



NOTE

Bacteriology

Prevalence of antimicrobial resistance in bacteria isolated from Great Cormorants (*Phalacrocorax carbo hanedae*) in Japan

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ABSTRACT. Wild birds are recognized as disseminators of antimicrobial-resistant (AMR) bacteria into the environment. Here, we isolated AMR indicator bacteria from 198 Great Cormorant cloacal swabs collected in Shiga ($n=90$), Oita ($n=52$), Gifu ($n=29$), and Gunma ($n=27$) Prefectures, Japan, in 2018 and 2019. In total, 198 *Aeromonas* spp. and 194 *Escherichia* spp. were isolated, and their antimicrobial susceptibility was examined. *Aeromonas* spp. were resistant to colistin (8.6%), nalidixic acid (4%), and other antimicrobials (<2%), with 3.0% positivity for *mcr-3*. *Escherichia* spp. showed resistance to colistin (3.1%), ampicillin (2.6%), tetracycline (2.1%), and other antimicrobials (<2%). This study shows the presence of AMR bacteria in Great Cormorants, indicating that these birds potentially disseminate AMR bacteria.

KEY WORDS: *Aeromonas* species, antimicrobial-resistant bacteria, *Escherichia* species, Great Cormorant

J. Vet. Med. Sci.

83(8): 1191–1195, 2021

doi: 10.1292/jvms.21-0108

Received: 22 February 2021

Accepted: 24 May 2021

Advanced Epub:

9 June 2021

The emergence and spread of antimicrobial-resistant (AMR) bacteria are an increasing threat to human and animal health worldwide [17]. AMR bacteria arising from human societies are disseminated into environments by wild animals. Although wild animals rarely encounter antimicrobial agents, they may contract AMR bacteria from the environment [3, 8]. As the One Health approach has been promoted in Japan with a national action plan to tackle antimicrobial resistance [17], integrated surveillance has been recognized as an effective strategy for understanding the prevalence of AMR bacteria in humans, animals, food, and the environment.

AMR bacteria can be released into the aquatic environment via effluents from farms, households, municipalities, and healthcare industries [26]. In Japan, several studies have reported AMR bacterial contamination in aquatic environments [2, 26]. The Great Cormorant (*Phalacrocorax carbo hanedae*), a wild bird with an increasing population in Japan, is causing severe damage to the aquatic industry and the environment, especially in the woods adjacent to the riverside, where it discharges large amounts of feces [11]. Because of the aquatic lifestyle of the Great Cormorant, the AMR bacteria that they harbor may be from aquatic environments. However, the prevalence of AMR indicator bacteria in the Great Cormorant has not been clarified. *Escherichia coli* is widespread in humans and animals and is used as an indicator bacterium to investigate antimicrobial resistance [12, 23]. Furthermore, *Aeromonas* spp. have been suggested to be suitable indicator bacteria for investigating antimicrobial resistance in aquatic environments [24]. Therefore, this study was conducted to clarify the prevalence of AMR indicator bacteria (*Aeromonas* spp. and *Escherichia* spp.) in the Great Cormorant.

Cloacal swabs were collected from Great Cormorants that were culled using the sharp-shooting method in the lower northern part (Gunma Prefecture, $n=27$), central part (Shiga and Gifu Prefectures, $n=90$ and 29, respectively), and southern part (Oita Prefecture, $n=52$) of Japan in 2018 and 2019 (Table 1). No ethical permission was required as the birds were not specifically killed for this study. For bacterial isolation, each cloacal swab was directly streaked on deoxycholate-hydrogen sulfide-lactose (DHL) plates (Eiken Chemical Co., Ltd., Tokyo, Japan) and aerobically incubated for 16–18 hr at 37°C. The isolates (three distinct isolates picked from each plate) were identified using the API-20E system (Sysmex BioMérieux, Tokyo, Japan) and polymerase chain reaction (PCR) to identify *Aeromonas* spp. and *Escherichia* spp. [13, 18]. Minimum inhibitory concentrations (MICs) of 12 antimicrobials were determined using the broth microdilution method with commercially available, custom-made plates (Eiken

