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Multidrug resistance of coagulase-negative staphylococci isolated from rescued wild animals

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Abstract

Wildlife is a bio-indicator of environmental pollution by antimicrobial resistant bacteria or genes, however, there is no information on antimicrobial resistance in wildlife-origin bacteria. This study aimed to investigate the normal microbiota of staphylococci and their antimicrobial resistance in wildlife that did not take any antimicrobials. After sampling and bacterial isolation/identification, antimicrobial resistance profiles were examined by broth microdilution test, Kirby-Bauer disc diffusion test and mecA gene-targeted PCR. Of 90 isolates from wildlife, 83 were coagulase-negative staphylococci while only 7 were coagulase-positive staphylococci. Methicillin-resistance was found in 63 (70%) isolates and 35 of 90 (38.9%) isolates were multidrug-resistant staphylococci. When considering that all of the animals did not take any medication or contacted any medical device before the sampling, the results indicate significantly high prevalence of antimicrobial resistance in wild environments. Further study would be necessary to investigate the transmission route of antimicrobial resistance.

Key words : Wildlife, Staphylococci, Antimicrobial resistance, Multidrug resistance

INTRODUCTION

Staphylococci are component organisms of the normal skin and mucosal microbiota in humans and animals. While normal microbiota may comprise coagulase-positive staphylococci, those organisms have been considered to be a major pathogen in human medicine. Moreover, coagulase-negative *S. epidermidis* or *S. haemolyticus* is now considered to be a significant pathogen in medical device or catheter-mediated infection (Becker et al, 2014). Various species of farm and companion animals host *Staphylococcus* spp. either as normal microbiota or pathogens (Lyskova et al, 2007), and in a variety of wild animals, coagulase-positive and coagulase-negative staphylococci were isolated as commensal organisms (Porrero et al, 2014; Sousa et al, 2016).

Investigation and management of antimicrobial-resistant staphylococci first focused on hospital cases of staphylococci infection, but has increasingly expanded to community-associated and livestock-associated staphylococci (Monaco et al, 2013). Antimicrobial-resistant bacteria and associated resistance genes are circulated among humans, animals, and the environment mainly by water (Allen et al, 2010; Radhouani et al, 2014). Wildlife that spend their entire life in their natural habitat are continuously exposed to several pollutants, toxicants, and antimicrobial-resistant bacteria or resistance genes, and are useful bio-indicators for evaluation of the severity of pollution and potential *in vivo* effects of exposure (Carroll et al, 2015). In the Republic of Korea, *Staphylococcus* species infection rates in carrier wildlife and the level of antimicrobial resistance in those remain unclear.

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This study aimed to identify the *Staphylococcus* species in the normal microbiota of antimicrobial-naïve wildlife and asses the antimicrobial resistance of the bacteria.

MATERIALS AND METHODS

Bacterial isolation and identification

Swab samples were collected from rescued wild animals at the Jeonbuk Wildlife Center and Wildlife Center of Chungbuk from December, 2016 to February, 2017. Sampling was performed in all animals before they contacted any medical device or received any medications. For mammals, samples were collected from the conjunctiva, nasal cavity, perianal area, and rectum, while for birds, they were collected from the conjunctiva, oral mucosa, pericloacal area, and cloaca. The swab samples were spread on trypticase soy agar plates containing 5% sheep blood, and subsequently, the plates were incubated at 37℃ for 24 to 48 hours. After incubation, staphylococci were isolated based on the colony morphology, hemolysis, Gram staining, conventional catalase test with 5% hydrogen peroxide, coagulase test with EDTA-treated rabbit plasma (BBL Coagulase Plasma, rabbit with EDTA; BD, Sparks, MD, USA), and DNase test with DNase test agar with methyl green (BD, Sparks, MD, USA). *S. aureus* strain ATCC 25923 (American Type Culture Collection [ATCC], Manassas, VA, USA) and a clinical isolate of *S. epidermidis* confirmed by species-specific polymerase chain reaction (PCR) (Martineau et al, 2000) and sequencing were used as positive and negative controls in the coagulase and DNase tests, respectively.

