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Occurrence of ESBL-Producing *Escherichia coli* ST131, Including the *H30*-Rx and C1-M27 Subclones, Among Urban Seagulls from the United Kingdom

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Antimicrobial resistance is a public health concern. Understanding any role that urban seagulls may have as a reservoir of resistant bacteria could be important for reducing transmission. This study investigated fecal *Escherichia coli* isolates from seagulls (herring gulls and lesser black-backed gulls) to determine the prevalence of extended-spectrum cephalosporin-resistant (ESC-R) and fluoroquinolone-resistant *E. coli* among gull species from two cities (Taunton and Birmingham) in the United Kingdom (UK). We characterized the genetic background and carriage of plasmid-mediated resistance genes in extended-spectrum β-lactamase (ESBL)-producing *E. coli* obtained from these birds. Sixty ESC-R *E. coli* isolates were obtained from 39 seagulls (39/78, 50%), of which 28 (28/60, 46.7%) were positive for plasmid-mediated CTX-M and/or AmpC β-lactamase resistance genes. Among these, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CMY-2}}$ predominated. Three isolates belonging to the B2-ST131 clone were detected, of which two harbored $bla_{\text{CTX-M-15}}$ (typed to C2/H30Rx) and one harbored $bla_{\text{CTX-M-27}}$ and was typed to C1/H30-R (recently described as the C1-M27 sublineage). The plasmid-mediated quinolone resistance (PMQR) gene carriage prevalence (11.7%) consisted of *aac(6′)-Ib-cr* and *qnrB* genes. No carbapenem or colistin resistance genes were detected. Urban seagulls in the UK are colonized and can spread major antimicrobial-resistant *E. coli* isolates harboring ESBL and PMQR determinants, including clinically important strains such as the pandemic clone B2-ST131 and the C1-M27 subclade. This is the first report of ST131-C1-M27 subclade in wildlife in the UK and in seagulls worldwide.

Keywords: *Escherichia coli*, B2-ST131, *Larus* spp., ESBLs, AmpC, PMQR, C1-M27

Introduction

In the past two decades, extraintestinal pathogenic *Escherichia coli* (ExPEC) has been the leading cause of difficult-to-treat human urinary tract infections (UTI), soft tissue and bloodstream infections, in both health care and community-onset settings.[1] Selection pressure from antibiotic use has shaped the rapid evolution and spread of antimicrobial resistance among *E. coli* strains no longer responding to conventional treatment and also to critically important antimicrobials such as third-generation cephalosporins.[2]

Antimicrobial resistance appears concentrated within specific bacterial genotypes, among which *E. coli* sequence type (ST) 131, with specific reference to the O25b:H4 serotype, has received particular attention owing to its rapid emergence and pandemic global spread.[3,4] Although the reasons behind the successful dissemination of *E. coli* B2-ST131-O25b:H4 have not been entirely elucidated, a large number of virulence-related genes combined with transferable antimicrobial resistance determinants carried on plasmids have been identified as possible factors.[5–10] In addition, *E. coli* ST131 isolates have been contributing to the recent CTX-M spread, leading to a global rise in Enterobacteriaceae harboring CTX-M-type extended-spectrum β-lactamases (ESBLs).[11]

There is now increasing evidence that the environment is a vast reservoir of resistant bacteria and their associated genes and that complex interactions exist between the environmental resistome and that of clinically significant

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pathogens.[12,13] In this context, current evidence suggests that wild birds, including seagulls, are contributing to the environmental dissemination of antibiotic resistance,[14] which is of great concern when considering highly pathogenic clones such as *E. coli* ST131.

Population genetic studies have given a new insight into the clonal structure of *E. coli* ST131 by revealing epidemiologically distinct sublineages nested within ST131 clade C/H30.[15] Clade C is known for expressing high-level ciprofloxacin resistance mutations resulting in distinctive *gyrA* and *parC* variants[16,17] and, nowadays, it accounts for the vast majority of *E. coli* ST131 worldwide.[18] Whole-genome sequencing enabled the characterization of the two main subclades within C/H30, namely C1/H30R (non-RX) and C2/H30Rx.[19,20] The latter is a clonal subset often associated with $bla_{\text{CTX-M-15}}$, whose clonal expansion was largely responsible for the *E. coli* ST131 pandemic and, along with it, to the substantial global rise in CTX-M-15 (currently the most widespread CTX-M-type ESBL enzyme in humans and animals worldwide).[17,21–23] Conversely, isolates belonging to the C1/H30R subclade are largely ESBL negative, although certain isolates harbor $bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-27}}$, the former being the second most common ESBL type occurring in humans globally, whereas the latter is an ESBL type more often recovered among animal and environmental ST131 strains.[24]

Notwithstanding *E. coli* ST131 being a major cause of disease in people, a few reports have described the detection of pandemic clone B2-ST131 carrying diverse CTX-M-type enzymes in animals, foodstuffs, and the environment.[3,25–30]

Although there are high-level similarities between certain human and animal ST131 isolates with regard to resistance characteristics, virulence factors, and genetic background, there is only limited evidence for direct interspecies transfer of ST131.[31] Given the importance of ST131 as a global public health issue, further investigation of animal-associated ST131 is warranted to clarify the ecology and capacity for cross-species transmission of these strains.

