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Molecular Characterization of Extended-Spectrum-Cephalosporin-Resistant *Enterobacteriaceae* from Wild Kelp Gulls in South America

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Extended-spectrum-cephalosporin-resistant *Enterobacteriaceae* are a public health concern due to limited treatment options. Here, we report on the occurrence and the molecular characteristics of extended-spectrum-cephalosporin-resistant *Enterobacteriaceae* recovered from wild birds (kelp gulls). Our results revealed kelp gulls as a reservoir of various extended-spectrum cephalosporinase genes associated with different genetic platforms. In addition, we report for the first time the presence of a known epidemic clone of *Salmonella enterica* serotype Heidelberg (JF6X01.0326/XbaI.1966) among wild birds.

Extended-spectrum-cephalosporinase-producing *Enterobacteriaceae* have been reported worldwide among isolates obtained from humans and from food-producing and companion animals, as well as from environmental sources (1). In spite of the limited number of studies regarding the occurrence of antibiotic resistance in natural environments, where animals do not naturally come into contact with antibiotics, the occurrence of extended-spectrum-cephalosporin-resistant (ESC^r) *Enterobacteriaceae* has been detected lately in wild birds, especially in populations of gulls (*Laridae*) (2–7). The kelp gull (*Larus dominicanus*) is a large gull species distributed in coastal areas through much of the Southern Hemisphere and is the only gull species inhabiting the Antarctic continent. It is known to be a food generalist, regularly feeding on food resulting from human activities (abattoirs, garbage, sewage outfalls, etc.) (8). This behavior makes it an interesting sentinel species for the study of the environmental spread of antibiotic-resistant bacteria. Our aim was to determine the occurrence and the molecular characteristics of ESC^r *Enterobacteriaceae* isolates recovered from kelp gulls, as this species could favor the dissemination of ESC^r *Enterobacteriaceae* in human populations and in the pristine Antarctic environment.

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During November 2012, fresh fecal specimens (n = 50) were collected from a flock of approximately 500 kelp gulls on a sandy beach where they were roosting in Ushuaia, in Argentina. All samples were enriched either in brain heart infusion broth (Becton-Dickinson, Franklin Lakes, NJ, USA), supplemented with 16 mg/liter vancomycin, or in buffered peptone water (SVA, Uppsala, Sweden) for 18 to 24 h in 37°C and subsequently inoculated on ChromID ESBL (bioMérieux, Solna, Sweden) for the selective isolation of extended-spectrum-β-lactamase (ESBL)-producing *Enterobacteriaceae* or on modified semisolid Rappaport Vassiliadis agar (SVA, Uppsala, Sweden) for the selective isolation of *Salmonella* species, respectively. Presumptive extended-spectrum-cephalosporinase-producing isolates were identified using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Brucker, Coventry, United Kingdom), while *Salmonella* isolates were further serotyped by the microtitration method. Antibiotic susceptibility of the isolates was assessed by broth microdilution and interpreted according the epidemiologic cutoff values recommended by the European Committee on Antimicrobial Susceptibility Testing (http://mic.eucast.org), whereas ESBL and/or AmpC production was evaluated by a combined disc test, as previously described (9).

Genes conferring the ESC^r phenotype were sought and their genetic location on either the chromosome or a plasmid was determined as previously described (9). Standard methods (PCR-based replicon [rep] typing, plasmid multilocus sequence typing [pMLST]/plasmid double-locus sequence typing [pDLST]/replicon sequence typing [RST], and S1 nuclease pulsed-field gel electrophoresis [PFGE]) were applied for further plasmid analysis, while the conjugal transferability of the extended-spectrum cephalosporinase genes and the presence of known insertion sequences (ISs) upstream of them were examined (9). Genetic relatedness among *Escherichia coli* and *Salmonella enterica* serotype Heidelberg isolates was assessed by MLST and XbaI-PFGE typing, respectively, as previously described (9, 10).