The isolates of staphylococci were identified by 16S ribosomal RNA (16S rRNA) and heat shock protein 60 (*hsp60*) analyses (Lane et al, 1985; Hill et al, 2006). All PCR amplicons were purified and sequenced, and subsequently aligned with a known *S. epidermidis* genomic sequence using BLAST software (National Center for Biotechnology Information [NCBI], USA) for nucleotide sequence homology. Finally, the source species of the isolates was confirmed by a multiple-PCR method (Sasaki et al, 2010).

Antimicrobial resistance testing

Antimicrobial resistance profiles of the isolates were tested by broth microdilution test with 34 antimicrobial agents as follows: clindamycin, tetracycline, rifampin, streptomycin, fusidate, penicillin, chloramphenicol, kanamycin, tiamulin, quinupristin/dalfopristin, vancomycin, gentamicin, trimethoprim, erythromycin, ciprofloxacin, cefoxitin, linezolid, mupirocin, sulfamethoxazole, ampicillin, amoxicillin/clavulanate, ticarcillin, trimethoprim/sulfamethoxazole, ceftriofur, enrofloxacin, cefovecin, amikacin, cefpodoxime, imipenem, marbofloxacin, oxacillin, ticarcillin/clavulanate, doxycycline, and cefazolin. Briefly, bacterial colonies were inoculated in broth and cultivated at 37℃ for 24 hours. The cultured broth was mixed with fresh Muller-Hinton broth, and the mixture was adjusted to 0.5 McFarland standard. Next, 50-μl mixture was inoculated in a 96-well antimicrobial-coated plate, and the plate was incubated at 37℃ for 24 hours. The results were evaluated according to Clinical and Laboratory Standard Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Multidrug-resistance (MDR) was evaluated according to the standard definition provided by the European Centre for Disease Prevention and Control (ECDC) and US Centers for Disease Control and Prevention (CDC) (Magiorakos et al, 2012). Methicillin resistance was confirmed by a combination of disc test (1-μg oxacillin disc and 30-μg cefoxitin disc; Oxoid, Hampshire, UK) and PCR assay for *mec*A. For quality control, methicillin-resistant *S. aureus* strain (ATCC 25923) was used as control in the disc test, and methicillin-susceptible *S. aureus* strain (ATCC 6538) was used as control strains in the PCR assay for *mecA*.

RESULTS

Isolated staphylococci

A total of 90 isolates of staphylococci was obtained from 44 wild animals (57 isolates from 25 wild birds and 33 isolates from 20 wild mammals). Of 90 isolates, 83 were coagulase-negative *Staphylococcus* species and

Table 1. Minimal inhibitory concentration of multidrug-resistant staphylococci against14 antimicrobials suggested by the guideline from European Centre for Disease Prevention and Control (ECDC) and Centers for Disease Control and Prevention (CDC)