Wild birds have been proposed as sentinels and potential vectors of antimicrobial resistance since many species, such as geese, mallards, cormorants, pigeons, corvids, gulls, and birds of prey, have been found carriers of diverse multidrug-resistant (MDR) bacteria, including *E. coli* strains resistant to extended-spectrum cephalosporins (ESCs) and fluoroquinolones (FQs).[32–41]

In this regard, members of the *Laridae* family of seabirds have become particularly studied as sentinels for monitoring the spread of antimicrobial resistance since their ecology is well understood and they possess several characteristics which make them suited to dissemination studies.[38] Furthermore, gull species are regarded as important bioindicators of environmental contamination by antibiotic resistance, with particular regard to surface waters and coastal areas.[42–44] In addition, gull species can migrate over long distances and they have proven capable of introducing MDR *E. coli* in remote areas dominated by ecosystems with little or no antibiotic resistance, as illustrated by the recovery of CTX-M-15-producing *E. coli* strain belonging to the pandemic clone B2-ST131 in the Russian Commander Islands.[45]

The aim of this study was dual; first, to determine the fecal prevalence of resistance to ESCs among seagull species from two cities in the United Kingdom (UK) (one coastal and one inland) and second, to characterize the genetic background as well as plasmid-mediated resistance genes, virulence genes, and plasmid incompatibility groups in ESBL-producing *E. coli* obtained from these birds.

Materials and Methods

Collection of fecal samples

Herring gull (*Larus argentatus*) and lesser black-backed gull (*Larus fuscus*) fecal samples were collected from two locations (L1: Taunton and L2: Birmingham) in England, UK, as part of Local Authority nonlethal pest-control programs designed to minimize public nuisance caused by the gulls. The method used involved replacing gull eggs in the nests with decoy eggs that encouraged gulls to remain on the nests and minimized the public nuisance caused by gulls scavenging for food within the cities. In response to being disturbed, gulls frequently produced a fecal sample that was collected into sterile universal tubes.

Sampling took place in May 2011 over a 2 days' period. For L1, 50 fecal samples were collected on the first day in the town center from $n=39$ herring gulls and $n=11$ lesser black-backed gulls. For L2, 28 fecal samples were collected on the second day in a single district of the city from $n=7$ herring gulls and $n=21$ lesser black-backed gulls. Upon collection, samples were placed in sterile Universal tubes and transported rapidly to the laboratory for immediate processing; when this was not possible, samples were refrigerated for next-day processing.

Bacterial isolates

Fecal samples (1–2 g) were placed in 10 mL of buffered peptone water (BPW; Thermo Scientific, Basingstoke, UK) and incubated overnight at 37°C.

To screen for ESC-resistant (ESC-R) *E. coli*, a small BPW inoculum (2–3 μL) was streaked onto eosin methylene blue agar (EMBA; Thermo Scientific) containing 1 μg/mL of cefotaxime (all antibiotics from Sigma-Aldrich Ltd., UK). If colonies were phenotypically different, each colony morphotype was selected.

Antimicrobial susceptibility testing

Any cefotaxime-resistant isolates obtained on the EMBA selective media, producing a characteristic *E. coli* green metallic sheen (and subsequently confirmed as *E. coli* by *uidA* and *uspA* polymerase chain reaction [PCR] assay) and for which plasmid-mediated ESC and/or FQ resistance was identified, were subjected to antimicrobial susceptibility testing.