Overall, we recovered 37 nonduplicate ESC^r *Enterobacteriaceae* isolates from 34 of the fecal samples included in the study. Among them, 91.9% (n = 34) were identified as *E. coli* and 8.1% (n = 3) as *S.* Heidelberg. The copresence of ESC^r *E. coli* and *S.* Heidelberg was documented in three fecal samples. The recovered isolates exhibited non-wild-type MICs mainly for ciprofloxacin (n = 27; 73.0%), nalidixic acid (n = 25; 67.6%), tetracycline (n = 22;

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TABLE 1 Characteristics of ESC^r *Enterobacteriaceae* isolates recovered from kelp gulls (*Larus dominicanus*) in Ushuaia, Argentina, in 2012[a]

Species (no. of isolates)	ST/PFGE type (no. of isolates)	Resistance phenotypes (no. of isolates)	ESBL/AmpC gene (no. of isolates)	Location (no. of isolates)	Plasmid characteristic(s)			
					Plasmid type/subtype (no. of isolates)	Size (kb)	Transferability	Upstream region
E. coli (34)	ST744 (6)	AMP, CAZ, CHL, CIP, CST, CTX, NAL, SMX, TET, TMP (1) AMP, AZM, CAZ, CHL, CIP, CTX, NAL, SMX, TET, TMP (2) AMP, CHL, CIP, CST, CTX, NAL, SMX, TET, TMP (3)	$bla_{CTX\text{-}M\text{-}14}$ (6)	Plasmid (6)	IncI1/ST80 (6)	105	Conjugative	—[e]
	ST617 (5)	AMP, CAZ, CHL, CIP, CTX, NAL, TET (4)	$bla_{SHV\text{-}2A}$ (4)	Plasmid (4)	IncI1/ST187 (3)	125	Conjugative	IS*26*
					IncF/F1:A1:B1 (1)	70	Conjugative	IS*26*
		AMP, CAZ, CIP, CTX, NAL, TET (1)	$bla_{CTX\text{-}M\text{-}14}$ (1)	Plasmid (1)	IncI1/ST80 (1)	105	Conjugative	—[e]
	ST57 (3)	AMP, CAZ, CIP, CTX, GEN, NAL, SMX, TET, TMP (3)	$bla_{CTX\text{-}M\text{-}2}$ (3)	Plasmid (3)	IncF/F18:A-:B1 (3)	208	Conjugative	IS*CR1*
	ST93 (3)	AMP, CAZ, CHL, CTX, SMX, TMP (1)	$bla_{CTX\text{-}M\text{-}2}$ (3)	Plasmid (1)	IncHI2/ST2 (1)	202	Conjugative	IS*CR1*
		AMP, CAZ, CIP, CTX, GEN, SMX (1)		Chromosome (2)	NA	NA	Nonconjugative[d]	IS*CR1*
		AMP, CAZ, CTX, SMX, TET (1)						
	ST4038 (3)	AMP, CAZ, CTX (3)	$bla_{SHV\text{-}2}$ (3)	Plasmid (3)	IncI1/ST12 (3)	145	Conjugative	IS*26*
	ST10 (2)	AMP, CAZ, CTX, TET (1)	$bla_{CTX\text{-}M\text{-}14}$ (2)	Plasmid (2)	IncI1/ST80 (2)	105	Conjugative	—[e]
		AMP, CHL, CIP, CTX (1)						
	STNew1 (2)[b]	AMP, CAZ, CIP, CTX, NAL (1)	$bla_{CTX\text{-}M\text{-}14}$ (1)	Plasmid (1)	IncI1/ST80 (1)	105	Conjugative	—[e]
		AMP, CAZ, CHL, CIP, CTX, GEN, NAL, SMX (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Chromosome (1)	NA	NA	Nonconjugative[d]	IS*CR1*
	ST69 (1)	AMP, CAZ, CTX, SMX, TET (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Chromosome (1)	NA	NA	Nonconjugative[d]	IS*CR1*
	ST88 (1)	AMP, CTX, TET (1)	$bla_{CTX\text{-}M\text{-}14}$ (1)	Plasmid (1)	IncI1/ST80 (1)	105	Conjugative	—[e]
	ST101 (1)	AMP, CAZ, CIP, CST, CTX, NAL, SMX, TET (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Plasmid (1)	IncA/C (1)	100	Nonconjugative[d]	IS*CR1*
	ST117 (1)	AMP, CAZ, CIP, CTX, NAL, SMX (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Plasmid (1)	NT (1)	132	Conjugative	IS*CR1*
	ST212 (1)	AMP, CAZ, CTX (1)	$bla_{SHV\text{-}2}$ (1)	Plasmid (1)	IncI1/ST12 (1)	121	Conjugative	IS*26*
	ST359 (1)	AMP, CAZ, CHL, CIP, CTX, NAL, SMX, TET (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Plasmid (1)	IncF/F24:A-:B1 (1)	170	Conjugative	IS*CR1*
	ST1011 (1)	AMP, CAZ, CIP, CTX, GEN, NAL, SMX (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Chromosome (1)	NA	NA	Nonconjugative[d]	IS*CR1*
	ST1193 (1)	AMP, CAZ, CIP, CTX, NAL, TET (1)	$bla_{CTX\text{-}M\text{-}15}$ (1)	Plasmid (1)	IncF/F1:A1:B1 (1)	87	Nonconjugative[d]	IS*Ecp1*
	ST2485 (1)	AMP, CAZ, CHL, CTX, SMX, TET (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Plasmid (1)	IncF/F24:A-:B10 (1)	205	Conjugative	IS*CR1*
	STNew2 (1)[b]	AMP, CAZ, CIP, CTX, NAL, SMX (1)	$bla_{CTX\text{-}M\text{-}2}$ (1)	Chromosome (1)	NA	NA	Nonconjugative[d]	IS*CR1*
S. Heidelberg (3)	JF6X01.0326 (3)[c]	AMP, CAZ, CIP, CTX, NAL (3)	$bla_{CMY\text{-}2}$ (3)	Plasmid (3)	IncI1/ST12 (3)	110	Conjugative	IS*Ecp1*

