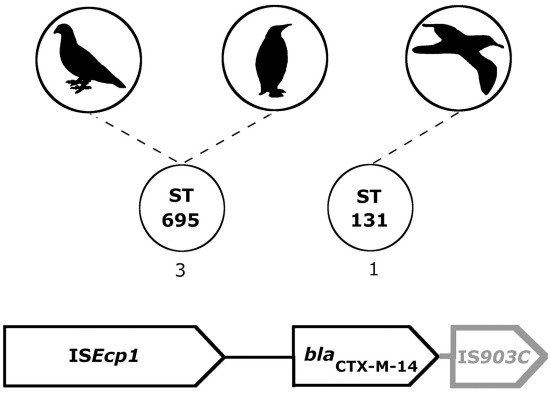


**FIG 5** Schematic diagram of mobile genetic element carrying $bla_{CTX\text{-}M\text{-}14}$, including sequence type information, numbers of isolates, and source animals. The region of the mobile genetic element shown in gray was present only in ST695 isolates.

associated mobile genetic element was not detected in any other isolates across the three bird species containing $bla_{CTX\text{-}M}$ genes. Four silver gulls carried $bla_{CMY\text{-}2}$; however, the ESC resistance gene was not detected in feral pigeons or little penguins.

**Plasmid transfer by conjugation.** The conjugation experiment undertaken to evaluate the potential for horizontal transfer of resistance by conjugation under laboratory conditions revealed that 43.8% of the selected representative isolates ($n = 16$) were capable of transferring ESC resistance to donor *E. coli* J53, with a conjugation efficiency ranging from $10^{-2}$ to $10^{-6}$ (Table 2). Phenotypic antimicrobial susceptibility testing of transconjugants confirmed resistance to ceftriaxone. Conjugation led to the successful transfer of ESC resistance genes, such as $bla_{CMY\text{-}2}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, and $bla_{CTX\text{-}M\text{-}27}$. Conjugation also led to the transfer of resistance to other classes of antimicrobials, such as second-generation cephalosporins (cefoxitin), tetracyclines, and penicillins (ampicillin) (Table 2). In addition, an elevated MIC for fluoroquinolones (ciprofloxacin) was observed in two of the transconjugants that acquired plasmids from donor isolates that carried a plasmid-mediated quinolone resistance gene, *qnrS* (143CIP and 157ESB). The ciprofloxacin MIC (0.25 mg/liter) for the two transconjugants was categorized by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) as non-wild type based on the epidemiological cutoff value (ECOFF) breakpoint compared to donor strain J53 (MIC $<$ 0.015 mg/liter) (Table 2).

## DISCUSSION

The key finding of this study was that in a discrete coastal environment where populations of four bird species exist in close cohabitation, three of them (silver gulls, feral pigeons, and little penguins) harbored *E. coli* strains resistant to CIAs while the remaining species (bridled terns) did not. The principal ecological species factor

**TABLE 2** Donor and transconjugant characteristics of ESC-resistant *E. coli* isolates subjected to conjugation under laboratory conditions

| | Characteristics | | | |
|---|---|---|---|---|
| | Donor | | Transconjugant | |
| Isolate ID | Species of origin | AMR gene(s) | Transfer efficiency | AMR phenotype[a] |
| 143CIP | Little penguin | $bla_{CTX\text{-}M\text{-}15}$, *qnrS*, $bla_{TEM\text{-}1}$, *tetA* | $1.16 \times 10^{-2}$ | AMP, CRO, CFT, CIP[b], TET |
| 130ESB | Silver gull | $bla_{CMY\text{-}2}$, $bla_{TEM\text{-}33}$ | $1.11 \times 10^{-2}$ | AMP, CRO, CFT, FOX |
| 157ESB | Silver gull | $bla_{CTXM\text{-}15}$, *qnrS* | $1.00 \times 10^{-6}$ | AMP, CRO, CFT, CIP[b] |
| 194ESB | Silver gull | $bla_{CTX\text{-}M\text{-}27}$, *strA*, *strB*, *sul2* | $4.16 \times 10^{-3}$ | AMP, CRO, CFT |
| 219ESB | Feral pigeon | $bla_{CTX\text{-}M\text{-}15}$ | $2.48 \times 10^{-3}$ | AMP, CRO, CFT |
| 233ESB | Little penguin | $bla_{CTX\text{-}M\text{-}14}$ | $1.08 \times 10^{-2}$ | AMP, CRO, CFT |
| 288ESB | Feral pigeon | $bla_{CTX\text{-}M\text{-}14}$ | $7.51 \times 10^{-4}$ | AMP, CRO, CFT |