The susceptibility testing was performed by disk diffusion on Mueller-Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute (CLSI) methodology 2018.[46] *E. coli* ATCC25922 was used as control for disk diffusion susceptibility testing for every new batch of isolates using a suspension of 0.5 McFarland to obtain a semiconfluent lawn on MHA. The antimicrobial panel comprised ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), cefoxitin (30 μg), cefpodoxime (10 μg), cefepime (30 μg), piperacillin/tazobactam (110 μg), aztreonam (30 μg), imipenem (10 μg), ertapenem (10 μg), nalidixic acid (30 μg), gentamicin (10 μg), amikacin (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), doxycycline (30 μg), and trimethoprim/

sulfamethoxazole (25 μg) (all disks and media from Oxoid, UK). Interpretation of results was performed according to the CLSI criteria 2018.[46]

Characterization of resistance genes

In order to identify the resistance genes carried by the ESC-R *E. coli* isolates, cell lysates were prepared by suspending the bacterial cells in 500 μL sterile distilled water followed by incubation at 95°C for 10 minutes and centrifugation at 10,000 *g* for 5 minutes.

All ESC-R isolates were subjected to PCR screening for the presence of bla_{SHV}, bla_{TEM}, $bla_{OXA1\text{-}like}$, and $bla_{CTX\text{-}M}$ genes, with a further multiplex PCR being used for the detection of family-specific plasmid-mediated AmpC β-lactamase genes.[47,48] Furthermore, plasmid-mediated quinolone resistance (PMQR) genes *qnrA*, *qnrB*, and *qnrS*, *qepA*, and the *aac(6′)-ib-cr* gene, which confers reduced susceptibility to aminoglycosides and FQs, were identified.[49,50] All isolates positive for *aac(6′)-ib* gene were sequenced with specific primers to confirm the presence of *aac(6′)-ib-cr* variant.[49] Additionally, genetic determinants encoding for resistance to carbapenems and colistin, were investigated according to the methods described by Dallenne *et al.*,[47] Poirel *et al.*,[51] Liu *et al.*,[52] and Rebelo *et al.*[53]

To confirm gene identity, DNA sequencing of both strands of PCR reaction products was done for representative isolates from groups displaying unique gene combinations (*e.g.*, $bla_{CTX\text{-}M}$, bla_{TEM}, *aac(6′)-ib-cr*) using the same sets of primers as in the original reactions (Eurofins MWG Operon). The resulting DNA sequences were compared using BLASTn against sequences in GenBank. All ESC-R *E. coli* isolates were selected for further molecular typing.

Characterization of virulence genes

ESC-R *E. coli* seagull isolates were screened by multiplex PCR for virulence determinants associated to diarrheagenic Shiga toxin-producing (STEC) strains, including Shiga-like toxins 1 and 2 (encoded by stx_1 *and* stx_2) and for accessory STEC virulence factors intimin (*eaeA*) and enterohemorrhagic (EHEC) plasmid-mediated enterohemolysin (*hlyA*) according to Paton and Paton[54] and Vidal *et al.*[55] EHEC belonging to serotypes displaying greater virulence, that is, O157 and O111, were investigated by multiplex PCR assay as previously described.[54] Furthermore, PCR targeting of virulence genes linked to uropathogenicity, including the cytotoxic necrotizing factor type 1 and 2 (*cnf1* and *2*) and various classes of adhesins, such as the pyelonephritis-associated pili (*pap*), S-fimbrial adhesin (*sfa*), and afimbrial adhesin (*afa*), was performed as described by Blanco *et al.*[56]

Molecular characterization of the ESC-R E. coli *isolates*

E. coli isolates were assigned to phylogenetic groups (PGs) A, B1, B2, C, D, E, F, and clade I) by multiplex PCR using two methods, the triplex and the revisited quadruplex methods proposed by Clermont *et al.*[57,58] The isolates identified as PG B2 were screened for the O25 group by a method based on an allele-specific PCR.[59] Positive isolates were further tested to identify members of the international clone B2-ST131 using primers for the genes *pabB* and *trpA*, as described by Clermont *et al.*[60] Following screening for the B2-ST131 clone, positive isolates carrying $bla_{CTX\text{-}M\text{-}27}$, $bla_{CTX\text{-}M\text{-}14}$, and $bla_{CTX\text{-}M\text{-}15}$ genes underwent further analysis consisting of multiplex conventional PCR to detect all ST131 clades (A, B, and C) as well as C subclades (C1-M27, C1-nM27 [C1-nonM27], and C2), as developed by Matsumura *et al.*[61]

Resistance transfer and PCR-based replicon typing

Transfer of resistance genes by conjugation was performed by broth mating to determine whether the ESBL resistance determinants were transferable. The conjugation assays were attempted for isolates carrying $bla_{CTX\text{-}M}$ genes using a streptomycin-resistant *E. coli* HB101 strain as a recipient as previously described by Oliver *et al.*[62] Transconjugants were selected on nutrient agar (Oxoid) supplemented with streptomycin (50 μg/mL) and cefotaxime (1 μg/mL). The resistance phenotype of transconjugants was determined by disk diffusion using the same antimicrobial panel as for parental strains. Plasmid replicons were determined on all $bla_{CTX\text{-}M}$-positive isolates and transconjugants, using a PCR-based replicon typing scheme as previously described.[63]

