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Multidrug-resistant *Escherichia coli* and Tetracycline-resistant *Enterococcus faecalis* in Wild Raptors of Alabama and Georgia, USA

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ABSTRACT: Wild birds inhabit in a wide variety of environments and can travel great distances. Thus, wild birds can possibly spread antimicrobial resistance along the way, and this may represent a potential public health concern. We characterized antimicrobial resistance in fecal *Escherichia coli* and *Enterococcus faecalis* in wild raptors in the southeastern US. Cloacal samples were collected from 118 wild raptors of 17 species from 18 counties in Alabama and 15 counties in Georgia. A total of 112 *E. coli* and 76 *E. faecalis* isolates were recovered, and we found significantly more antimicrobial-resistant *E. coli* (20/112, 18%) than *E. faecalis* (6/76, 8%; P=0.05). Five antimicrobial-resistant genes: *blaTEM-1*, *blaCTX-M-1*, *tet*(M), *cmlA*, *cat*, and *gyrA*, were identified in antimicrobial-resistant *E. coli* isolates. Five of 13 (38%) ampicillin-resistant *E. coli* harbored both *blaTEM-1* and *blaCTX-M-1* genes, indicating they are extended-spectrum β-lactamase–carrying strains. Both of the tetracycline resistance genes, *tet*(M) and *tet*(L), were identified in *E. faecalis* isolates. Wild raptors seem to be a reservoir host of antimicrobial-resistant *E. coli* and *E. faecalis* and may represent a hazard to animal and human health by transmission of these isolates.

Key words: Antimicrobial resistance, *Enterococcus faecalis*, *Escherichia coli*, extended-spectrum β-lactamase producing, raptors.

Antimicrobial resistance remains one of the largest threats to public health, despite decades of efforts to slow down the selection and transfer of resistance through judicious use of antimicrobials (Davies 2007). Understanding the mode of dissemination of antimicrobial-resistant microbes and their resistance genes is crucial in formulating strategies to prevent and control the rising threat of antibiotic resistance. Free-living raptors fly great distances and inhabit a variety of environments and thus can potentially serve as a reservoir and as a means of dissemination of antimicrobial resistance. However, there is only one report of antimicrobial resistance in raptors in the US, which showed that enterococci were resistant to multiple antibiotics (Marrow et al. 2009). To examine the potential role of wild raptors as a reservoir and a vector of antimicrobial resistance, we investigated the prevalence of antimicrobial-resistant *Escherichia coli* and *Enterococcus faecalis*, enteric bacteria capable of rapidly acquiring resistance, in wild raptors.

A total of 118 free-living raptors of 17 species were brought to the Southeastern Raptor Center (Auburn, Alabama, US) between January 2013 and April 2014 from 18 Alabama and 15 Georgia counties. The birds were suffering from emaciation or a variety of injuries, including gunshots, severe lacerations, or fractures. At admission, a cloacal swab was obtained from each bird and transferred to the laboratory in Amies transport medium (Thermo Scientific, Grand Island, New York, USA). Swabs were processed within 2 h of collection onto 5% bovine blood agar and MacConkey agar (BD Biosciences, San Jose, California, USA). One colony with morphologies typical for *E. coli* and *E. faecalis* was selected from the cultures from each bird and identified by conventional biochemical methods.

For antimicrobial susceptibility testing, we selected antibiotics (ampicillin, chloramphenicol, ciprofloxacin, and tetracycline) representing four major classes of drugs widely used in both medicine and agriculture. All four antibiotics were appropriate for testing against both *E. faecalis* and *E. coli*, and

TABLE 1. Primers and probes used to amplify antimicrobial-resistant gene targets. The PCRs amplifying *blaTEM-1*, *blaCTX-M-1*, *tet*(M), and *tet*(L) were run in a SYBR Green PCR system, while PCRs for *gyrA*, *cmlA*, and *cat* were performed in a fluorescence resonance energy transfer–PCR platform. The established PCRs were verified for their sensitivity and specificity.