[a]AMP, ampicillin; CRO, ceftriaxone; CFT, ceftiofur; FOX, cefoxitin; CIP, ciprofloxacin; TET, tetracycline.
[b]Phenotypic resistance was categorized based on non-wild-type classification by ECOFFs.

explaining this difference is the distinctly different feeding behavior of bridled terns, which consists of foraging on natural food sources at sea distant from human populations.

This study further validates the findings from an earlier Australian study that described high levels of CIA-resistant *E. coli* carriage in gulls. Antimicrobial susceptibility testing confirmed that 36% (34/94) of *E. coli* isolates from gulls were resistant to ciprofloxacin, 43% (40/94) were resistant to ceftriaxone, and 12% (11/94) of isolates were resistant to both. These findings are similar to those of the previous survey. The frequencies of resistance to FQ and ESC among the *E. coli* isolates from feral pigeons were 25% (4/16) of isolates resistant to FQ or ESC and 38% (6/16) resistant to both antimicrobials. Resistance to either FQ or ESC antimicrobials was demonstrated by 33% (3/9) of the *E. coli* isolates from little penguins, while another 33% (3/9) of isolates were resistant to both FQ and ESC. Resistance to FQs was likely mediated by SNP accumulations in the QRDRs, as outlined in Fig. S2. The main resistance gene associated with ESC resistance isolated from all the bird species was $bla_{\text{CTX-M-15}}$, followed by $bla_{\text{CTX-M-27}}$ and $bla_{\text{CTX-M-14}}$. Other, less prevalent ESC resistance genes included $bla_{\text{CTX-M-3}}$ and $bla_{\text{CMY-2}}$. Other genes associated with resistance to less important antimicrobial classes were scattered among isolates, with no clear pattern based on species of origin or phylotype, and may have played some role in coselection for $\beta$-lactamase-harboring isolates.

Bioinformatics analysis revealed that each ESC resistance gene always occurred within the context of a mobile genetic element, with all CTX-M-type and CMY-type $\beta$-lactamase genes found downstream from an IS*Ecp1* insertion sequence and CTX-M-type $\beta$-lactamase genes associated with IS*26* insertion sequences. While not a direct indication of interspecies transmission, examination of these elements could be used as a proxy for mapping the movement of resistance, indicating that even without direct clonal transmission, horizontal transmission of the elements carrying the resistance markers may be responsible for the profiles seen across bird species.

Two mobile genetic elements carrying $bla_{\text{CTX-M-15}}$ were identified among silver gulls, feral pigeons, and little penguins and appear to be promiscuous based on their ability to be incorporated into multiple *E. coli* lineages. The most prevalent of these mobile genetic elements was found in all three bird species, while the other was found in two out of the three bird species. While ST648 harboring MGE 1 was common to gulls and penguins, the remaining MGEs were not clonally shared between species, further indicating that horizontal transmission of MGEs may be responsible for resistance profiles, rather than direct transfer and colonization of clones already harboring these elements. One mobile genetic element carrying $bla_{\text{CTX-M-27}}$ was found in all $bla_{\text{CTX-M-27}}$-positive silver gull origin *E. coli* isolates, while two different mobile genetic elements also carrying $bla_{\text{CTX-M-27}}$ were found in feral pigeon origin *E. coli* isolates. Two similar mobile genetic elements carrying $bla_{\text{CTX-M-14}}$ were found in the $bla_{\text{CTX-M-14}}$-positive silver gull origin *E. coli* isolates. A third distinct but structurally similar mobile genetic element carrying $bla_{\text{CTX-M-14}}$ was common to all $bla_{\text{CTX-M-14}}$-positive feral pigeons and little penguins. These findings provide further evidence suggesting transmission of AMR between the three bird species may be occurring through the horizontal gene transfer of mobile genetic elements in an interclonal and interspecies pathway. Further evidence of interspecies transmission of the CIA-resistant *E. coli* strains is the sharing of human-associated ExPEC clones, like ST131, ST69, ST95, and ST10, among the three affected bird species (17). This finding is consistent with those of an earlier study in which a high prevalence of ST131, ST69, and ST10 strains was observed among *E. coli* isolates from Australian silver gulls (14).