Results

Bacterial isolates and antimicrobial susceptibility testing

Screening of the 78 seagull fecal samples collected from both locations identified 60 ESC-R *E. coli* isolates obtained from 39 fecal samples, resulting in 50% ESC-R *E. coli* carriage in the seagull population investigated; 55 ESC-R *E. coli* isolates were obtained from L1 (from 34 seagulls) and five isolates from L2 (from five seagulls). Antimicrobial susceptibility testing showed high levels of resistance to ampicillin (100%), cefpodoxime (100%), aztreonam (91.6%), amoxicillin/clavulanic acid (83.8%), streptomycin (70.3%), cefoxitin (62.2%), tetracycline (56.8%), and trimethoprim/sulfamethoxazole (51.4%). In addition, 10.8% and 5.4% of the *E. coli* isolates were resistant to ertapenem and imipenem, respectively.

Characterization of resistance and virulence genes

Screening for ESBL and AmpC genetic determinants identified 46.7% (28/60) of isolates positive for plasmid-mediated CTX-M and/or AmpC β-lactamase resistance genes. The $bla_{CTX\text{-}M}$ genes were identified in 21/60 (35%) of the seagull ESC-R *E. coli* isolates. Of these 21, 12 belonged to the CTX-M group 1 (57.1%) and sequencing identified $bla_{CTX\text{-}M\text{-}15}$ to be the most prevalent resistance gene carried by 11 isolates (91.7%), whereas only one isolate harbored $bla_{CTX\text{-}M\text{-}1}$. Also nine of the 21 *E. coli* $bla_{CTX\text{-}M}$-positive isolates (42.9%) carried CTX-M group 9, with six of the nine harboring $bla_{CTX\text{-}M\text{-}14}$ (66.7%) and $bla_{CTX\text{-}M\text{-}27}$ being present in the remaining three isolates (33.3%). Resistance genes encoding for CTX-M group 2, 8, and 25 were not identified. It is important to note that none of the investigated seagull isolates harbored the bla_{SHV} gene.

A variety of gene combinations was found in the CTX-M-producing *E. coli* isolates with $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}14}$, and $bla_{CTX\text{-}M\text{-}27}$ carried alone or in combination with bla_{TEM}, $bla_{OXA1\text{-}like}$, and *aac(6′)-ib-cr* gene (Table 1). When screened for plasmid-mediated AmpC-type β-lactamase,

TABLE 1. PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF SEAGULL EXTENDED-SPECTRUM CEPHALOSPORIN-RESISTANT *ESCHERICHIA COLI* ISOLATES

