

Antimicrobial Susceptibility of *Escherichia coli* Isolated from Feces of Wild Cranes Migrating to Kagoshima, Japan

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ABSTRACT. Susceptibility to 13 antimicrobial agents was examined for 138 *Escherichia coli* isolates obtained from 192 fecal samples of wild cranes that migrated for wintering to the Izumi plain, Kagoshima prefecture in Japan. The numbers of isolates that were resistant to the antimicrobials used in this study are as follows: oxytetracycline (OTC), 22 isolates; minocycline, 7 isolates; ampicillin (ABPC), 4 isolates; nalidixic acid, 4 isolates; enrofloxacin, 2 isolates; kanamycin, one isolate. Multidrug resistant isolates exhibiting 2–4 drug resistances were obtained. All of the OTC-resistant isolates carried either the *tet(A)* or *tet(B)* gene. The *bla_{TEM}* gene was found in all of the ABPC-resistant isolates.

KEY WORDS: antimicrobial-resistant *Escherichia coli*, migratory bird, wild crane.

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From the middle of October every year, more than ten thousand cranes migrate from Siberia and north China to the Izumi plain, Kagoshima prefecture in Japan for wintering (http://www.city.izumi.kagoshima.jp/izumi_crane/default.asp). The cranes on the plain are fed rice and wheat to prevent them from intruding into nearby farms. Thus, many cranes crowd there for food. The cranes mainly consist of the hooded crane (*Grus monacha*) and white-naped crane (*Grus vipio*) which are designated as vulnerable species by the Ministry of the Environment, Japan.

Recent studies have shown that antimicrobial-resistant bacteria are present in many kinds of wild animals that have not been exposed to antibiotics [5, 6, 17]. Kanai *et al.* [9] suggested that wild birds might become carriers of resistant strains and might be responsible for the spread of R plasmids over in a wide area. The resistance genes of bacteria from wild birds may result in increased numbers of resistant bacteria in livestock and humans.

In the present study, we investigated the frequencies of antimicrobial-resistant strains among *Escherichia coli* (*E. coli*) isolates obtained from the wild crane feces.

In November 2007 and January 2008, a total of 192 samples of freshly dropped crane feces were collected, and processed within 24 hr. The fecal material was suspended in sterile saline (about 10% of final concentration), spread on MacConkey agar plates, and incubated for 24 hr at 37°C. One or two colonies per sample with typical *E. coli* morphology were picked, screened by biochemical methods

(Gram-stain, catalase, oxidase, indole, methyl red, Voges Proskauer, citrate), and identified using the API 20E system (BioMérieux Co.).

Susceptibility tests were performed by the agar dilution method (Mueller Hinton agar, Becton, Dickinson and Co.) to determine the minimum inhibitory concentrations (MICs) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [3] for 13 antimicrobials: ampicillin (ABPC; Sigma Chemical Co.; Sigma), cefazolin (CEZ; Sigma), chloramphenicol (CP; Sigma), oxytetracycline (OTC; Sigma), minocycline (MINO; Sigma), kanamycin (KM; Sigma), gentamicin (GM; Sigma), nalidixic acid (NAL; Sigma), orbifloxacin (OBFX; Dinippon Sumitomo Pharma. Co., Ltd.), oxolinic acid (OXA; Sigma), enrofloxacin (ERFX; Sigma), colistin (CL; Wako Pure Chemical Industries Ltd), and fosfomycin (FOM; Sigma). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as control organisms to confirm MIC as suitable. Breakpoints established by the CLSI [4] were used for the following antimicrobial agents: ABPC, CEZ, CP, MINO, KM, GM, NAL, and FOM. MICs of ERFX, OTC and CL were interpreted using the resistance breakpoints reported by Kojima *et al.* [13]. No breakpoint is available for OXA and OBFX.