SNP-based analysis of each mobile genetic element of interest determined that very limited sequence diversity was observed, further supporting the hypothesis that common genetic elements are circulating throughout the ecosystem. However, comparison of each mobile genetic element of interest detected in the avian population on Penguin Island to similar mobile genetic elements found that SNPs were not common within these mobile genetic elements, regardless of geographical origin, with MGEs 1,

4, and 5 demonstrating high levels of similarity to previously recorded elements (≥99% query coverage and ≥99% similarity). The presence of these (or highly similar) circulating elements in international isolates (predominantly human clinical and livestock-associated *E. coli* isolates) precludes the ability to state that these elements circulate only in the Penguin Island birds. However, of the four bird species studied, only the bridled tern is migratory, and no resistant isolates were detected in the species, indicating circulation of these elements throughout the ecosystem is a more likely occurrence than migratory bird species introducing them from overseas. In contrast to MGEs 1, 4, and 5, MGEs 2 and 3 showed greater diversity than international elements, with similarity over 93 to 95% query coverage, giving further weight to the theory that these mobile elements are being transferred between selected bird species in the studied ecosystem.

Various investigations worldwide implicate gulls as reservoirs of high levels of CIA-resistant *E. coli*, which is primarily attributed to their scavenging behavior. This study found that carriage of CIA-resistant bacteria was significantly greater in gulls than in the other three species, making them a keystone species in AMR transmission throughout the environment. Although gulls are naturally opportunistic shoreline foragers around cities and towns, they have adapted to feeding primarily from landfill sites, sewage treatment plants, and urban wetlands, which elevates their risk of exposure to the CIA-resistant bacteria (18). In this case, the disposal of human refuse contaminated with human fecal flora (sanitary products) at landfill sites is hypothesized to be the most likely source of initial transference from human to bird populations, although other explanations, such as overflow of sewage effluents into coastal environments during periods of high rainfall, are also possible. Proximity to human activities has been shown to have a substantial impact on the intestinal flora of gulls and other wildlife (19–21).

Studies have demonstrated that *E. coli*, once released into the external environment through fecal deposition on the soil, sediments near marine coastal areas and can successfully replicate and survive for prolonged periods outside the host body in the secondary environment (22). The absence of resistant *E. coli* in bridled terns suggests that transmission may be occurring via contaminated water. The little penguins make landfall through the poorly mixed shallows around Penguin Island, where gulls concentrate, wash, and defecate, and these birds may be ingesting CIA-resistant *E. coli* bacteria from the shallow water at their landfalls or from contaminated colony soil. Coastal freshwater resources in artificial and natural wetlands used by both gulls and pigeons are the most likely location for transmission to feral pigeons.

The findings from this study have significant ecological implications, with the potential leapfrog movement of resistant *E. coli* mobile elements within a semicontained environment likely to be related to the feeding habits of different species and the ecological niche that supports them. In addition, it is reasonable to propose that more complex environments, such as those on the major avian flyways, would precipitate further mixing of species and transfer of resistance components originating from multiple countries, followed by dissemination. While this study did not investigate offshore dissemination of resistance, pigeons and gulls also have proximity to humans, livestock, and companion animals, which further increases the risk of transmission. It is noteworthy that the transfer of CIA-resistant *E. coli* from the pigeons and gulls is not confined or limited to the near-coastal areas, as these birds are frequent visitors to urban and human residential areas. Fecal droppings of pigeons and gulls carrying CIA-resistant *E. coli* strains can contaminate the surface water, as well as agricultural lands, and could be further transmitted to livestock or food-producing animals, humans, and other bird species.