					Other beta-lactamase					*Associated virulence factors*							
No. of isolates (n=60)	*Location*	*Old PG*	*New PG*	*CTX-M type*	*TEM SHV/OXA$_{1\text{-}like}$*	*Plasmid-mediated AmpC*	*PMQR*	*PBRT*	*Resistance profile*	pap*1/2*	sfa*1/2*	afa*1/2*	pap*3/4*	cnf*1a/b*	eaeA	Ecp1	*IS*26
1	L1	A	CLADE I	CTX-M-1				FIA	AMP, ATM, CPD, DO							IS*Ecp1*	
1	L1	B1	D	CTX-M-15	TEM			NT	AMP, ATM, CPD							IS*Ecp1*	
1	L1	B1	U	CTX-M-15	TEM			I1	AMP, ATM, CPD, ETP, FOX, IMP, NA, S								
1	L1	B2	B2-ST131	CTX-M-15	TEM			I1, FIA	AMC, AMP, ATM, C, CPD, DO, FOX, NA, S, STX							IS*Ecp1*	
1	L1	B2	B2	CTX-M-15	TEM			FIA	AMC, AMP, ATM, C, CPD, DO, NA, S, STX							IS*Ecp1*	
1	L1	B2	B1	CTX-M-15	TEM			F, FIB	AMC, AMP, ATM, C, CN, CPD, DO, FEP, FOX, NA, PTZ, S, STX	*pap*1/2		*afa*1/2	*pap*3/4			IS*Ecp1*	IS*26*
1	L1	B2	B2	CTX-M-15	TEM			FIB, F	AMC, AMP, ATM, CN, CPD, DO, FEP, NA, S, STX			*afa*1/2				IS*Ecp1*	
1	L1	D	B2	CTX-M-15	TEM, OXA$_{1\text{-}like}$	CMY-2	*aac(6′)-ib-cr*	HI1, FIA	AMP, ATM, CPD, NA								
1	L1	A	F	CTX-M-15	TEM, OXA$_{1\text{-}like}$		*aac(6′)-ib-cr qnrB*	F, FIA	AMC, AMP, ATM, CPD, FOX, S, STX							IS*Ecp1*	
1	L1	D	F	CTX-M-15	TEM, OXA$_{1\text{-}like}$		*aac(6′)-ib-cr*	FIA	AMP, ATM, CPD, NA, STX							IS*Ecp1*	
1	L1	D	D	CTX-M-15	TEM, OXA$_{1\text{-}like}$		*aac(6′)-ib-cr*	FIA	AMC, AMP, ATM, CN, CPD, FEP, FOX, NA, PTZ, STX				*pap*3/4			IS*Ecp1*	
1	L1	B2	B2-ST131	CTX-M-15	TEM, OXA$_{1\text{-}like}$		*aac(6′)-ib-cr*	FIA, Y	AMC, AMP, ATM, CPD, NA, PTZ, STX			*afa*1/2				IS*Ecp1*	IS*26*
1	L1	D	D	CTX-M-14	TEM			NT	AMC, AMP, ATM, C, CPD, DO, NA, S, STX				*pap*3/4				
1	L1	D	D	CTX-M-14	TEM			NT	AK, AMC, AMP, ATM, C, CPD, DO, ETP, FEP, FOX, NA, S, STX				*pap*3/4				
1	L1	D	D	CTX-M-14	TEM			FIB	AMC, AMP, C, CPD, DO, FEP, S, STX				*pap*3/4				
1	L1	D	D	CTX-M-14	TEM			FIB	AMC, AMP, ATM, C, CPD, DO, FEP, FOX, S, STX				*pap*3/4				
1	L1	D	D	CTX-M-14	TEM			FIB	AMC, AMP, ATM, C, CPD, DO, FEP, S, STX				*pap*3/4				
1	L1	D	F	CTX-M-14				F, FIB	AMC, AMP, ATM, C, CPD, DO, FEP, FOX, S, STX								
1	L1	D	D	CTX-M-27	TEM			FIB, FIA	AMC, AMP, ATM, CPD, FOX, NA, STX								
1	L1	B2	B2-ST131	CTX-M-27				FIA, FIB, F	AMP, ATM. CPD, DO, NA, S, STX								
1	L1	D	D	CTX-M-27				F, FIB	AMP, ATM, CPD, DO, S, STX								
1	L1	A	D		TEM		*qnrB*	B/O	AMC, AMP, ATM, CPD, FOX, STX								
1	L1	A	F			CMY-2	*qnrB*	NT	AMC, AMP, CPD, FOX, IMP, PTZ								
1	L1	B1	B1			CMY-2		NT	AMC, AMP, ATM, CPD, FOX, PTZ								

(*continued*)

TABLE 1. (CONTINUED)

No. of isolates (n=60)	*Location*	*Old PG*	*New PG*	*CTX-M type*	*Other beta-lactamase*		*PMQR*	*PBRT*	*Resistance profile*	*Associated virulence factors*							
					TEM SHV/OXA$_{1\text{-}like}$	*Plasmid-mediated AmpC*				pap*1/2*	sfa*1/2*	afa*1/2*	pap*3/4*	cnf*1a/b*	eaeA	Ecp1	*IS*26
1	L1	B2	D			CMY-2		FIB	AMC, AMP, ATM, C, CPD, FOX, S	*pap*1/2					*eaeA*		
1	L1	B1	B1			CMY-2		Y	AMC, AMP, ATM, CPD, FOX, S								
1	L1	B1	C			CMY-2		Y	AMC, AMP, CPD, NA, S								
1	L1	A	B1			CMY-2		NT	AK, AMC, AMP, ATM, CPD, DO, FOX, S								
1	L1	A	A		TEM	CMY-2		NT	AMC, AMP, ATM, CPD, FOX								
1	L1	B1	D		TEM			FIA, FIB	AK, AMC, AMP, ATM, C, CPD, DO, ETP, FOX, N, PTZ, S	*pap*1/2							
1	L1	B1	B1		TEM			FIA, FIB	AMC, AMP, ATM, C, CPD, DO, PTZ, S	*pap*1/2							
1	L1	B1	B1		TEM			FIA, FIB	AK, AMC, AMP, ATM, C, CPD, DO, ETP, FEP, PTZ, S	*pap*1/2							
3	L1	B1	B1		TEM			FIB, FIA	AMC, AMP, ATM, C, CPD, DO, FOX, PTZ, S	*pap*1/2							
1	L1	B1	B1		TEM			FIB, FIA	AMC, AMP, ATM, C, CN, CPD, FOX, NA, PTZ, S, STX	*pap*1/2							
1	L1	B1	B1		TEM			FIB, FIA	AMC, AMP, C, CPD, DO, S	*pap*1/2							
4	L1 (2), L2 (2)	A	U														
1	L1	B1	B1														
1	L1	B2	B2								*sfa*1/2			*cnf*1a/b			
2	L2	B2	B2								*sfa*1/2			*cnf*1a/b			
2	L1	B2	B2														
1	L1	B2	B2														
1	L1	A	A							*pap*1/2							
2	L1	A	A														
6	L1	A	B1														
1	L1	D	C														
1	L1	D	E														
1	L2	D	CLADE I or II								*sfa*1/2						