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Chemical Co., Ltd.) following the manufacturer's instructions, as previously described [3]. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used for quality control, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [5]. The resistance breakpoints for all antimicrobial agents (except for colistin (CST)) were determined based on the CLSI guidelines for *Aeromonas* spp. [6] and *Escherichia* spp. [5]. The resistance breakpoint of CST was based on the criteria of the European Committee on Antimicrobial Susceptibility Testing [9]. Isolates resistant to three or more antimicrobial classes were identified as multi-drug resistant [14]. The tetracycline (TET)-resistance genes *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*, and *tet(G)* were investigated in all TET-resistant isolates by PCR using primers described elsewhere [10]. Additionally, the plasmid-mediated mobile colistin resistance (*mcr*) genes *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7*, *mcr-8*, *mcr-9*, and *mcr-10* were investigated in all CST-resistant isolates using primers described elsewhere [4, 20, 25, 28]. The obtained data were analyzed using Fisher's exact test with a 2×4 table to assess statistical differences among the four sampling sites, and then Holm correction method was used to adjust for multiple testing using the statistical software R version 3.6.2 [19]. Differences were considered significant if the resultant *P* value was ≤ 0.05 .

In total, 149 *A. veronii* and 49 *A. hydrophila* isolates were recovered from 76 and 32 cloacal swabs of the Great Cormorants, respectively. However, 11 samples from Shiga (4), Oita (4), and Gifu (3) Prefectures were positive for both *A. veronii* and *A. hydrophila*. In contrast, 144 *E. coli* and 50 *E. albertii* isolates were recovered from 72 and 32 swabs, respectively (Table 1), with 16 samples from Shiga (4), Oita (8), Gifu (1), and Gunma (3) Prefectures positive for both *E. coli* and *E. albertii*. The frequent occurrence of *A. veronii* and *A. hydrophila* observed in this study is consistent with the finding of Miyagi *et al.* [15], who detected *A. veronii* and *A. hydrophila* predominantly in freshwater in Okinawa Prefecture, Japan. In this study, *E. albertii* was found in the four prefectures in addition to *E. coli*. Murakami *et al.* [16] also reported the isolation of *E. albertii* from wild birds across Japan, further suggesting its prevalence in free-living birds.

Thirty-two samples were positive for AMR *Aeromonas* spp. (21 samples) and *Escherichia* spp. (11 samples). Among the samples positive for *Aeromonas* spp. twenty samples were positive for AMR *A. veronii* and one was positive for AMR *A. hydrophila*. Among the samples positive for *Escherichia* spp., seven samples were positive for AMR *E. coli* and four were positive for *E. albertii*. Antimicrobial resistance was observed in *A. veronii* (27 isolates from 20 samples) from Shiga (5 isolates from 3 samples), Oita (2 isolates from 2 samples), Gifu (13 isolates from 9 samples), and Gunma (7 isolates from 6 samples) Prefectures. Regarding antimicrobial resistance in *A. hydrophila*, one isolate from a sample from Gifu was resistant. In *E. coli*, antimicrobial resistance was found in 8 isolates from 7 samples from Shiga (2 isolates from 2 samples), Oita (2 isolates from 2 samples), Gifu (1 isolate from 1 sample), and Gunma (3 isolates from 2 samples) Prefectures. Antimicrobial resistance was observed in *E. albertii* (5 isolates from 4 samples) from Shiga (3 isolates from 2 samples) and Gunma (2 isolates from 2 samples) Prefectures. No sample was positive for both AMR *Aeromonas* spp. and *Escherichia* spp. All isolates of *Aeromonas* spp. were susceptible to cefotaxime, chloramphenicol (CHL), ciprofloxacin (CIP), sulfamethoxazole-trimethoprim (SXT), gentamicin, and meropenem (Table 2). Furthermore, *A. veronii* was resistant to CST (16 isolates, 10.7%), nalidixic acid (NAL; 8 isolates, 5.4%), TET (3 isolates, 2.0%), and kanamycin (KAN; 2 isolates, 1.3%). *Aeromonas hydrophila* was resistant to CST (1 isolate, 2.0%). In a previous study in Thailand, *Aeromonas* spp. from inland-cultured shrimps were resistant to NAL (22%) and TET (18%) [27]. *Aeromonas hydrophila* (80.8%) isolated from water bodies in the United States of America were resistant to both CIP and TET [22]. In this study, all three TET-resistant *A. veronii* isolates possessed *tet(E)*. The *tet(E)* has been found to be responsible for TET resistance in *Aeromonas* spp., more than any other class of TET resistance genes [1]. Of the 16 CST-resistant *A. veronii* isolates, 6 possessed the *mcr-3* gene, but no *mcr* gene was detected in the CST-resistant *A. hydrophila* (Table 3). In China, the prevalence of *mcr-3* responsible for CST resistance in *Aeromonas* spp. from aquatic environments is 10% [21]. Although acquired resistance genes were observed in *Aeromonas* spp. isolated from freshwater in Japan [2], the resistance rate of *Aeromonas* spp. in this study was low compared with that in other countries.