[a] ST, sequence type; AMP, ampicillin; AZM, azithromycin; CAZ, ceftazidime; CIP, ciprofloxacin; CHL, chloramphenicol; CST, colistin; CTX, cefotaxime; GEN, gentamicin; MEM, meropenem; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim; NA, not applicable.

[b] Assignment to a specific ST could not be performed, as uploading new sequences and STs based on AB1 files is no longer supported by the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

[c] PFGE pattern numbers correspond to the PulseNet database.

[d] No transconjugants were obtained after liquid mating experiments, suggesting either the presence of nonconjugative plasmids or conjugation frequencies below the detection limit ($\leq 1 \times 10^{-9}$).

[e] IS*Ecp1*, IS*CR1*, or IS*26* insertion sequences were not found upstream of the ESBL genes for these STs.

59.5%), sulfamethoxazole ($n = 20$; 54.0%), and chloramphenicol ($n = 15$; 40.5%). All isolates were susceptible to meropenem and tigecycline, whereas they exhibited non-wild-type MICs for the remaining tested agents (ranging from 5.4% to 27.0%). All *E. coli* isolates exhibited an ESBL phenotype and carried $bla_{CTX\text{-}M\text{-}2}$ ($n = 14$; 41.2%), $bla_{CTX\text{-}M\text{-}14}$ ($n = 11$; 32.3%), $bla_{SHV\text{-}2}$ ($n = 4$; 11.8%), $bla_{SHV\text{-}2A}$ ($n = 4$; 11.8%), and $bla_{CTX\text{-}M\text{-}15}$ ($n = 1$; 2.9%) genes, whereas all *S.* Heidelberg isolates exhibited an AmpC phenotype and carried the $bla_{CMY\text{-}2}$ gene.

The broad-host-range IncI1 ($n = 21$; 67.7%) and narrow-host-range IncF ($n = 7$; 22.6%) plasmids were by far the most common rep types accounted for the ESC^r phenotype among the recovered isolates. The $bla_{CTX\text{-}M\text{-}2}$ gene was found mainly on the chromosome ($n = 6$; 42.9%) or on plasmids of different replicon types, including IncF plasmids with fused FIB-FII replicons ($n = 5$; 35.7%), IncHI2 ($n = 1$; 7.1%), IncA/C ($n = 1$; 7.1%), and nontypeable ones ($n = 1$; 7.14%). The $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$ genes were identified exclusively on IncI1/sequence type 80 (ST80) and IncFIA-FIB plasmids, respectively. The $bla_{SHV\text{-}2}$ gene was associated with IncI1/ST12 and $bla_{SHV\text{-}2A}$ with IncI1/ST187 and IncFIA-FIB plasmids, whereas the $bla_{CMY\text{-}2}$ gene was located on IncI1/ST12 plasmids. Detailed results regarding the subtyping, the size, and the transferability of the plasmids are summarized in Table 1.

Three insertion sequence elements previously associated with the mobilization and support of extended-spectrum cephalosporinase genes were identified. Briefly, in all isolates carrying $bla_{CTX\text{-}M\text{-}2}$, the gene was accompanied upstream by a copy of IS*CR1* in the same orientation as the resistance gene, regardless of the plasmid replicon type or whether the gene was chromosomally located (Table 1). Similarly, IS*Ecp1* was found upstream of the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$ genes, while IS*26* was found upstream of the $bla_{SHV\text{-}2}$ and $bla_{SHV\text{-}2A}$ genes. IS*Ecp1*, IS*CR1*, or IS*26* insertion sequences were not found upstream of $bla_{CTX\text{-}M\text{-}14}$ gene (Table 1).