AK, amikacin; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; ATM, aztreonam; C, chloramphenicol; CN, gentamicin; CPD, cefpodoxime; DO, doxycycline; ETP, ertapenem; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; NA, nalidixic acid; NT, not typable by PBRT; PBRT, polymerase chain reaction-based replicon typing; PG, phylogenetic group; PMQR, plasmid-mediated quinolone resistance; PTZ, piperacillin/tazobactam; S, streptomycin; STX, trimethoprim/sulfamethoxazole.

13.3% (8/60) of seagull ESC-R *E. coli* isolates carried bla_{AmpC} resistance genes, borne singularly or in various combinations with $bla_{\text{CTX-M-15}}$, bla_{TEM}, $bla_{\text{OXA1-like}}$, *aac(6′)-ib-cr*, and *qnrB*. Sequencing identified all bla_{AmpC} genes as $bla_{\text{CMY-2}}$. The overall PMQR gene prevalence among seagull ESC-R *E. coli* isolates was 11.7% (7/60) and these consisted of five *aac(6′)-ib-cr* and three *qnrB*-positive strains, one of which coharbored both determinants. None of the investigated isolates carried carbapenem resistance genes nor *mcr*-1, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* determinants conferring resistance to colistin.

A PCR assay targeting STEC-associated *stx1* and *stx2* genes and *E. coli* serotypes O111 and O157 was negative for all *E. coli* seagull isolates. One isolate carried the gene encoding for intimin (*eaeA*), whereas none was positive for plasmid-encoded enterohemolysin (*hlyA*). The vast majority of uropathogenic genes were carried individually in *E. coli* seagull isolates, with just two virulence gene combinations occurring in four isolates (Table 1). Of the 23 *E. coli* isolates carrying urovirulence determinants (mainly *pap1/2*$^{+}$), 78.2% (18/23) also harbored AMR genes, whereas 21.8% (5/23) was negative for any of the AMR genes tested.

Molecular typing of ESC-R E. coli

All *E. coli* seagull isolates initially assigned to PGs using the triplex PCR method, were subsequently retested using the extended quadruplex approach. While there was consistency for allocation of the isolates within PGs B1, B2, and D between the two methods, the quadruplex method allowed to redistribute a substantial fraction of the isolates formerly ascribed to phylogroup A into the later recognized phylogroups C, E, F, and clade I as follows: A ($n=4$), B1 ($n=18$), B2 ($n=12$), C ($n=2$), D ($n=12$), E ($n=1$), F ($n=4$), clade I ($n=2$), and unassignable ($n=5$).

Overall, phylogroup B1 was the most represented among the seagull isolates (30%); nonetheless, the proportion of isolates belonging to phylogroup B2, which are more commonly associated with extraintestinal clinical infection,[64,65] appears high (20%). Furthermore, our study indicates that $\sim$36% and 21% of all ESBL isolates ($n=28$) belonged to D and B2 phylogroups, respectively, whereas both phylogroups B1 and F accounted for 14% of all ESBL seagull isolates.

Among the seagull *E. coli* isolates belonging to phylogroup B2, three (25%) have been typed to the international clone B2-ST131-O25b based on the detection of *pabB* and *trpA* genes. This was subsequently confirmed using the multiplex conventional PCR targeting all ST131 clades and the C subclades, which typed two of the isolates carrying $bla_{\text{CTX-M-15}}$ to the C2/H30Rx subclade, whereas one isolate carrying $bla_{\text{CTX-M-27}}$ belonged to the C1-M27 subclade of ST131.