E. coli was resistant to ampicillin (AMP; 5 isolates, 3.5%), TET (4 isolates, 2.8%), CHL (2 isolates, 1.4%), NAL (2 isolates, 1.4%), SXT (2 isolates, 1.4%), CIP (1 isolate, 0.7%), and CST (1 isolate, 0.7%) (Table 2). All four TET-resistant *E. coli* isolates possessed *tet(A)* gene (Table 3). *E. albertii* isolates were susceptible to all the antimicrobial agents tested, except for CST (5 isolates, 10%). None of the isolates had the *mcr* genes tested (Table 3). In the Czech Republic, *E. coli* isolated from black-headed gulls were resistant to TET (19.1%) and AMP (11.7%) [8]. Moreover, *E. coli* isolated from free-living Canada geese in a lagoon near a swine housing facility was resistant to TET (64%) and AMP (20%) [7]. A study conducted on wild cranes in 2010 in Japan reported that *E. coli* was resistant to AMP (2.9%), NAL (2.9%), and oxytetracycline (15.9%) [12]. A follow-up study reported that *E. coli* isolated from wild cranes was resistant to AMP (3.1% in 2016, 4.4% in 2017), NAL (2.9% in 2016, 7.7% in 2017), and oxytetracycline (10.9% in both 2016 and 2017) [23]. In this study, one isolate (0.7%) of *E. coli* showed resistance to fluoroquinolone (CIP). Kitadai *et al.* (2012) [12] reported that 1.4% of *E. coli* isolated from wild cranes showed resistance to fluoroquinolone (enrofloxacin). These results suggest the low prevalence of AMR *E. coli* in wild waterfowl in Japan.

Regarding regional differences, the antimicrobial resistance rates in *A. veronii* from Gifu and Oita, Gifu and Gunma, and Shiga and Oita were not significantly different ($P > 0.05$). However, antimicrobial resistance in *A. veronii* from Gifu was significantly higher ($P \leq 0.05$) than that in *A. veronii* from Shiga, whereas antimicrobial resistance in *A. veronii* from Gunma was significantly higher ($P \leq 0.05$) than that in *A. veronii* from both Shiga and Oita. In contrast, antimicrobial resistance in *A. hydrophila*, *E. coli*, and *E. albertii* did not differ ($P > 0.05$) among the locations. However, multi-drug resistant *E. coli* was found in the Great Cormorants collected from Shiga (AMP-CHL-TET), Oita (AMP-NAL-SXT), and Gunma (AMP-CHL-NAL-CIP-SXT) Prefectures (Table 3). TET-resistant *E. coli* harboring *tet(A)* was found in Gunma, Oita, and Shiga Prefectures, whereas TET-resistant *Aeromonas* spp. harboring *tet(E)* were found in Gunma and Shiga Prefectures. Furthermore, of the six isolates carrying *mcr-3*, five were from Gifu