Strain Number	Animal	Sampling site	*Staphylococcus* species	Abbreviation of antimicrobial name*													
				OXA	SXT	CLI	DOX	RIF	ERY	TET	CIP	LZD	SYN	VAN	FUS	CHL	GEN
1	Raccoon dog	Perianal area	*S. haemolyticus*	>4	>2/38	>4	<2	>2	>8	2	>8	<1	4	2	>4	8	>16
42	Raccoon dog	Perianal area	*S. haemolyticus*	>4	>2/38	>4	<2	<1	>8	2	>8	<1	1	2	>4	8	>16
47	Black-tailed gull	Conjunctiva	*S. sciuri*	>4	>2/38	>4	<2	<1	>8	8	0.5	2	2	<1	4	<4	<1
48	Black-tailed gull	Oral mucosa	*S. sciuri*	>4	>2/38	>4	<2	<1	>8	8	0.5	2	2	<1	4	8	<1
49	Black-tailed gull	Oral mucosa	*S. sciuri*	>4	>2/38	>4	8	<1	>8	>16	>8	4	4	<1	4	64	<1
50	Black-tailed gull	Oral mucosa	*S. haemolyticus*	>4	<0.5/9.5	>4	<2	>2	>8	2	>8	<1	1	2	<0.5	8	>16
51	Black-tailed gull	Cloaca	*S. haemolyticus*	>4	1/19	>4	<2	>2	>8	2	>8	<1	1	2	<0.5	8	>16
53	Brown hawk owl	Oral mucosa	*S. cohnii*	0.5	<0.5/9.5	>4	<2	<1	>8	1	0.5	2	1	2	>4	16	<1
54	Brown hawk owl	Oral mucosa	*S. sciuri*	>4	<0.5/9.5	<0.5	<2	>2	0.5	1	0.5	3	4	2	>4	8	8
55	Brown hawk owl	Cloaca	*S. epidermidis*	>4	<0.5/9.5	>4	4	<1	>8	8	2	2	1	2	>4	16	>16
57	Brown hawk owl	Perianal area	*S. xylosus*	<0.25	<0.5/9.5	>4	8	<1	>8	>16	<0.25	2	4	2	1	8	<1
59	Brown hawk owl	Cloaca	*S. xylosus*	<0.25	<0.5/9.5	>4	4	<1	>8	>16	0.5	2	4	2	1	16	<1
60	Brown hawk owl	Oral mucosa	*S. haemolyticus*	>4	2/38	>4	<2	>2	>8	2	8	<1	1	2	<0.5	8	>16
61	Brown hawk owl	Oral mucosa	*S. cohnii*	0.5	<0.5/9.5	>4	<2	<1	>8	<0.5	0.5	<1	1	2	>4	16	8
62	Brown hawk owl	Oral mucosa	*S. sciuri*	>4	<0.5/9.5	1	<2	>2	0.5	1	0.5	4	4	2	>4	8	8
65	Brown hawk owl	Conjunctiva	*S. sciuri*	>4	<0.5/9.5	>4	<2	<1	>8	1	1	2	4	<1	>4	32	<1
66	Brown hawk owl	Conjunctiva	*S. cohnii*	2	<0.5/9.5	>4	<2	<1	>8	<0.5	0.5	<1	1	2	>4	16	<1
67	Brown hawk owl	Oral mucosa	*S. haemolyticus*	>4	1/19	>4	<2	>2	>8	2	>8	<1	1	2	<0.5	8	>16
68	Brown hawk owl	Perianal area	*S. haemolyticus*	>4	2/38	>4	<2	>2	>8	2	8	<1	1	2	<0.5	8	>16
69	Brown hawk owl	Cloaca	*S. haemolyticus*	>4	2/38	>4	<2	>2	>8	2	8	<1	1	2	<0.5	8	>16
84	Buzzard	Oral mucosa	*S. cohnii*	4	<0.5/9.5	>4	<2	<1	>8	<0.5	0.5	2	1	2	>4	16	16
104	Black-tailed gull	Oral mucosa	*S. sciuri*	>4	<0.5/9.5	1	4	<1	0.5	>16	1	8	2	<1	>4	8	8
107	Korean water deer	Rectum	*S. muscae*	0.5	<0.5/9.5	>4	8	>2	8	>16	>8	2	>4	2	>4	8	>16
108	Buzzard	Oral mucosa	*S. warneri*	0.5	>2/38	>4	>8	>2	>8	>16	>8	2	>4	2	>4	8	>16
109	Buzzard	Oral mucosa	*S. sciuri*	1	<0.5/9.5	>4	8	<1	8	>16	>8	8	4	8	>4	8	>16
111	Buzzard	Conjunctiva	*S. delphini*	<0.25	<0.5/9.5	>4	4	<1	>8	16	>8	2	2	2	2	8	>16
113	Korean water deer	Conjunctiva	*S. warneri*	<0.25	<0.5/9.5	>4	8	<1	>8	>16	>8	2	>4	2	4	8	>16
114	Korean water deer	Conjunctiva	*S. sciuri*	>4	<0.5/9.5	1	4	<1	4	>16	4	2	4	2	>4	8	16
115	Korean water deer	Conjunctiva	*S. delphini*	4	<0.5/9.5	>4	4	<1	>8	8	>8	<1	>4	2	4	<4	>16
116	Korean water der	Nasal cavity	*S. haemolyticus*	>4	2/38	>4	4	>2	>8	>16	8	<1	>4	8	>4	8	>16
117	Korean water deer	Conjunctiva	*S. delphini*	0.5	<0.5/9.5	<0.5	8	<1	8	>16	8	2	>4	2	4	8	>16
125	Pheasant	Oral mucosa	*S. sciuri*	1	<0.5/9.5	<0.5	<2	<1	8	16	4	2	4	2	4	8	>16
126	Owl	Conjunctiva	*S. xylosus*	<0.25	<0.5/9.5	1	<2	<1	>8	>16	>8	2	>4	8	4	8	>16
127	Owl	Oral mucosa	*S. vitulus*	<0.25	<0.5/9.5	>4	8	<1	>8	>16	>8	<1	>4	2	4	8	>16
131	Turtle dove	Oral mucosa	*S. sciuri*	0.5	<0.5/9.5	>4	>8	<1	>8	>16	>8	2	>4	2	>4	8	>16

