Short Communication

High prevalence of a gene cluster conferring resistance to streptomycin, sulfonamide, and tetracycline in *Escherichia coli* isolated from indigenous wild birds

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A total of 116 *Escherichia coli* isolates from cecal contents of 81 indigenous wild birds in Korea were tested for antimicrobial susceptibility. Seventy-one isolates from sparrows (*Passer montanus*) and one isolate from doves (*Columba livia*) were resistant to three antimicrobials, including streptomycin, sulfonamide, and tetracycline (SSuT). PCR and subsequent sequence analysis revealed the SSuT gene cluster region (approximately 13 kb) harboring genes encoding resistance to streptomycin (*strA* and *strB*), sulfonamide (*sul2*), and tetracycline (*tetB*, *tetC*, *tetD*, and *tetR*). In particular, tetracycline resistance genes were located on the transposon Tn10-like element. The SSuT element-harboring *E. coli* can be an important source of the transmission of antimicrobial resistance to other pathogenic bacteria. Therefore, strict sanitary measures in human and animal environments are necessary to prevent the spread of resistant bacteria through fecal residues of wild birds.

Key Words: antimicrobial resistance; *Escherichia coli*; streptomycin; sulfonamide; tetracycline; wild birds

Antimicrobial-resistant *Escherichia coli* strains have been frequently found and disseminated in animals, humans, and various environments (Sjölund et al., 2009). In particular, the emergence of multiple drug-resistant (MDR) *E. coli* poses a significant challenge to animal production and public health (Kaesbohrer et al., 2012). It is commonly

known that the extensive use of antimicrobials has led to the dissemination of resistant bacteria and resistance genes in animals and humans (van den Bogaard and Stobberingh, 2000). Otherwise, bacteria may become resistant to the antimicrobials by acquiring antimicrobial resistance determinants without direct exposure to antimicrobials.

A variety of mobile genetic elements, including plasmids, integrons, and transposons, can be implicated in the horizontal transmission of resistance genes between bacteria (Middleton and Ambrose, 2005). Commensal bacteria such as *E. coli* can easily acquire and transmit resistance genes (Kaesbohrer et al., 2012). Therefore, monitoring of antimicrobial resistance in commensal *E. coli* from healthy animals facilitates early detection of emergence and possible spread of resistance to other animals and humans (Kaesbohrer et al., 2012).

Wild birds have been suspected as reservoirs and potential spreaders of antimicrobial-resistant bacteria (Pinto et al., 2015). Previous studies strongly suggest that wild birds harbor and spread resistant bacteria, which can be transmitted from birds to other animals and humans and vice versa (Shobrak and Abo-Amer, 2015). In the present study, we analyzed the level and genetic basis of antimicrobial resistance in commensal *E. coli* isolated from indigenous wild birds in Korea.

This study tested 116 *E. coli* isolates from cecal contents of 81 dead wild birds indigenous to the Chungcheongnam-do province of South Korea, submitted to the Animal and Plant Quarantine Agency, South Korea for diagnosis in 2016. The province is located in the southwest of the Korean Peninsula. Wild birds of which cecal samples were used in this study included 5 common kestrels (*Falco tinnunculus*), 29 sparrows (*Passer*

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Table 1. Frequency of the SSuT resistance phenotype in *E. coli* isolated from indigenous wild birds in Korea.

Common name	Latin name	No. of birds	No. of <i>E. coli</i> isolates	No. (%) with SSuT resistance phenotype*
Common kestrel	<i>Falco tinnunculus</i>	5	8	0 (0.0)
Sparrow	<i>Passer montanus</i>	29	73	71 (97.3)
Dove	<i>Columba livia</i>	14	10	1 (10.0)
Great tit	<i>Parus major</i>	9	0	0 (0.0)
Black kite	<i>Milvus migrans</i>	1	0	0 (0.0)
Common kingfisher	<i>Alcedo atthis</i>	2	3	0 (0.0)
Black-billed magpie	<i>Pica pica</i>	3	10	0 (0.0)
Grey starling	<i>Sturnus cineraceus</i>	2	0	0 (0.0)
Great spotted woodpecker	<i>Dendrocopos major</i>	1	5	0 (0.0)
Upland buzzard	<i>Buteo hemilasius</i>	1	5	0 (0.0)
Yellow-throated bunting	<i>Emberiza elegans</i>	14	2	0 (0.0)
Total		81	116	72 (62.1)

*All isolates except for SSuT-resistant isolates were susceptible to all the 18 antimicrobials tested.