Table 1. Bacterial isolates collected from cloacal swabs of Great Cormorants

Location	Sample no.	Positive sample No. (% positive)			Positive sample No. (% positive)		
		<i>A. veronii</i>	<i>A. hydrophila</i>	Total*	<i>E. coli</i>	<i>E. albertii</i>	Total*
Shiga Prefecture	90	37 (41.1)	16 (17.8)	49 (54.4)	24 (26.7)	11 (12.2)	31 (34.4)
Oita Prefecture	52	15 (28.8)	6 (11.5)	17 (32.7)	29 (55.8)	12 (23.1)	33 (63.5)
Gifu Prefecture	29	16 (55.2)	10 (34.5)	23 (79.3)	7 (24.1)	2 (6.9)	8 (27.6)
Gunma Prefecture	27	8 (29.6)	0	8 (29.6)	12 (44.4)	7 (25.9)	16 (59.3)
Total	198	76 (38.4)	32 (16.2)	97 (49.0)	72 (36.4)	32 (16.2)	88 (44.4)

*Samples positive for both bacteria were counted once in the total. Eleven samples from Shiga (4), Oita (4), and Gifu (3) were positive for both *Aeromonas veronii* and *A. hydrophila*. Sixteen samples from Shiga (4), Oita (8), Gifu (1), and Gunma (3) were positive for both *Escherichia coli* and *E. albertii*.

Table 2. Distribution of the minimum inhibitory concentration (MIC) of antimicrobial agents to the *Aeromonas veronii* (n=149), *A. hydrophila* (n=49), *Escherichia coli* (n=144), and *E. albertii* (n=50) isolated from Great Cormorants

Antimicrobial agents*	Breakpoints	Bacteria species	MIC (µg/ml)/ No. of isolates										No. resistant isolates (%)
			≤0.5	1	2	4	8	16	32	64	128	>128	
AMP	≥32	<i>E. coli</i>			60	73	5	1			1	4	5 (3.5)
	≥32	<i>E. albertii</i>		1	20	29							
CFZ	≥32	<i>E. coli</i>		90	50	3		1					
	≥32	<i>E. albertii</i>		36	14								
CTX	≥4	<i>A. veronii</i>	148	1									
	≥4	<i>A. hydrophila</i>	48	1									
	≥4	<i>E. coli</i>	144										
	≥4	<i>E. albertii</i>	50										
MEM	≥4	<i>A. veronii</i>	95	53	1								
	≥4	<i>A. hydrophila</i>	34	15									
	≥4	<i>E. coli</i>	144										
	≥4	<i>E. albertii</i>	50										
GEN	≥16	<i>A. veronii</i>		25	80	38	6						
	≥16	<i>A. hydrophila</i>		14	28	7							
	≥16	<i>E. coli</i>	14	24	31	67	8						
	≥16	<i>E. albertii</i>	7	21	17	5							
KAN	≥64	<i>A. veronii</i>				5	66	67	9	1		1	2 (1.3)
	≥64	<i>A. hydrophila</i>				2	28	19					
	≥64	<i>E. coli</i>			2	30	39	70	3				
	≥64	<i>E. albertii</i>			10	26	14						
TET	≥16	<i>A. veronii</i>	135	6	1	2	2	3					3 (2.0)
	≥16	<i>A. hydrophila</i>	48	1									
	≥16	<i>E. coli</i>	5	72	62	1			2	2			4 (2.8)
	≥16	<i>E. albertii</i>	43	5	1		1						
NAL	≥32	<i>A. veronii</i>		135	4	2					1	7	8 (5.4)
	≥32	<i>A. hydrophila</i>		48	1								
	≥32	<i>E. coli</i>		8	107	26	1				1	1	2 (1.4)
	≥32	<i>E. albertii</i>		39	7	4							
CIP	≥4	<i>A. veronii</i>	147	2									
	≥4	<i>A. hydrophila</i>	49										
	≥4	<i>E. coli</i>	143			1							1 (0.7)
	≥4	<i>E. albertii</i>	50										
CST	≥8	<i>A. veronii</i>	3	56	49	25	3	13					16 (10.7)
	≥8	<i>A. hydrophila</i>	1	29	17	1		1					1 (2.0)
	≥4	<i>E. coli</i>	59	66	18	1							1 (0.7)
	≥4	<i>E. albertii</i>	7	21	17	5							5 (10)
CHL	≥32	<i>A. veronii</i>		139	3	2	4	1					
	≥32	<i>A. hydrophila</i>		47	1		1						
	≥32	<i>E. coli</i>			3	45	94			1	1		2 (1.4)
	≥32	<i>E. albertii</i>		1	28	21							
SXT			≤2.38/0.12	4.75/0.25	9.5/0.5	19/1	38/2	76/4	152/8				
	≥76/4	<i>A. veronii</i>	27	103	17	1	1						
	≥76/4	<i>A. hydrophila</i>	8	35	6								
	≥76/4	<i>E. coli</i>	111	26	4	1			2				2 (1.4)
	≥76/4	<i>E. albertii</i>	50										

*AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; MEM, meropenem; GEN, gentamicin; KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim.

Table 3. Resistance phenotypes of *Aeromonas* and *Escherichia* species isolated from Great Cormorants

Bacteria species	Resistance profile*	No. of isolates per location (Pos. res. gene No.)				Resistance gene detected	Total (Pos. res. gene No.)
		Shiga	Oita	Gifu	Gunma		
<i>A. veronii</i>	CST-NAL			1		None	1
	KAN-NAL				1	ND	1
	CST	2	2 (1)	8 (5)	3	<i>mcr-3</i>	15 (6)
	KAN				1	ND	1
	NAL	1		4	1	ND	6
	TET	2 (2)			1 (1)	<i>tet(E)</i>	3 (3)
	Susceptible	65	21	25	11		122
Subtotal		70 (2)	23 (1)	38 (5)	18 (1)		149 (9)
<i>A. hydrophila</i>	CST			1		none	1
	Susceptible	27	7	14	0		48
	Subtotal	27	7	15	0		49
<i>E. coli</i>	AMP-CHL-NAL-CIP-SXT				1	ND	1
	AMP-NAL-SXT		1			ND	1
	AMP-CHL-TET	1 (1)				<i>tet(A)</i>	1 (1)
	AMP-CST			1		None	1
	AMP	1				ND	1
	TET		1 (1)		2 (2)	<i>tet(A)</i>	3 (3)
	Susceptible	46	58	11	21		136
Subtotal	48 (1)	60 (1)	12	24 (2)		144 (4)	
<i>E. albertii</i>	CST	3			2	none	5
	Susceptible	16	13	2	14		45
	Subtotal	19	13	2	16		50
Total		164 (3)	103 (2)	67 (5)	58 (3)		392 (13)

*AMP, ampicillin; KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim. ND, not determined; Pos. res. gene No., Positive resistance gene number.

prefecture. Thus, no clear trends in regional differences of AMR bacteria and resistance genes were seen across the locations. This observation highlights the complexity of AMR bacteria; hence, further investigation is needed to elucidate the contributing factors to AMR bacteria prevalence.

In conclusion, this study provides evidence of the presence of AMR bacteria in Great Cormorants. Therefore, these birds have the potential to harbor AMR bacteria and resistance genes and to disseminate them into the environment.

CONFLICT OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENTS. This study was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant number 18H04073), and JOO is a beneficiary of a Japanese Government (MEXT) scholarship.

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