Target gene	Oligonucleotide sequence (5′-3′)[a]	Amplicon size (bp)
blaCTX-M-1	F: ATGGTTAAAAAATCACTGCGTC	418
	R: ATTCATCGCCACGTTATCG	
blaTEM-1	F: CTGGTGAAAGTAAAAGATGCT	401
	R: TCAAGGCGAGTTACATGATC	
cat	F: CACCGTTGATATATCCCAATGGCAT	231
	R: CGTCTTTCATTGTCATACGGAATTC	
	6-fam: TCAGGCCAAGAATGTGAATAA-6-fam	
	LCRed640: LCRed640-AAAAATAAGCACAAGTTTTATCCGGCC-phos	
cmlA	F: GACAACGTACTTGGTCATGATTGGT	212
	R: GCGTAAATGTCACGTACTGTTGCAA	
	6-fam: CCTGTCAGCTCTTGTTTGGACCT-6-fam	
	LCRed640: LCRed640-TATCTGACCGACTGGGGCTCC-phos	
tet(M)	F: CCACCGAATCCTTTCTGGGC	401
	R: ATCCGAAAATCTGCTGGGGTACT	
tet(L)	F: ATGGTTTTGAACGTCTCATT	414
	R: CCAATGAATATAATGGGCTA	

[a] F = forward primer; R = reverse primer; 6-fam = 6-carboxyfluoresein probe; LCRed640 = LightCycler 640 probe (Roche Diagnostics, Indianapolis, Indiana, USA); phos = phosphorylation.

neither organism exhibits intrinsic resistance to any of these antibiotics. Minimal inhibitory concentrations (MICs) were determined by using the ETEST (AB Biodisk, bioMérieux, Inc., St. Louis, Missouri, USA) according to the manufacturer's recommendations. Multidrug-resistant bacteria was defined as resistance to three or more classes of antimicrobials (Magiorakos et al. 2012).

Bacterial genomic DNA was extracted by boiling isolates at 100 C, followed by flash cooling. The extracted DNA was stored at –80 C for molecular detection. The representative nucleotide sequences for antimicrobial resistance genes were obtained from GenBank and were aligned by using Clustal multiple alignment (Vector NTI, Invitrogen, Grand Island, New York, USA) to identify regions to design primers and probes (Table 1). The PCRs for β-lactamases, *blaTEM-1*, *blaCTX-M-1*, and tetracycline resistance genes, *tet*(M), *tet*(L), were run in a SYBR Green PCR system, while PCRs for *gyrA* and two chloramphenicol-resistant genes (*cmlA*, *cat*) were in a fluorescence resonance energy transfer–PCR platform, as previously described (Yang et al. 2014; Zhang et al. 2018). All PCRs in this study were performed in a Roche LightCycler 1.5 PCR system (Roche Diagnostics, Indianapolis, Indiana, USA). Sensitivity of PCRs was determined by amplification of standards with 10^4, 10^3, 10^2, and 10^1 copies per reaction. The specificity of established PCRs was verified by gel electrophoresis and DNA sequencing of PCR products for all positive samples.

The statistical analysis was done by using Statistica version 8.0 (StatSoft, Inc., Tulsa, Oklahoma, USA). A chi-squared test was performed to compare the difference in overall prevalence of antibiotic resistance between *E. coli* and *E. faecalis* isolates. Difference was considered significant at $P \leq 0.05$.

From the 118 wild raptors, we isolated a total of 112 *E. coli* and 76 *E. faecalis*. The rate of isolation for *E. coil* was 95 and 64% for *E. faecalis*, with 74 (63%) of the birds being culture positive for both (Table 2). Among the *E. coli i*solates, the most prevalent resistance was to tetracycline, with 16% of the strains

TABLE 2. Antimicrobial resistance pattern and AMR genes identified in *Escherichia coli* and *Enterococcus faecalis* isolated from raptors. A total of 118 free-living raptors of 17 species were brought to the Southeastern Raptor Center between 2013 and 2014 from Alabama and Georgia, USA. A cloacal swab was obtained from each bird and transported to the laboratory for isolation of *E. coli* and *E. faecalis*. Subsequently, the minimum inhibitory concentrations of four antibiotics was performed on these isolates, followed by PCRs to identify the presence of antimicrobial resistance genes in the isolates.[a]