Characterization of antimicrobial-resistance genes was investigated using polymerase chain reaction (PCR). The following genes were analyzed by PCR: *bla_{TEM}*, *bla_{SHV}* and *bla_{OXA-1}* (in ABPC-resistant isolates); *tet(A-E)* and *tet(M)* (in OTC-resistant isolates); *aphA1* (in a KM-resistant isolate). The PCR procedures were carried out using primers for *bla_{TEM}*, *bla_{SHV}*, *bla_{OXA}*, *tet(A)*, *tet(C)*, *tet(E)* and *aphA1* as described by Maynard *et al.* [14] and for *tet(B)*, *tet(D)* and *tet(M)* as described by Costa *et al.* [5].

The quinolone resistance-determining region of the *gyrA* gene, as well as the analogous region of the *parC* gene, was

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Table 1. Distribution of the MICs of antimicrobial agents of 138 *E. coli* strains isolated from cranes wintering in Japan

Antimicrobial agent*	MIC ($\mu\text{g/ml}$) and No. of isolates													Break-point**	No. of resistant isolates (%)
	≤ 0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	>128		
ABPC		54	56	23			1					2	2	32	4 (2.9)
CEZ					53	85								32	
MINO					50	81				3	4			16	7 (5.1)
OTC				35	80	1					15	7		16	22 (15.9)
KM				41	90	5				1			1	64	1 (0.7)
GM			48	85	1	4								16	
FOM								5	75	55	3			256	
CP					4	41	90	2	1					32	
CL			115	22	1									16	
OXA		15	115	2	1	1	2					2			
NAL						1	62	46	25		1	3		32	4 (2.9)
OBFX	30	91	9	2	2	3					1				
ERFX	132	2		1	1	1			1					2	2 (1.4)

* ABPC: ampicillin, CEZ: cefazolin, MINO: minocycline, OTC: oxytetracycline, KM: kanamycin, GM: gentamicin, FOM: fosfomycin, CP: chloramphenicol, CL: colistin, OXA: oxolinic acid, NAL: nalidixic acid, OBFX: orbifloxacin, ERFX: enrofloxacin.

** MICs were interpreted using the resistance breakpoints defined by CLSI [4] or reported by Kojima *et al.* [13]. No breakpoint is available for OXA and OBFX.

amplified by PCR [16, 21] for quinolone-resistant *E. coli* isolates. Amplified fragments were purified and both strands were sequenced using the same primers as for the PCR amplifications. The sequences obtained were compared with those previously reported for the *gyrA* (Gene Bank accession number X06373) and *parC* genes (M58408).

A total of 138 *E. coli* isolates were recovered from 138 (71.9%) out of 192 samples, and from which antimicrobial-resistant strains against 6 drugs were found at rates of 0.7%-15.9% (Table 1). Twenty-two OTC-resistant isolates were obtained followed by seven MINO-resistant isolates, four ABPC- and NAL-resistant isolates, 2 ERFX-resistant isolates, and one isolate resistant to KM. Nine isolates resistant to MINO or NAL were also resistant to OTC (Table 2). An isolate resistant to ERFX was also resistant to NAL and showed the highest MICs of OBFX (64 $\mu\text{g/ml}$) and OXA (128 $\mu\text{g/ml}$) among the isolates studied.

The *tet (A)* gene was detected in 15 (68.2%) of the 22 OTC-resistant isolates, and the remaining 7 isolates (31.8%) carried the *tet (B)* gene. The MINO-resistant strains with MIC value of 32 or 64 $\mu\text{g/ml}$ isolated in the present study harbored the *tet(B)* gene as well as the previous study [20]. The *bla_{TEM}* gene was detected in all four ABPC-resistant isolates. The *aphA1* gene, which is one of the representative genes conferring resistance to KM [18], was detected in our KM-resistant *E. coli* isolate. These resistance genes have been widely found in the resistant isolates from humans, food and wild animals [2, 5, 7, 18, 20].

