

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 μ L) 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 CHROMagarTM 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 Bio-

typer 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*_{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 (BactoTM, 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 \times 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,

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
<i>bla</i> 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.

and effective countermeasures therefore are important.

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.

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LITERATURE CITED

- Asai T, Usui M, Sugiyama M, Izumi K, Ikeda T, Andoh M. 2020. Antimicrobial susceptibility of *Escherichia coli* isolates obtained from wild mammals between 2013 and 2017 in Japan. *J Vet Med Sci* 82:345–349.
- Benskin CMWH, Wilson K, Jones K, Hartley IR. 2009. Bacterial pathogens in wild birds: A review of the frequency and effects of infection. *Biol Rev* 84:349–373.
- Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons M-N, et al. 2015. Tackling antibiotic resistance: The environmental framework. *Nat Rev Microbiol* 13:310–317.
- Clinical and Laboratory Standards Institute. 2020. *Performance standards for antimicrobial susceptibility testing*. 30th Ed. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, 293 pp.
- Cohen NR, Lobritz MA, Collins JJ. 2013. Microbial persistence and the road to drug resistance. *Cell Host Microbe* 13:632–642.
- Dierig A, Frei R, Egli A. 2015. The fast route to microbe identification: Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). *Pediatr Infect Dis J* 34:97–99.
- Dorado-García A, Smid JH, van Pelt W, Bonten MJM, Fluit AC, van den Bunt G, Wagenaar JA, Hordijk J, Dierikx CM, Veldman KT, et al. 2018. Molecular relatedness of ESBL/AmpC-producing *Escherichia coli* from humans, animals, food and the environment: A pooled analysis. *J Antimicrob Chemother* 73:339–347.
- Fukuda A, Usui M, Okubo T, Tagaki C, Sukpanyatham N, Tamura Y. 2018. Co-harboring of cephalosporin (*bla*)/colistin (*mcr*) resistance genes among Enterobacteriaceae from flies in Thailand. *FEMS Microbiol Lett* 365:fny178.
- Guenther S, Ewers C, Wieler LH. 2011. Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? *Front Microbiol* 2:246.
- Guyonard-Rabenirina S, Reynaud Y, Pot M, Albina E, Couvin D, Ducat C, Gruel G, Ferdinand S, Legreneur P, Le Hello S, et al. 2020. Antimicrobial resistance in wildlife in Guadeloupe (French West Indies): distribution of a single *bla*_{CTX-M-1/Inc11/ST3} plasmid among humans and wild animals. *Front Microbiol* 11:1524.
- Kitadai N, Obi T, Yamashita S, Murase T, Takase K. 2012. Antimicrobial susceptibility of *Escherichia coli* isolated from feces of wild cranes migrating to Kagoshima, Japan. *J Vet Med Sci* 74:395–397.
- Kudo H, Usui M, Nagafuji W, Oka K, Takahashi M, Yamaguchi H, Tamura Y. 2019. Inhibition effect of flavophospholipol on conjugative transfer of the extended-spectrum β -lactamase and *vanA* genes. *J Antibiot (Tokyo)* 72:79–85.
- Minamoto T, Hayami K, Sakata MK, Imamura A. 2019. Real-time polymerase chain reaction assays for environmental DNA detection of three salmonid fish in Hokkaido, Japan: Application to winter surveys. *Ecol Res* 34:237–242.
- Miró E, Mirelis B, Navarro F, Matas L, Giménez M, Rabaza C. 2005. *Escherichia coli* producing an ACC-1 class C β -lactamase isolated in Barcelona, Spain. *Antimicrob Agents Chemother* 49:866–867.
- Moriguchi S, Amano T, Ushiyama K, Fujita G, Higuchi H. 2010. Seasonal and sexual differences in migration timing and fat deposition in the Greater White-fronted Goose. *Ornithol Sci* 9:75–82.
- National Center for Biotechnology Information. 2021. *Basic local alignment search tool*. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. Accessed May 2021.
- Nippon AMR One Health Report. 2020. *A statistical information site that deepens the understanding of AMR (drug resistance) and One Health*. <https://amr-onehealth.ncgm.go.jp/en/>. Accessed January 2021.
- Pérez-Pérez FJ, Hanson ND. 2002. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 40:2153–2162.
- Ramey AM, Ahlstrom CA. 2020. Antibiotic resistant bacteria in wildlife: Perspectives on trends, acquisition and dissemination, data gaps, and future directions. *J Wildl Dis* 56:1–15.

- Ramey AM, Hernandez J, Tyrlöv V, Uher-Koch BD, Schmutz JA, Atterby C, Järhult JD, Bonnedahl J. 2018. Antibiotic-resistant *Escherichia coli* in migratory birds inhabiting remote Alaska. *EcoHealth* 15:72–81.
- Sakata MK, Yamamoto S, Gotoh RO, Miya M, Yamanaka H, Minamoto T. 2020. Sedimentary eDNA provides different information on timescale and fish species composition compared with aqueous eDNA. *Environ DNA* 2:505–518.
- Sayah RS, Kaneene JB, Johnson Y, Miller RA. 2005. Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic and wild-animal fecal samples, human septage, and surface water. *Appl Environ Microbiol* 71:1394–1404.
- Sen K, Berglund T, Soares MA, Taheri B, Ma Y, Khalil L, Fridge M, Lu J, Turner RJ. 2019. Antibiotic resistance of *E. coli* isolated from a constructed wetland dominated by a crow roost, with emphasis on ESBL and AmpC containing *E. coli*. *Front Microbiol* 10:1034.
- Ushio M, Murata K, Sado T, Nishiumi I, Takeshita M, Iwasaki W, Miya M. 2018. Demonstration of the potential of environmental DNA as a tool for the detection of avian species. *Sci Rep* 8:4493.
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