Resistance transfer and PCR-based replicon typing

Conjugation experiments demonstrated transferability of $bla_{\text{CTX-M-15}}$ as well as of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-27}}$ to the *E. coli* recipient in seagull isolates. In addition, PCR-based replicon typing (PBRT) showed that IncFIA and Y were associated with transfer of ESBL genes. IncFIA was the most common replicon found in the seagull *E. coli* transconjugants, being recovered from four out of six transconjugants in which the CTX-M genes were successfully transferred. In the case of one $bla_{\text{CTX-M-15}}$ transconjugant, the recovered plasmids were not typeable using PBRT, whereas for $bla_{\text{CTX-M-1}}$ the transconjugant obtained lacked IncFIA identified in the original isolate. For $bla_{\text{CTX-M-27}}$, both IncFIA and Y replicon types were recovered from the single successful transconjugant. None of the $bla_{\text{CTX-M-14}}$ genes was transferred, indicating that they may be located on a nonconjugative plasmid or on the chromosome.

Discussion

The results described in this study provide further evidence that wild seagulls in Europe may be a reservoir and contribute to dissemination of ESC-R and FQ-R *E. coli.* This is in agreement with previous studies indicating the ability of gulls to carry and disseminate *E. coli* strains harboring several TEM and CTX-M types of ESBL[41,66–72] alone or combined with AmpC β-lactamase[73,74] and, more recently, with VIM-type carbapenemase.[75,76]

In this study, we report a high prevalence (50%) of ESC-R *E. coli* carriage in the UK urban seagull population investigated, among which $bla_{\text{CTX-M-15}}$ was the predominant ESBL genotype. This is much higher than the ESC-R *E. coli* carriage identified in a recent study in silver gulls from Australia (21.7%)[77] or among North American gulls and pelicans (14%)[73] and European gulls (28.7%).[72] In the European study, ESBL-producing *E. coli* from English gulls were detected at a prevalence of 27.1%, with Spain only displaying higher prevalence than the UK (74.8%).[72] Consequently, seagulls can act as sentinels and bioindicators of antimicrobial resistance and should be regarded as vehicles and potential long-term reservoirs of multidrug-resistant bacteria.

Current global antimicrobial drug resistance data indicate that environmental reservoirs are likely to contribute to the selection and spread of resistance determinants and antimicrobial-resistant bacteria.[78] Despite not being directly exposed to clinically employed antimicrobial agents, wild animals can be an underestimated vector of antimicrobial resistance as they may acquire it through contact with human-influenced habitats of any sort, such as manure-fertilized meadows, livestock farms, landfills, hospital sewage systems, wastewater treatment facilities, recreational waters, and others.[79] Their feeding behavior and association with water environments are epidemiologically relevant for the gulls' capacity to spread antibiotic resistance and both coastal and urban environments provide potentially diverse sources of food. Furthermore, levels of resistance seem to correlate with the extent of association to human activities.[80]

A few studies have shown gulls' uptake of MDR *E. coli* from human waste and sewage facilities (including ST131 strains and, notably, one *mcr-1*-positive isolate)[81–83] and their ability to acquire antibiotic resistance from these sources and release it into beach waters or other aquatic environment.[71,74,84,85] This is particularly relevant for transmission since gulls are migratory birds and can convey antimicrobial-resistant bacteria and associated genes into distant environments and populations, including humans, interconnecting environmental niches of different antimicrobial resistance levels. Moreover, clusters of genetically

comparable ESBL-producing *E. coli* have been simultaneously isolated from humans and gulls in France,[67] Sweden,[86] and Chile,[87] indicating that transfer between human populations and the environment, including wild birds, is likely to occur. One Alaskan study described the increased detection of MDR *E. coli* from gulls specifically in urban areas rather than rural ones.[88]

The pandemic virulent *E. coli* B2-ST131 clone was rare among the ESC-R *E. coli* isolates analyzed (5%), with 2/3 isolates harboring $bla_{\text{CTX-M-15}}$, whereas 1/3 harbored $bla_{\text{CTX-M-27}}$. B2-ST131 has been widely shown to be an important driver of the increase in antimicrobial resistance and to have contributed to the dissemination of the CTX-M β-lactamase worldwide.[15] Recent epidemiological and genomic studies have shown that particular *E. coli* ST131 subclades, such as the C2/H30Rx have been largely responsible for the dissemination of CTX-M-15 β-lactamase worldwide,[19] whereas the C1/H30R (recently described as the C1-M27) was involved in the epidemic spread of ST131-CTX-M-27 in Asia.[24]

This distinct *E. coli* ST131subclade, carrying $bla_{\text{CTX-M-27}}$ and defined by unique genomic characteristics, has recently emerged within C1/H30R and has been rapidly established as the predominant ST131 type and most important vehicle of ESBL-producing ExPEC in Japan.[89] Following its recognition in other Asian countries,[90] this novel ST131 subclade, now commonly referred to as C1-M27, has been increasingly reported also in Europe, with some countries, such as France and Switzerland, having disclosed alarmingly high prevalence rates.[91–94]

