DOI: 10.7589/JWD-D-21-00006

Journal of Wildlife Diseases, 57(4), 2021, pp. 954–958

Prevalence of Antimicrobial-resistant *Escherichia coli* in Migratory Greater White-fronted Geese (*Anser albifrons*) and Their Habitat in Miyajimanuma, Japan

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ABSTRACT: The spread of antimicrobial-resistant bacteria (ARB) in natural environments including wild animals is a concern for public health. Birds cover large areas, and some fly across borders to migrate in large flocks. As a migratory bird, the Greater White-fronted Goose (*Anser albifrons*) travels to Miyajimanuma, North Japan, each spring and autumn. To investigate the ARB in migratory birds and their surroundings, we collected 110 fecal samples of *A. albifrons* and 18 water samples from Miyajimanuma in spring and autumn of 2019. Isolation of *Escherichia coli* was performed using selective agars with or without antimicrobials (cefazolin and nalidixic acid). Isolates of *E. coli* were recovered from 56 fecal samples (50.9%) and five water samples (27.8%) on agars without antimicrobials. No isolates were recovered on agars with antimicrobials. One *E. coli* isolate derived from a fecal sample exhibited resistance to β-lactams (ampicillin and cefazolin), whereas all other isolates exhibited susceptibility to all tested antimicrobials. The resistant isolate harbored bla_{ACC}, which could be transferred to other bacteria and confer resistance to β-lactams. These results suggest a low prevalence of antimicrobial resistance in wild migratory birds and their living environments; however, wild migratory birds sometimes carry ARB harboring transferrable antimicrobial resistance genes and therefore present a risk of spreading antimicrobial resistance.

Key words: Antimicrobial resistance, eDNA, *Escherichia coli*, migratory bird, transferrable element.

The use of antimicrobials creates a selective pressure, and the emergence of antimicrobial-resistant bacteria (ARB) make it difficult to treat bacterial infections in both human and veterinary clinical settings. These settings are thought to be hot spots of ARB, causing the spread to surroundings (Guenther et al. 2011). Surveillance of ARB is conducted in clinical settings; however, ARB in natural environments and wild animals are not surveyed sufficiently because of a wide variety of species and ecologies (Ramey and Ahlstrom 2020).

As an indicator bacterium of antimicrobial resistance (AMR) in warm-blooded animals, *Escherichia coli* is commonly used in national surveillance of clinical settings and research on wild animals (Berendonk et al. 2015; Asai et al. 2020). Moreover, *E. coli* is regarded as the indicator bacteria of fecal contamination from humans and animals in environments (Sen et al. 2019). Thus, *E. coli* is considered an appropriate indicator to estimate the prevalence of AMR derived from local humans and animals in various environments.

Environmental DNA (eDNA) is derived from organisms living there and is used to detect the presence of macro-organisms (Sakata et al. 2020). In aquatic ecosystems, detecting the presence of animals is possible regardless of species, because animals generally need frequent opportunities to come in contact with water (Ushio et al. 2018).

Recently, research on the prevalence of AMR in wild animals has been reported, especially in wild birds (Sen et al. 2019). Many birds fly and move freely in large areas, and some species migrate across borders in large

flocks. Migratory birds pose a risk of disseminating ARB worldwide. In southern Japan, more than 10% of cranes harbor antimicrobial-resistant *E. coli*, including resistance to important antimicrobials in human clinical settings, such as β-lactams and quinolones (Kitadai et al. 2012). Ecological aspects such as feeding, resting areas, and migrating routes, vary among species of migratory birds. The prevalence of ARB in the Greater White-fronted Goose (*Anser albifrons*) roosting in Miyajimanuma, Northern Japan has not been researched. Our study aimed to investigate the prevalence of AMR in these migratory birds and their surroundings in Northern Japan.