		E. coli			*E. faecalis*		
Species[b] (*n*)	Primary diet	*n* resistant/ *n* recovered	AMR pattern[c] (*n*)	AMR gene	*n* recovered/ *n* resistant	AMR pattern (*n*)	AMR gene
American Kestrel (1)	Mammals	1/1	AMP-CHL-CIP-TET	*blaTEM-1*, *blaCTX-M-1*, *cmlA*, *cat*, *gyrA*, *tet*(M)	—	—	—
Barn Owl (1)		0/1	—	—	0/1	—	—
Barred Owl (21)		4/19	AMP-CHL-TET	*blaTEM-1*, *cat*, *tet*(M)	0/14	—	—
			AMP-CIP-TET	*blaCTX-M-1*, *gyrA*		—	—
			AMP-TET	*blaTEM-1*,		—	—
			AMP	*blaTEM-1*, *blaCTX-M-1*		—	—
Eastern Screech Owl (5)		3/5	AMP-CHL-CIP-TET	*blaCTX-M-1*, *cat*	0/4	—	—
			CHL-TET	*cat*		—	—
			AMP	*blaTEM-1*		—	—
Golden Eagle (1)		1/1	AMP-CHL-TET	*cat*	—	—	—
Great-horned Owl (6)		0/6	—	—	1/2	TET	*tet*(M)
Red-shouldered Hawk (11)		2/9	AMP-CHL-CIP-TET	*blaTEM-1*, *blaCT-XM-1*, *cmlA*, *cat*, *gyrA*	0/8	—	—
			TET	—	—	—	—
Red-tailed Hawk (37)		4/36	AMP-CHL-CIP-TET	*blaTEM-1*, *blaCTX-M-1*, *cmlA*, *cat*, *gyrA*	2/28	TET	*tet*(M), *tet*(L)
			AMP-CIP-TET	*blaTEM-1*, *blaCTX-M-1*, *gyrA*, *tet*(M)		TET	*tet*(M)
			TET	*tet*(M)		—	—
			TET	—		—	—
Broad-winged Hawk (8)	Mixed	1/8	AMP-TET	*blaCTX-M-1*	0/4	—	—
Cooper's Hawk (7)	Birds	1/6	TET	—	1/4	TET	*tet*(M), *tet*(L)
Gyrfalcon (1)		0/1	—	—	—	—	—
Sharp-shinned Hawk (1)		0/1	—	—	—	—	—
Black Vulture (4)	Carrion	2/4	TET	—	1/1	TET	*tet*(M), *tet*(L)
			TET	—	—	—	—
Turkey Vulture (4)		0/4	—	—	1/1	TET	*tet*(M)
Bald Eagle (8)	Fish	1/8	AMP-CHL-TET	*cat*, *tet*(M)	0/6	—	—
Osprey (3)		0/1	—	—	0/2	—	—

TABLE 2. Continued.

Species[b] (*n*)	Primary diet	*E. coli*			*E. faecalis*		
		n resistant/ *n* recovered	AMR pattern[c] (*n*)	AMR gene	*n* recovered/ *n* resistant	AMR pattern (*n*)	AMR gene
Mississippi Kite (1)	Insect	0/1	—	—	0/1	—	—

[a] — = not present.

[b] American Kestrel (*Falco sparverius*); Barn Owl (*Tyto alba*); Barred Owl (*Strix varia*); Eastern Screech Owl (*Megascops asio*); Golden Eagle (*Aquila chrysaetos*); Great-horned Owl (*Bubo virginianus*); Red-shouldered Hawk (*Buteo lineatus*); Red-tailed Hawk (*Buteo jamaicensis*); Broad-winged Hawk (*Buteo platypterus*); Cooper's Hawk (*Accipiter cooperii*); Gyrfalcon (*Falco rusticolus*); Sharp-shinned Hawk (*Accipiter striatus*); Black Vulture (*Coragyps atratus*); Turkey Vulture (*Cathartes aura*); Bald Eagle (*Haliaeetus leucocephalus*); Osprey (*Pandion haliaetus*); and Mississippi Kite (*Ictinia mississippiensis*).