Little data are available regarding the presence of *E. coli* ST131 C1-M27 in the veterinary field. Sporadic reports of *E. coli* ST131 carrying $bla_{\text{CTX-M-27}}$ in animals include clinical specimens from dogs and cats, mostly suffering from UTIs, in Japan and Europe[95–99] and fecal samples from wild birds, namely two great cormorants, identified in the Czech Republic[39] and Switzerland.[100] CTX-M-27-producing ST131 isolates have also been detected in diverse aquatic environments in Asia and Europe, including hospital wastewater treatment plants,[101] rivers and lakes,[102,103] and fish.[27] Furthermore, a recent study from the Czech Republic has demonstrated that ST131 harboring $bla_{\text{CTX-M-27}}$ from different sources, including wild water birds, wastewater, and hospital isolates, shared extensive genetic commonalities between them and with isolates previously reported from Japanese hospitals.[26] To date, coastal and river waters in the UK have been found polluted with B2-ST131 *E. coli* producing CTX-M-15 and CTX-M-14 enzymes but not CTX-M-27.[104–106] To the best of our knowledge, this is the first report of an *E. coli* ST131 strain belonging to C1-M27 in seagulls and in UK wildlife.

In our study, the CTX-M-15-type enzyme was also prevalent among non-ST131 ESBL-producing *E. coli*, followed by CTX-M-14 type. This is in agreement with previous findings of non-ST131 *E. coli* CTX-M-15- and CTX-M-14-producing ESBL types being frequently isolated from wild gulls (and other wild bird species).[41] Conversely, we report a lower prevalence of $bla_{\text{CTX-M-1}}$ among non-ST131 ESBL-producing *E. coli* in our UK seagull population than was previously described by other studies.[72]

As prevalence rates of *E. coli* ST131 in people appear consistently higher than those found in animals in diverse geographic areas, it is generally presumed that humans are more important hosts of ST131 clinical strains compared with animals[3,107] and that spill-over has later occurred into the veterinary field. Consistent distribution of ESBL genotypes, including ST131, has generally been described in humans, whereas that observed in animals appears more diversified and incongruent (also geographically), with partial overlap of human and animal genotypes. In contrast to domesticated animals, the presence and epidemiology of ESC-R and FQ-R *E. coli*, including medically important and globally widespread clonal lineages like B2-O25b-ST131, have been infrequently addressed in wildlife and the extent and pathways through which these pathogenic bacteria are spread between different ecological niches remain to be elucidated.

In the present study, seagulls colonized with ESBL *E. coli*, including B2-ST131 isolates, were associated with urban areas in a coastal city and we presume they might have acquired them from those environments. The countryside surrounding Taunton stretches up to the coast and offers numerous water bodies and reservoirs serving as overnight roosting sites as well as various agricultural food sources while Birmingham is a much larger and populated inland city, placed at greater distance from water bodies but with abundance of landfills and establishments to provide urban gulls with food. The reasons behind the lack of isolation of ESBL-producing *E. coli* among seagulls sampled in the inland city could be partially explained by the smaller sample size or it may be that coastal areas in the UK provide greater opportunities for seagulls to become colonized than those offered by urban environments without close proximity to waterfronts.

The limitations of this study are the small scale and lack of a far-reaching geographical systemic approach and the absence of parallel investigations into human or environmental ESBL *E. coli*, including ST131-C1-M27 strains, in the same areas as well as the possible relatedness of our C1-M27 isolate with Asian reference strains.

Conclusions

Wild seagulls in the UK are colonized, and therefore can spread, major antimicrobial-resistant *E. coli* isolates, including clinically important strains such as the pandemic B2-ST131 clone, and the emerging C2/H30Rx and the C1-M27 subclades.

To the best of our knowledge, this is the first report of the ST131-C1-M27 in wildlife in the UK and in seagulls worldwide. Findings from this study support the concept that carriage of these multidrug-resistant strains is widespread among wild gulls, at least in some countries. Further studies are needed to explore the implications of such strains' carriage by wild seabirds and to unravel the potential for ongoing interspecies transmission between humans and urban opportunistic birds. As available evidence is pointing toward humans as the original source for ESBL, AmpC, and PMQR enrichment of *E. coli* strains in wild species, priority must be given to break the transmission chain between humans and the environment, including wildlife, to prevent further dissemination of MDR bacteria at a global level through these birds' migrations.

Disclosure Statement

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