Anser albifrons migrate between their breeding grounds in far East Russia and wintering grounds in Northern Japan. The freshwater lake Miyajimanuma in a rural area of Hokkaido prefecture, Northern Japan, is a stop-over site during spring and autumn migrations (Moriguchi et al. 2010). More than 60,000 *A. albifrons* fly to Miyajimanuma yearly, and this wetland is registered under the Ramsar Convention. We collected 110 fecal samples in 2019, 60 in April, and 50 in October, from *A. albifrons* roosting in Miyajimanuma; at the same times, 18 water samples were collected from the lake (10 in April, eight in October). Samples of voided fecal matter and surface water were collected in sterile tubes and stored at 4 C.

The fecal sample (0.2 g) or water sample (100 μL) was inoculated into 5 mL of Luria-Bertani Broth (Invitrogen, Carlsbad, California, USA), and incubated at 37 C overnight. This culture was streaked onto deoxycholate-hydrogen sulfate-lactose agar (Nissui Pharmaceutical, Tokyo, Japan) and CHROMagar™ ECC (CHROMagar Microbiology, Paris, France) with and without cefazolin (25 mg/L) or nalidixic acid (25 mg/L; Sigma-Aldrich, St. Louis, Missouri, USA), and incubated at 37 C overnight. Up to three colonies on each plate that were suspected as *E. coli* were subcultured for further analysis. The isolates were identified as *E. coli* via matrix-assisted laser desorption-ionization time of flight mass spectrometry using the Bruker MALDI Biotyper system (Bruker Daltonics, Bremen, Germany; Dierig et al. 2015).

We determined minimal inhibitory concentrations using the agar dilution method according to the Clinical Laboratory Standards Institute guidelines (2020). We tested ampicillin, cefazolin, cefotaxime, kanamycin, gentamicin, streptomycin, tetracycline, nalidixic acid, and ciprofloxacin (all from Sigma-Aldrich). Resistance breakpoints were defined following the Clinical Laboratory Standards Institute guidelines. *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as controls.

For β-lactam-resistant isolates, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and AmpC β-lactamase genes were analyzed using PCR (Pérez-Pérez and Hanson 2002; Fukuda et al. 2018).

Transferability of the β-lactam resistance gene was determined using the previously described filter-mating assay with a slight modification (Kudo et al. 2019). Briefly, the recipient was a rifampicin-resistant *E. coli* ML4909 strain. The transconjugant was selected on Tryptic Soy Agar (Bacto™, Franklin Lakes, New Jersey USA) supplemented with 50 mg/L rifampicin (Sigma-Aldrich) and 16 mg/L cefazolin, and was tested for susceptibility to antimicrobials and the presence of β-lactam resistance gene as described above.

For each water sample, eDNA was extracted from 45 mL. After ethanol precipitation, the eDNA was extracted as reported previously (Minamoto et al. 2019). First-round PCR (1st PCR) for amplification of avian eDNA was performed (Ushio et al. 2018), and PCR was performed in four replications. Purification of the 1st PCR products and subsequent steps were performed according to the previous study with slight modification (Sakata et al. 2020). Sequencing was conducted using an Illumina iSeq with 2×150 base pair-end kits (Illumina, San Diego, California, USA), and the species identification was conducted by online BLAST queries with default settings (National Center for Biotechnology Information 2021).

From agars without antimicrobials, 160 *E. coli* isolates were recovered from 56 fecal samples of *A. albifrons* (50.9%) and 15 from

five water samples (27.8%). Water samples were not concentrated, thus false-negatives for the isolation of *E. coli* might have occurred because of the small amount of inoculum. Moreover, for the isolation of ARB, we used selective agars with cefazolin or nalidixic acid, which are important antimicrobials in clinical settings. The use of selective agars with antimicrobials improves the isolation efficiency of ARB (Ramey et al. 2018); however, no *E. coli* was isolated from selective agars with antimicrobials. In environments not directly exposed to antimicrobials, ARB are a minority because of their inferior fitness to the wild types (Cohen et al. 2013). In natural environments including wild animals, which are rarely exposed to antimicrobial selection, ARB would be kept in the minority (Sayah et al. 2005; Ramey and Ahlstrom 2020).