[c] AMR = antimicrobial resistance; AMP = ampicillin; CHL = chloramphenicol; CIP = ciprofloxacin; TET = tetracycline.

TABLE 3. Minimal inhibitory concentration (MIC) of the antimicrobial-resistant *Escherichia coli* (20/112, 17.8%) and *Enterococcus faecalis* (6/76, 7.9%) isolated from raptors. The ETEST was used to determine MIC of *E. coli* and *E. faecalis* isolates for antibiotics (ampicillin, chloramphenicol, ciprofloxacin, and tetracycline) representing four major classes of drugs widely used in both medicine and agriculture.

Antimicrobials	MIC (μg/mL)[a]			
	n	*E. coli*	*n*	*E. faecalis*
Ampicillin	13	>256	0	—
Chloramphenicol	1	32	0	—
	1	>64	0	—
	6	>256	0	—
Ciprofloxacin	6	>32	0	—
Tetracycline	1	>128	1	24
	4	>192	1	32
	13	>256	2	48
	—	—	1	>64
	—	—	1	>96

[a] *n* = number of resistant isolate(s); — = not applicable.

having a MIC>128 μg/mL. Ampicillin resistance was found at a rate of 12% (Table 3), with all of the resistant strains having an MIC>256 μg/mL. Of the four drugs tested, the *E. faecalis* isolates only exhibited resistance to tetracycline at a rate of 8% (Table 3), with MIC levels ranging from 24 μg/mL to >64 μg/mL. The percentage of isolates resistant to at least one of the antibiotics tested was higher (P=0.052) in *E. coli* (20/112, 18%) than in *E. faecalis* (6/76, 8%; Table 2).

Five of the six tested antimicrobial resistance genes, *blaTEM-1*, *blaCTX-M-1*, *tet*(M), *cmlA*, *cat*, as well as mutations in *gyrA* were identified in the resistant *E. coli* isolates (Table 2). In addition, we identified *tet*(M) and *tet*(L) in the tetracycline-resistant isolates of *E. faecalis* (Table 2). The nucleotide sequences for antimicrobial resistance genes obtained in this study were deposited to the GenBank with the following gene accession numbers: MH033833 and MH033834, *blaTEM-1*; MH033835, *tet*(L); MH033836, *tet*(M); MH085993, MH085994, and MH085995, *cat*; and MH085992, *cmlA* (Table 2).

Ampicillin resistance in *E. coli* was primarily mediated by *blaTEM-1* and *blaCTX-M-1*. Five of the resistant *E. coli* possessed both genes, while four only carried the *blaTEM-1* gene, and another three only contained *blaCTX-M-1*. Two ampicillin-resistant *E. coli* strains were negative for both *blaTEM-1* and *blaCTX-M-1*, suggesting a different β-lactamase is involved. Of the 18 tetracycline-resistant *E. coli*, only five possessed the *tet*(M) gene. Chloramphenicol resistance in *E. coli* was mediated by *cat* (n=8), with three also possessing *cmlA*. Of interest is the observation that all of the chloramphenicol-resistant *E. coli* were also resistant to tetracycline. In the six ciprofloxacin-resistant *E. coli* isolates, we identified five isolates with double mutations of *gyrA* (serine-83→leucine and aspartic acid-87→asparagine). Three of six tetracycline-resistant *E. faecalis* isolates harbored both *tet*(M) and *tet*(L) genes, but only *tet*(M) was found in the remaining three isolates (Table 2).

Wild birds of prey can cover great distances, have wide dietary choices, and inhabit many diverse environments. Because of their predatory nature, the discovery of any drug resistance in bacteria recovered from their gut flora is most likely acquired from their food sources or environmental exposure. As such, wild raptors can signal environmental contamination with antimicrobial-resistant bacteria, but of even more concern, they may also serve as a reservoir for commingling of drug-resistant bacteria and resistance genes and, ultimately, as a means of dispersal of such agents.