*OXA, oxacillin; SXT, trimethoprim/sulfamethoxazole; CLI, clindamycin; DOX, doxycycline; RIF, rifampicin; ERY, erythromycin; TET, tetracycline; CIP, ciprofloxacin; LZD, linezolid; SYN, quinupristin/dalfopristin; VAN, vancomycin; FUS, fusidic acid; CHL, chloramphenicol; GEN, gentamicin.

7 were coagulase-positive *Staphylococcus* species (*S. aureus* and *S. pseudintermedius*). In wild birds, *S. sciuri* was the most common species among 10 *Staphylococcus species* isolated; whereas, in wild mammals of homogenously mixed species, 13 *Staphylococcus* species were isolated. Of 33 isolates from wild mammals, 6 isolates were coagulase-positive *S. pseudintermedius* (3 isolates from 2 raccoon dogs; *S. aureus*: 3 isolates from 2 Korean water deer), and only 1 isolate was coagulase-positive *S. aureus* (1 isolate from 1 wild gray heron). All coagulase-negative staphylococci were adapted from our previous study (Lee et al, 2019).

Antimicrobial resistance profiles

Of 33 isolates from wild mammals, 23 (60.6%) were methicillin resistant, while of 57 isolates in wild birds, 40 (70.2%) were methicillin resistant, which yielded a total of 63 methicillin-resistant strains. Among coagulase-positive isolates, *S. aureus* from the single wild gray heron was methicillin resistant, while 3 each *S. pseudintermedius* and *S. aureus* from the 4 wild mammals were methicillin susceptible.

In the broth microdilution test, 35 isolates (38.9%) were MDR staphylococci comprising 27 of 57 (47.4%) from wild birds and 8 of 33 (24.2%) from wild mammals, which indicates a higher prevalence of MDR in wild birds. Of 35 MDR bacteria, 29 isolates (82.9%) were methicillin resistant, and of these, 25 (86.2%) were erythromycin resistant; All eight MDR bacteria from wild mammals were ciprofloxacin resistant. Table 1 summarize the origin and antimicrobial resistance profiles of MDR staphylococci.

DISCUSSION

This study revealed a higher prevalence of methicillin resistance (70.2% cases) and MDR (47.4% cases) in wild birds than in wild mammals (60.6% and 24.2% cases respectively). This phenomenon may be due to the area of activity of wildlife: Wild birds usually stay at one location for a short duration before moving to another location; in contrast, wild mammals usually remain in the same area of activity for their entire life, which reduces the risk of exposure to resistant bacteria or resistance genes when the environment of their territory is uncontaminated.

To prevent artificial transmission of antimicrobial bacteria or genes, we performed all sampling before administering medication or protocol with any medical device to the wildlife; nevertheless, the isolates of staphylococci showed high methicillin resistance compared to the results in previous reports of 65.5% in clinically healthy dogs, 26.8% in hospitalized dogs, and 33.8% in dogs with bacterial pyoderma in the Republic of Korea (Yoo et al, 2010; Moon et al, 2012; Han et al, 2016). Moreover, coagulase-negative staphylococci comprised the majority of total isolates, and erythromycin-resistant strain comprised the majority of methicillin-resistant staphylococci, which is a contrasting finding to those of a previous study on resistance to ampicillin and penicillin (Han et al, 2016). Based on these collective findings, we ruled out by-product of companion animals as a source of methicillin resistance in the wildlife included in our study. A study to investigate the epidemiologic route of resistance in wildlife is needed.

In conclusion, wildlife showed high prevalence of methicillin resistance and MDR. The source of antimicrobial resistance may include continuous environmental contamination with antimicrobial-resistant bacteria or resistance genes or natural transmission from the environment to humans or animals. A large-scale study is needed to clarify the mechanism of higher prevalence of antimicrobial resistance in wildlife.

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