There is no indication of health issues affecting seabirds harboring resistant bacteria; however, the study location at Penguin Island is a popular tourist destination and has a high number of visitors for coastal recreational activities. The fact that a commonly visited destination is contaminated with clinically significant, human-associated pathogenic, and CIA-resistant *E. coli* strains warrants further investigation to determine the

sources of contamination that the gulls encounter and to further clarify the process of amplification and transmission of these CIA-resistant *E. coli* strains within the adjacent bird colonies. In addition, larger-scale studies to determine the effectiveness of surveying MGEs as a historical roadmap of interspecies bacterial transmission will also assist in ongoing ecosystem-wide studies. Future studies should include large-scale resistome studies of indigenous microflora of the bird species and environmental samples, including freshwater, to evaluate the carriage and potential transfer of AMR genes.

## MATERIALS AND METHODS

**Sample collection.** All sampling procedures were carried out under the approval of the Murdoch University Animal Ethics Committee (permit no. R3060/18). A total of 311 cloacal swabs were taken from the four bird species nesting or roosting on Penguin Island. The swabs were collected over a 6-month period (May to October 2018). The gulls and terns were captured using walk-in nest traps, while pigeons and penguins were captured at night, using headlamps and long-handled nets for the pigeons. Sampling was performed indoors wherever possible, and the birds were released into their colony areas within 5 min. Samples included cloacal swabs from 100 silver gulls, 57 little penguins, 102 feral pigeons, and 52 bridled terns. The swabs were collected into charcoal medium (Copan) and transported to the Murdoch University Antimicrobial Resistance and Infectious Diseases Laboratory in Perth, Western Australia, within 24 h of collection. To resuscitate the bacteria, the swabs were incubated for 4 h in 3 ml of buffered peptone water (Oxoid, ThermoFisher Scientific).

**Bacterial isolation.** Following nonselective enrichment in buffered peptone water, *E. coli* isolates resistant to FQ and ESC were identified by streaking the swabs onto two selective agar plates: MacConkey agar supplemented with 1 mg/liter ciprofloxacin (ThermoFisher Scientific) and Brilliance ESBL agar (ThermoFisher Scientific). Presumptive *E. coli* isolates (one colony per plate) identified on the selective agar after overnight incubation at 37°C were subcultured onto sheep blood agar (Micromedia, Australia), and pure colonies were obtained. Species identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Microflex; Bruker). Confirmed *E. coli* isolates were selected for further characterization. A flow diagram illustrating the sequence of experiments used in isolating and characterizing *E. coli* isolates resistant to critically important antimicrobials from the birds is shown in Fig. S1 in the supplemental material.

**Phenotypic antimicrobial susceptibility testing.** Antimicrobial disc diffusion testing using Clinical and Laboratory Standards Institute (CLSI) guidelines and recommended breakpoints for interpreting phenotypic AMR (23) was performed on all identified *E. coli* isolates ($n = 126$). The 12 antimicrobials used were ampicillin, ciprofloxacin, imipenem, meropenem, trimethoprim-sulfamethoxazole, tetracycline, gentamicin, ceftriaxone, streptomycin, cefoxitin, amoxicillin-clavulanate, and chloramphenicol. *E. coli* ATCC 25922 was used as a control according to CLSI guidelines during antimicrobial susceptibility testing.

**Phylogenetic analysis using PCR.** DNA extraction was performed as described previously using 6% Chelex (Bio-Rad) (24). A multiplex sequence-type-specific PCR (ST PCR) was performed to identify clinically significant human-associated *E. coli* ST131, ST69, and ST95 (25). Electrophoresis was performed using 2% agarose gels containing 0.01% SYBRsafe dye (Invitrogen).

A multiplex PCR-based assay described previously (26) was performed to determine the phylogenetic groups of the resistant *E. coli* isolates. Following phylogenetic grouping, isolates belonging to each group were further delineated using a random amplified polymorphic DNA (RAPD) assay (27), and whole-genome sequencing (WGS) was performed on a subset with unique profiles.