These resistance genes are occasionally located on mobile elements, such as plasmids and transposons [18]. Wild birds may act as reservoirs of antimicrobial-resistant bacteria [9]. Of 156 *Salmonella* isolates recovered from crane fecal samples collected in the Izumi plain during the period between 2002 and 2008, however, all the isolates except for one each isolate were susceptible to ABPC and GM [8, 12]. The resistance genes detected in the present

Table 2. Resistance phenotypes of *E. coli* isolates recovered from cranes wintering in Japan

Resistance phenotype	No. of isolates (%)
ABPC*	2 (1.4)
OTC	13 (9.4)
OTC-MINO	6 (4.3)
OTC-NAL	1 (0.7)
NAL-ERFX	2 (1.4)
ABPC-OTC-NAL	1 (0.7)
ABPC-OTC-MINO-KM	1 (0.7)
Total	26 (18.8)

* Abbreviation: See Table 1.

study might not be easily transferred to *Salmonella*. Nakamura *et al.* [15] reported that wild birds play a less important role in spreading R plasmids than do domestic animals. Alternatively, Kinjo *et al.* [10] indicated that wild serows were likely to carry antimicrobial-resistant *E. coli* soon after they were reared in human areas even in the absence of direct exposure to antimicrobials. An actual examination of conjugative R plasmids might be needed to further characterize our resistant *E. coli* isolates.

Fluoroquinolone-resistant *E. coli* from wild animals has already been reported [5]. In the present study, one isolate of fluoroquinolone-resistant *E. coli* with high MIC values (OBFX 64 $\mu\text{g/ml}$, ERFX 16 $\mu\text{g/ml}$) was detected. Resistances to quinolones and fluoroquinolones have been explained by mutations in the *gyr* and *par* genes located on the chromosomal DNA [16, 21]. In 2 isolates resistant to NAL and one isolate resistant to both NAL and ERFX (MIC: 2 $\mu\text{g/ml}$), a single nucleotide change at nucleotide 248 (cytosine→thymine) in the *gyrA* gene resulted in the amino acid replacement Ser83Leu in the GyrA protein. Nucleotide changes at positions 248 and 260 (adenine→thymine) in the *gyrA* gene accompanied by two amino acid replacements, Ser83Lue and Asp87Asn, and a single nucle-

otide change at position 241 (cytosine→guanine) in the *parC* gene accompanied by the amino acid replacement Ser80Ile in the ParC protein were identified in the isolate resistant to both NAL and ERFX (MIC: 16 µg/ml) (data not shown).

In livestock of our country, OTC-resistant *E. coli* isolates were most frequently detected compared with other drugs. The reason for this prevalence was explained by the heavy usage of tetracycline [1]. Our result of 15.9% OTC resistance was lower than those for pigs (66.8%), broiler chickens (69.4%) and cattle (25.3%) reported by Kijima-Tanaka *et al.* [11]. Such a tendency was also recognized in ABPC and NAL.

The frequencies in other wild bird reports [5, 9, 15, 20] tended to be a little lower than that of our present results. Tsubokura *et al.* [19] reported that whistling swans harbored multi-antimicrobial-resistant *E. coli* at a higher rate (80%) than pintail (22%) and black-tailed gulls (46%) among migratory waterfowl in Japan. Thus, the distribution of resistant bacteria in wild animals varies according to the geographic location and animal species [8, 17]. In the present study, we used selective agar plates without any antimicrobial agents; nevertheless, many antimicrobial-resistant *E. coli* strains were isolated. If we used plates with target antimicrobial agents, the isolation rates of antimicrobial-resistant *E. coli* might be higher.

The existence of antimicrobial-resistant strains has been observed among wild birds, which may further spread antimicrobial-resistant strains or R plasmids to remote areas [10]. Continuous study utilizing molecular epidemiological methods will be required to confirm whether these resistant *E. coli* strains will be detected every time consistently and whether there is a specific transmission route for antimicrobial resistance genes among the wild birds on this pain.

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