Multiple studies have been conducted in Europe and elsewhere surveying the level of antimicrobial resistance in various avian species (Bonnedahl and Järhult 2014). Limited information is available in the US to indicate if there is a similar pattern of drug resistance among the normal flora of wild birds. A majority of the *E. coli* (82%) and *E. faecalis* (92%) isolates obtained from raptors in this study were susceptible to all four antibiotics tested (Table 2). However, of the 20 (18%) *E. coli* isolates expressing some level of resistance, nine expressed a multidrug-resistant phenotype of which four were resistant to all four classes of antibiotics. Despite a low overall prevalence of antimicrobial-resistant bacteria, finding nearly half of the resistant *E. coli* to be multidrug resistant is alarming, especially in birds that should be antibiotic naive.

Among 13 ampicillin-resistant *E. coli*, the resistant genes, *blaTEM-1* and *blaCTX-M-1* were relatively common: *blaTEM-1* and *blaCTX-M-1* were each found in eight (62%) isolates. The gene *blaTEM-1* encodes the TEM type β-lactamase and confers resistance to penicillin and first-generation cephalosporin and is widely found among gram-negative bacteria (Palzkill 2018). The *blaCTX-M-1* provides high-level resistance to third-generation cephalosporins, and they confer variable levels of resistance to fourth-generation cephalosporins (Cantón et al. 2012). The presence of these two genes only among birds that have mammals in their diet in this study is supportive of the role food source has in spreading antimicrobial resistance in remote areas.

Tetracycline resistance was prominent among the *E. coli* isolates, with all 18 isolates expressing very high MICs. Of the two common genes *tet*(M) and *tet*(L) tested, we identified only *tet*(M) in just five isolates, indicating presence of other resistance mechanism. Previous publications reported that *tet*(M) confers only low levels of resistance in *E. coli* (Bryan et al. 2004), whereas our five *tet*(M)-positive isolates had exceptionally high MIC values (16 times the resistant break point). Most resistance mechanisms to chloramphenicol are efflux pumps (*cmlA*), as well as inactivating enzymes, such as chloramphenicol acetyltransferase (*cat*; Frye and Jackson 2013). Chloramphenicol resistance was detected in eight birds; the *cat* gene was found in all eight, while the *cmlA* gene was found in only three.

Ample evidence suggests that wild birds can host antimicrobial-resistant bacteria (Bonnedahl and Järhult 2014); however, few studies are from the US. In a study conducted in the midwestern region of the US (Marrow et al. 2009), *E. faecalis* was isolated from 21 wild

and four captive raptors, including hawks, owls, and kestrels. Multiple isolates were recovered from each bird and demonstrated resistance to several antibiotics, including chloramphenicol (39%), enrofloxacin (41%), and tetracycline (33%), but no resistance to ampicillin was detected. The current study differed from Marrow et al. (2009) in that single isolates of *E. coli* and *E. faecalis* were collected from wild raptors, and the sampled population included birds with more diverse feeding habits. By selecting birds with limited human contact, these birds were more likely to represent the natural population, which allowed us to determine the level of environmental contamination due to drug resistant bacteria. Furthermore, by testing only one isolate per bird in a limited number of population over a 15-mo period, we assured that the level of drug resistance in the wild population was not artificially inflated, and we may even have underestimated the level of antimicrobial resistance.

Our findings indicated that wild raptors could be an important indicator of environmental contamination with antimicrobial-resistant bacteria, and even more importantly, that they could be potential vectors in the dissemination of antibiotic resistance. Future studies should expand the area for sampling, including wild raptors from different geographic areas, distinguishing between congested urban areas versus remote locations and birds with different dietary choices. During surveillance, it will be important to expand gene testing to determine what new resistance genes wild birds are already absorbing and the degree with which they are shedding such genes. Our findings reflected the blurring of lines separating human and animal populations and the environment. These results should initiate a discussion into the development of practices that will slow the spread of drug resistance, reducing its impact on human and animal health.

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