**Molecular characterization using whole-genome sequencing.** Whole-genome sequencing was performed on all CIA-resistant *E. coli* isolates from little penguins and feral pigeons and on a subset of isolates from silver gulls ($n = 41$; 43.6%) selected based on RAPD, ST PCR, and AMR profiles. Sequencing was performed as previously described on an Illumina NextSeq 500 platform using a V2 midoutput 2-by-150 flow cell (24). FASTQ files underwent adapter trimming using BaseSpace (Illumina), and quality assessment was performed using FastQC v0.11.7 (28). FASTQ files that failed quality control were trimmed with Trimmomatic v0.38 (29) using the SLIDINGWINDOW setting, and reads where average quality dipped below a phred score of 25 across a 5-bp window were trimmed. *De novo* assembly was performed with SPAdes genome assembler v3.12.0 (30) using default settings. Contig files were uploaded to the Center for Genomic Epidemiology (http://www.genomicepidemiology.org) and parsed through the Bacterial Analysis Pipeline (31) to detect AMR genes and determine isolate multilocus sequence typing (MLST). SNPs within the QRDRs were detected using ABRicate (https://github.com/tseemann/abricate). Heatmap in R studio was used to map AMR gene presence and absence. Contigs containing AMR genes were uploaded to Galileo AMR (32; https://www.arcbio.com/amr) to annotate AMR-associated mobile genetic elements. Mobile genetic elements conferring resistance to ESCs were extracted and used for reference to mapping of the FASTQ read data using Bowtie2 v2.3.4.1 (33), and consensus sequences were collected using SAMtools v1.9 (34). Contigs were also uploaded to MLplasmids v1.0.0 (35) to infer whether each contig containing a mobile genetic element conferring resistance to ESCs formed part of the chromosome or plasmid. A phylogenetic tree was constructed to investigate the clonal similarity among CIA-resistant *E. coli* isolates from Penguin Island, first by producing a core SNP alignment using Snippy v4.1.0 (available from https://github.com/tseemann/snippy), using the *E. coli* K12 MG1655 chromosome (GenBank accession number NC_000913.3) as a reference (36). The core SNP alignment was then used for maximum-likelihood phylogenetic-tree construction with RAxML v8.2.11

(37) after regions of recombination were removed using ClonalFrameML v1.11 (37, 38). The final phylogenetic tree was annotated using iTOL v4.3.3 (39).

Key mobile genetic elements were aligned using MUSCLE and investigated for intraelement variations (SNPs) between isolates before being aligned against BLAST hits using the NCBI nr/nt nucleotide database to determine their presence and variation in international *E. coli* isolates.

**Plasmid transfer by conjugation.** Transfer of the resistance plasmid was performed by bacterial conjugation with the sodium azide-resistant *E. coli* J53 recipient strain, using a representative subset of ESC-resistant *E. coli* isolates ($n = 16$) carrying various $\beta$-lactamase gene variants of $bla_{CTXM}$ and $bla_{CMY-2}$ from all three bird species. Conjugation was performed using overnight culture of donor and recipient *E. coli* isolates grown in Luria broth (LB) (Thermo Fisher) at 37°C. Strains were mixed at a ratio of 1:2 (donor/recipient) and incubated for 4 h. Transconjugants were selected by serially diluting the donor-recipient mixture and immediately inoculating 5 $\mu$l (in triplicate) of serial dilutions ($10^{-1}$ to $10^{-6}$) onto MacConkey agar containing sodium azide (150 mg/liter) and/or ceftriaxone (8 mg/liter). The transconjugants were subcultured again onto MacConkey agar containing sodium azide (150 mg/liter) and ceftriaxone (8 mg/liter) and blood agar and subjected to MIC testing as previously described (24). The isolates were tested against the following 13 antimicrobials: amoxicillin-clavulanate, ampicillin, cefoxitin, ceftiofur, chloramphenicol, ciprofloxacin, colistin, ceftriaxone, florfenicol, gentamicin, streptomycin, tetracycline, and trimethoprim-sulfamethoxazole. MICs were interpreted using clinical breakpoints recommended by CLSI (23), and ECOFFs were interpreted using EUCAST guidelines (https://www.eucast.org/organization/). Conjugation efficiency was calculated by dividing the total colony count of transconjugants by the colony counts of donor cells.

**Statistical analysis.** Comparison of proportions of isolates and samples expressing resistance were made using contingency table analysis and Fisher's exact test in Stata SE v16.0 (StataCorp LLC, College Station, TX, USA).

**Data availability.** All sequence data were deposited in the NCBI database under accession number PRJNA613145.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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