All except one of the isolates were susceptible to all tested antimicrobials. The detection rate of the antimicrobial resistant *E. coli* isolate from *A. albifrons* was 0.9% (1/110). In other studies on migratory birds, detection rates of antimicrobial-resistant *E. coli* was 0.7% in Alaska, and resistance to β-lactams and tetracyclines were 2.9% and 15.9%, respectively, in Southern Japan (Kitadai et al. 2012; Ramey et al. 2018). In livestock of Japan, tetracyclines have been frequently used and tetracycline-resistant isolates have been most frequently detected (Nippon AMR One Health Report 2020). The prevalence of ARB in wild animals in close contact with humans and livestock habitats is higher than that in animals that have less contact (Sayah et al. 2005; Guyomard-Rabenirina et al. 2020; Ramey and Ahlstrom 2020). Surrounding environments affect the prevalence of ARB in natural environments of wild animals, and the effluent of ARB from humans and livestock habitats especially is a risk factor (Berendonk et al. 2015; Asai et al. 2020). Additionally, from 13/18 (72.2%) water samples, *A. albifrons* DNA was detected with eDNA, suggesting that *A. albifrons* affects the water environment. Inhibiting the effluent of ARB from and to wild animals, revealing diffusion pathways and ecologies of wildlife, and effective countermeasures therefore are important.

TABLE 1. Characterization of the β-lactams-resistant *Escherichia coli* isolate from a fecal sample of a Greater White-fronted Goose (*Anser albifrons*), collected in Miyajimanuma, North Japan, harboring the bla_{ACC} gene. Boldface type indicates resistance judged by the Clinical and Laboratory Standards Institute (2020).

	Donor	Recipient	Transconjugant
Isolate no.	19EM82	ML4909	19EM82-ML
bla gene	ACC	Not detected	ACC
Antimicrobials (MIC[a]; mg/L)			
Ampicillin	**64**	1	**64**
Cefazolin	**>128**	0.5	**>128**
Cefotaxime	1	<0.25	1
Kanamycin	16	8	8
Gentamicin	4	<0.5	<0.5
Streptomycin	16	8	8
Tetracycline	4	1	1
Nalidixic acid	2	2	2
Ciprofloxacin	0.06	<0.06	<0.06

[a] MIC = minimum inhibitory concentration.

One isolate (19EM82) derived from the fecal sample of *A. albifrons* showed resistance to ampicillin and cefazolin and harbored the bla_{ACC} gene (Table 1). Using the conjugation experiment, the bla_{ACC} gene was transferred to other bacteria and it conferred resistance to ampicillin and cefazolin. The AmpC-type of β-lactamase genes, including bla_{ACC}, have been detected in various environments and clinical settings (Miró et al. 2005; Dorado-García et al. 2018). Previous studies reported that wild animals carry the clinically crucial ARB with antimicrobial resistance genes on transmissible elements and pathogenic bacteria (Benskin et al. 2009; Guenther et al. 2011; Guyomard-Rabenirina et al. 2020). These results suggest that pathogenic bacteria are able to gain transferable antimicrobial resistance genes in wild animals.

In conclusion, our study showed a low prevalence of ARB in migratory Greater White-fronted Geese and their environment; however, migratory birds do carry ARB harbouring transferrable antimicrobial resis-

tance genes. To maintain a low prevalence of antimicrobial resistance in natural environments and wild animals, monitoring is required. Migratory birds could be particularly important for the dissemination of antimicrobial resistance because they fly across borders in large flocks, and continued surveillance of the prevalence of ARB in migratory birds and their environments is recommended

Author contributions are as follows: Conceptualization, M.U., Y.T. and T.A.; methodology, F.A., M.U., M.S. and T.M.; formal analysis, F.A. and M.U.; investigation, F.A., M.U., D.S., N.H., M.S. and K.M.; resources, M.U., K.U. and O.Y.; data curation, F.A. and M.U.; writing—original draft preparation, F.A.; writing—review and editing, M.U.; funding acquisition, T.A. All authors have read and agreed to the published version of the article. This research was funded by The Ministry of Education, Culture, Sports, Science, and Technology (MEXT) KAKENHI grant 18H04073. The authors declare no conflict of interest. Some of the instruments used in the analysis were supported by the Wild Animal Medical Center.

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Submitted for publication 20 January 2021.
Accepted 9 April 2021.