Fig. 1. The SSuT gene cluster and flanking regions (13 kb) identified in SSuT-resistant isolates of *E. coli*.

Tetracycline resistance genes (*tetB*, *tetC*, *tetD*, and *tetR*) are located on the transposable element containing two transposase genes (*tnpL* and *tnpR*) on its both ends. Streptomycin resistance genes (*strA* and *strB*) and the sulfonamide resistance gene (*sul2*) are located on the region flanking the left end of the transposable element. HindIII and SacI restriction sites are presented with vertical dotted lines. The arrow line below the map indicates the 11-kb region amplified by PCR for restriction digestion analysis. IRL, left inverted repeat; IRR, right inverted repeat.

montanus), 14 doves (*Columba livia*), 9 great tits (*Parus major*), 1 black kite (*Milvus migrans*), 2 common kingfishers (*Alcedo atthis*), 3 black-billed magpies (*Pica pica*), 2 grey starlings (*Sturnus cineraceus*), 1 great spotted woodpecker (*Dendrocopos major*), 1 upland buzzard (*Buteo hemilasius*), and 14 yellow-throated buntings (*Emberiza elegans*) (Table 1). It was determined that all birds died of traumatic injuries in rural areas of the province. For *E. coli* isolation, up to five suspected isolates per bird were selected and subjected to conventional biochemical tests (Ewing, 1986) or VITEK 2 (bioMérieux Inc., Hazelwood, MO, USA) analysis to identify *E. coli*. Antimicrobial susceptibilities were tested using the Kirby-Bauer agar diffusion method described previously (CLSI, 2016) with 18 antimicrobials: ampicillin (10 µg), amoxicillin-clavulanic acid (20 to 10 µg), cephalothin (30 µg), cefepime (30 µg), gentamicin (10 µg), neomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), trimethoprim-sulfamethoxazole (1.25 to 23.75 µg), chloramphenicol (30 µg), cefoxitin (30 µg), sulfisoxazole (250 µg), ceftiofur (30 µg), apramycin (15 µg), enrofloxacin (5 µg), and

Fig. 2. PCR and restriction digestion analysis of the SSuT gene cluster region of all SSuT-resistant *E. coli* isolates.

The 11-kb internal regions of the gene cluster region (Fig. 1) were amplified by PCR and digested with HindIII and SacI. The restriction fragments (4.6 kb, 2.8 kb, 2.1 kb, 1.0 kb, and 0.5 kb) were separated by 1% agarose gel electrophoresis. Lanes M, 1-kb DNA marker (Intron Inc., Gyeonggi, Korea); lane 1, *E. coli* 16W-EC08-1; lane 2, *E. coli* 16W-EC09-1; lane 3, *E. coli* 16W-EC11-1; and lane 4, *E. coli* 16W-EC12-1.

florfenicol (30 µg). *E. coli* isolates resistant to three antimicrobials, including streptomycin, sulfonamide, and tetracycline (SSuT) were genetically characterized. PCR was performed with genomic DNA of each SSuT-resistant isolate to detect genes encoding resistance to streptomycin (*strA* and *strB*), sulfonamide (*sul2*), and tetracycline (*tetA* and *tetB*) as described previously (Khachatryan et al., 2008). Two long-range PCRs were conducted to amplify the SSuT gene cluster region of a representative isolate, 16W-EC08-1, with two primer sets described previously (Ziebell et al., 2011). The two targets were amplified using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) on the conditions including initial denaturation for 1 min at 94°C followed by 30 cycles of 15 s at 94°C, 30 s at 55°C, and 7 min at 68°C. The amplified product was cloned and sequenced (Macrogen Inc., Seoul, Korea). The sequence data were assembled and analyzed to generate the entire SSuT gene