High diversity of genotypes was observed among the *E. coli* isolates, resulting in 17 different STs, each comprised of one to six isolates. The most predominant genotypes were ST744 ($n = 6$; 17.6%), ST617 ($n = 5$; 14.7%), ST57 ($n = 3$; 8.8%), ST93 ($n = 3$; 8.8%), and ST4038 ($n = 3$; 8.8%), while isolates belonging to ST10, ST69, ST88, ST101, ST117, ST212, ST359, ST1011, ST1193, ST2485, STNew1, and STNew2 were also identified. All *S.* Heidelberg isolates belonged to epidemic clone JF6X01.0326/XbaI.1966 (PulseNet database). Different ESBL determinants were found among isolates with the same genotype; conversely, different genotypes carrying the same ESBL determinants were identified (Table 1).

Several studies have documented the occurrence of ESC^r *Enterobacteriaceae* isolates among wild birds at prevalences ranging from 0% to 37% (4, 11–15). However, our study revealed a higher occurrence among kelp gulls in accordance with studies regarding Brown-headed gulls and Franklin's gulls (5). Although the resistance gene families described in this study are similar to those reported previously (2, 4, 5, 12, 14–19), we documented for the first time the presence of $bla_{SHV\text{-}2A}$ and the predominance of $bla_{CTX\text{-}M\text{-}2}$ among wild birds. The latter mirrors the situation observed for nosocomial infections in Argentinian hospitals (20, 21), confirming the endemicity of $bla_{CTX\text{-}M\text{-}2}$ within this area and its potential transmission from humans to wild birds and/or vice versa. Of note was the association of $bla_{CTX\text{-}M\text{-}2}$ gene with IS*CR1* on four different plasmid replicon types associated with six different *E. coli* STs and on the chromosome of five other different *E. coli* STs, underscoring that IS*CR1* has probably played a significant role in the capture of this gene by conjugative plasmids and in its further interreplicon and interclone dissemination. Moreover, our data suggest the horizontal transfer of a conjugative IncI1/ST80 plasmid (105 kb) carrying $bla_{CTX\text{-}M\text{-}14}$ among five different *E. coli* STs, underscoring the dissemination of this gene owing to a successful plasmid-gene combination.

Among the 17 different STs detected here, we identified several, namely, ST10, ST69, ST101, ST117, ST167, ST617, and ST744, that have been previously reported from ESC^r *E. coli* isolates of human and animal origin (1, 5, 12, 15). Interestingly, some of the identified STs (ST10, ST117, ST157, ST359, ST617, and ST744) have been previously reported among wild birds as well, but they have been found to harbor different extended-spectrum cephalosporinase genes, suggesting that avian commensal *E. coli* strains play a role in the maintenance and dissemination of these genes (1, 5, 12, 15). In contrast with the solely ESC^r *S.* Heidelberg isolate carrying $bla_{CMY\text{-}2}$ on a 97-kb IncN plasmid reported previously from an Argentinian adult inpatient (22), here we documented for the first time the presence in wild birds of a known epidemic ESC^r *S.* Heidelberg clone (JF6X01.0326/XbaI.1966), carrying $bla_{CMY\text{-}2}$ on a 110-kb IncI1/ST12 plasmid. This PFGE type, circulating in the United States and recently introduced to Europe (9), has been documented to cause outbreaks and exhibit potency for bloodstream infections (23).

In conclusion, although there are few studies on the presence of resistance genes conferring the ESC^r phenotype among *Enterobacteriaceae* from wild birds, to our knowledge this is the first report presenting a detailed characterization of ESC^r *Enterobacteriaceae*, including the underlying antibiotic resistance gene content and its genetic support (plasmids and IS elements). Our data imply that kelp gulls act as reservoirs of a variety of extended-spectrum cephalosporinase genes associated with different genetic platforms that could facilitate their horizontal transfer. In addition, our findings underscore the potential role of kelp gulls as a bridge species for transfer of ESC^r *Enterobacteriaceae* between humans and wildlife and as a spreader of these isolates among human populations and naturally antibiotic-resistant-bacterium-free environments (Antarctic continent) via their movement and migration.

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