cluster region using Vector NTI software (Invitrogen). A primer set (SSuT-I-L, 5'-CTCTCAGTGCATTGCCTCCA-3', and 5'-SSuT-I-R, GATATTCGCGGTTTTCCAGA-3') was designed to amplify an 11-kb internal region of the SSuT gene cluster. The 11-kb internal region of all SSuT-resistant isolates was amplified and digested with HindIII (Promega, Madison, WI, USA) and SacI (Promega). The restriction fragments of the internal region were separated by 1% agarose gel electrophoresis. Phylogenetic groups (A, B1, B2, and D) of all SSuT-resistant isolates were determined based on the presence of the *chuA*, *yjaA*, and TSpE4 by triplex PCR assay as described previously (Clermont et al., 2000).

A total of 116 *E. coli* isolates from cecal contents of 81 indigenous wild birds were selected in order to include at most five isolates per bird and tested for susceptibility to 18 antimicrobials. Seventy-one isolates from 21 sparrows (*Passer montanus*) and one isolate from a dove (*Columba livia*) were resistant to three antimicrobials, including streptomycin, sulfonamide, and tetracycline (Table 1). The other isolates were susceptible to all the antimicrobials tested. The presence of genes encoding resistance to streptomycin (*strA* and *strB*), sulfonamide (*sul2*), and tetracycline (*tetA* and *tetB*) was determined by PCR with genomic DNA of 72 SSuT-resistant isolates of *E. coli*. All isolates carried resistance genes, including *strA*, *strB*, *sul2*, and *tetB*, but not *tetA*. To determine the location of the resistance genes, the plasmid DNA was extracted by the alkaline lysis method (Birnboim, 1983) and analyzed by Southern blot hybridization with digoxigenin-labeled resistance gene probes as described previously (Ziebell et al., 2011). Briefly, plasmids were extracted by the alkaline lysis method. The analysis did not show positive reactions with each probe in plasmid DNA, indicating that the resistance genes are located in the chromosome.

Two long-range PCRs with the genomic DNA of 16W-EC08-1 and subsequent sequence analysis revealed the SSuT gene cluster region (approximately 13 kb) harboring all the resistance genes detected (Fig. 1). Tetracycline resistance genes (*tetB*, *tetC*, *tetD*, and *tetR*) were located on the transposon Tn10-like element carrying two oppositely oriented insertion sequences that harbor a transposase gene (*tnpL* or *tnpR*) and two terminal inverted repeats IRL (left inverted repeat) and IRR (right inverted repeat) flanked at its ends (Fig. 1). Streptomycin resistance genes (*strA* and *strB*) and the sulfonamide resistance gene (*sul2*) were located on the region flanking the left end of the transposable element (Fig. 1). Other open reading frames (ORFs) included hypothetical proteins, adhesin, ArsR family transcriptional regulator (*ybdA*), amino acid-binding protein (*ybeA*), antibiotic biosynthesis monooxygenase (*byeB*), sodium glutamate symporter (*ybfA*), and phosphoglucosamine mutase (*glmM*) (Fig. 1). The sequence including the SSuT gene cluster region determined in this study was deposited under the GenBank accession number MF630619.

All 72 SSuT-resistant isolates were positive for PCR to amplify the 11-kb internal region of the SSuT gene cluster. Subsequent restriction digestion analysis showed that all the 72 SSuT-resistant isolates produced five restriction fragments (4.6 kb, 2.8 kb, 2.1 kb, 1.0 kb, and 0.5 kb)

specific to the SSuT gene cluster (Fig. 2). All the 72 SSuT-resistant isolates of *E. coli* were PCR-negative for *chuA* and TspE4.C2 and grouped into phylogenetic group A.

Previous studies on antimicrobial resistance in *E. coli* of migratory and non-migratory wild birds showed that most isolates were resistant to approximately 3–10 antimicrobials (Pinto et al., 2015; Shobrak and Abo-Amer, 2014). *E. coli* isolates that produce extended-spectrum β -lactamases and AmpC β -lactamases have also been found in wild birds worldwide (Alcalá et al., 2016; Hasan et al., 2012). Nevertheless, it was also reported that MDR was higher in commensal *E. coli* isolates from migratory birds compared to those from non-migratory birds (Shobrak and Abo-Amer, 2014), and especially the migratory birds might play a role on the environmental dissemination of antimicrobial resistance (Bonnedaahl and Jarhult, 2014; Shobrak and Abo-Amer, 2014). This study showed that many commensal *E. coli* isolates from non-migratory indigenous species of wild birds exhibited a pansusceptible phenotype to all the antimicrobials tested. Antimicrobial resistance was found only in most isolates of *E. coli* from sparrows and one isolate of *E. coli* from doves. Interestingly, all of these resistant isolates had the SSuT resistance phenotype showing resistance to streptomycin, sulfonamide, and tetracycline.

The SSuT resistance phenotype has been reported in clinical and commensal isolates of *E. coli* from humans and food animals, such as poultry, swine, and cattle, worldwide (Khachatryan et al., 2008; Ziebell et al., 2011). Also, the gene cluster regions harboring *strA*, *strB*, *sul2*, and *tetB* were found in *E. coli* isolates with the SSuT resistance phenotype (Bean et al., 2009; Khachatryan et al., 2008; Ziebell et al., 2011). The present study also demonstrated the presence of the 13-kb SSuT gene cluster in the chromosome of commensal *E. coli* isolated from indigenous sparrows and doves in Korea. This gene cluster region includes a transposon Tn10-like element and other flanking regions, suggesting that at least a part of the gene cluster can be an important source of the transmission of antimicrobial resistance.

The SSuT resistance phenotype was highly prevalent in *E. coli* isolated from sparrows in the present study. This MDR phenotype may confer a selective advantage for isolates in the host that are exposed to the antimicrobials although this may not be the case in wild birds. Previous studies suggested that genetic factors associated with the SSuT resistance phenotype confer a selective advantage for bacteria in the host, resulting in the maintenance of resistance genes in these *E. coli* populations (Khachatryan et al., 2008; Ziebell et al., 2011). Also, the possible mobility of the resistance gene element may have contributed to the persistence of SSuT resistance genes in the *E. coli* populations (Bean et al., 2009). Based on the results of the present study, it is not clear as for why the SSuT resistance phenotype is highly prevalent only in *E. coli* isolated from sparrows. Contaminated habitats that antimicrobial-resistant bacteria have been commonly isolated are considered as important sources of antimicrobial resistance in a certain geographic location (Bonnedaahl and Jarhult, 2014). It is believed that sparrows might have come into extensive contact with human or animal sources

contaminated with resistant bacteria. Because wild birds are seldom exposed continuously to the antimicrobials, it is plausible that *E. coli* isolates with the SSuT resistance phenotype can persist in birds such as sparrows without antimicrobial selection pressure.

The present study shows that all *E. coli* isolates with the SSuT resistance phenotype from wild birds belong to phylogenetic group A. This finding indicates that SSuT-resistant isolates of *E. coli* may represent clonal expansion of bacteria that have been abundant in sparrows from a single region to which they are indigenous. Also, the phylogenetic group does not include virulent strains of *E. coli*. Therefore, *E. coli* isolates with the SSuT resistance phenotype identified in the present study may not directly cause diseases in humans and animals. Nevertheless, the resistance trait of the *E. coli* isolates from wild birds may be transmitted to other pathogenic bacteria by the transposon movement, resulting in the emergence of pathogenic and resistant bacteria. Therefore, strict sanitary measures such as physical separation are necessary for human environments and animal production facilities to prevent the spread of the resistant bacteria by environmental contamination through fecal residues of wild birds.

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