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ARTICLE

Species Distribution, Antimicrobial Resistance, and Enterotoxin Profiles of Non-*aureus* Staphylococci Isolated from Poultry Slaughterhouses in Korea

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Abstract Although non-*aureus* staphylococci (NAS), such as coagulase-negative staphylococci (CoNS), can substantially affect human and animal health, information on NAS species distribution in poultry slaughterhouses and their antimicrobial resistance (AMR) profiles is limited. In this study, we analyzed the prevalence of NAS species and AMR profiles of NAS isolates collected from poultry slaughterhouses, including chicken carcasses and facility environments. In total, 100 NAS isolates were collected from six poultry slaughterhouses in Korea. The AMR patterns of the NAS species and the major genetic elements associated with AMR phenotypes, particularly methicillin and fluoroquinolone resistance, were determined. In addition, the prevalence of classical staphylococcal enterotoxin (SE, *sea-see*) and toxic shock syndrome toxin-1 (*tst-1*) genes among NAS isolates was examined. Among the 10 NAS species, coagulase-negative *Staphylococcus simulans* (n=49, 49%) was the most dominant species, followed by *Staphylococcus agnetis* (n=16, 16%). The multiple drug resistance phenotype was identified in 67% (n=67) of the NAS isolates, with the highest resistance to erythromycin (66%) and clindamycin (62%). Furthermore, fluoroquinolone resistance was confirmed in 34 (34%) NAS isolates. Fifteen NAS isolates were *mecA*-positive, harboring staphylococcal cassette chromosome *mec* (SCC*mec*) I (n=2), SCC*mec* IV (n=1), or non-typeable SCC*mec* types (n=12). Carriage of SE genes was detected in none of the NAS isolates, and *tst-1* was detected in only two CoNS strains. Our results suggest that NAS in poultry slaughterhouses can have potential role in the maintenance and transmission of AMR.

Keywords non-*aureus* staphylococci, poultry slaughterhouse, species profiles, antimicrobial resistance, fluoroquinolone resistance

Introduction

Staphylococci are commensal bacteria that colonize on the skin and mucous membranes of humans and animals (Becker et al., 2014; Casey et al., 2007). However, they are

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occasionally implicated in local and systemic infections such as scalded skin syndrome, gastroenteritis, and toxic shock syndrome (Ladhani et al., 2004; Lowy, 1998). Although *Staphylococcus aureus* is most frequently associated with disease outbreaks, recent studies have revealed that non-*aureus* staphylococci (NAS) substantially affect human and animal health (Adkins et al., 2018; Osman et al., 2017; Wuytack et al., 2020). The consumption or close contact with raw or undercooked meat and other food products contaminated with bacteria are the most common transmission routes from livestock to humans (Osman et al., 2016; Osman et al., 2017; Podkowik et al., 2012). As a zoonotic bacterial pathogen, *S. aureus* is characterized by the (i) production of coagulase, which converts fibrinogen to fibrin, and (ii) secretion of toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs), which cause staphylococcal food poisoning (SFP; Argudín et al., 2010; Dellaripa, 2000). Although some NAS strains, particularly coagulase-negative staphylococci (CoNS), have one or more genes encoding various SEs, their pathophysiological roles in SFP remain unclear (Podkowik et al., 2013; Wiśniewski et al., 2023). However, antimicrobial resistance (AMR) genes in NAS can be horizontally transferred to confer AMR phenotype in other staphylococci. Notably, studies have revealed a high prevalence of methicillin-resistant *S. aureus* and MR-NAS in livestock farms, slaughterhouses, and retail meat (Huber et al., 2011; Lim et al., 2010; Schnitt et al., 2021; Van Cleef et al., 2010). The *mecA*-containing staphylococcal cassette chromosome *mec* (SCC*mec*) and other mobile genetic elements (MGEs) carrying AMR genes can be transferred between *S. aureus* and NAS, which normally co-colonize in livestock such as cattle, pigs, goats, sheep, and poultry (Bhargava and Zhang, 2012; Pyzik et al., 2019; Ray et al., 2016).

Poultry carcasses have been associated with various foodborne pathogens such as *Salmonella* spp., *Campylobacter* spp., and *Staphylococcus aureus* (Crețu et al., 2011; Crețu et al., 2012; Crețu et al., 2015). Moreover, poultry is one of the principal reservoirs for antimicrobial-resistant staphylococci owing to the excessive use of antibiotics in poultry meat production (Apata, 2009; Diarra and Malouin, 2014). High fluoroquinolone (FQ) resistance in chicken-associated staphylococci has led to therapeutic dilemmas in both human and veterinary medicine (Dalhoff, 2012). Although the AMR profiles of *S. aureus* isolates from poultry and retail chicken meat are annually monitored in several countries including Korea, the USA, and the European Union (Abdalrahman et al., 2015; Feßler et al., 2011; Lee et al., 2022; Normanno et al., 2007), the AMR data for NAS isolates are relatively limited. Several previous studies have revealed various NAS species with AMR, including *Staphylococcus gallinarum*, *Staphylococcus xylosus*, *Staphylococcus simulans*, *Staphylococcus arlettae*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, and *Staphylococcus lentus* in the poultry food chain (Osman et al., 2016; Pimenta et al., 2021; Pyzik et al., 2019). In addition to AMR, most genes encoding SEs located on MGEs can be transferred between NAS and *S. aureus*, thereby increasing the morbidity and mortality rates of staphylococci (Alibayov et al., 2014).

Previously, we reported the AMR and SE profiles of NAS isolates from healthy broilers (Park et al., 2023) and retail chicken meat (Lee et al., 2020) in Korea. However, NAS species distribution in poultry slaughterhouses and their AMR profiles remain unreported. Therefore, in the present study, we analyzed the species prevalence, AMR phenotypes, and SE gene distribution of NAS isolates obtained from poultry slaughterhouses, including chicken carcasses and facility environments. Furthermore, the major genetic factors associated with methicillin and FQ resistance phenotypes were examined using SCC*mec* typing and quinolone-resistance determining region (QRDR) sequencing.

Materials and Methods

Sample preparation

In total, 270 swab samples were collected from six poultry slaughterhouses located in six Korean provinces from March to

December 2019. Swab samples were obtained from chicken carcasses (n=240) within 8 h of slaughter before a chilling process; and slaughterhouse environments (n=30), including cutting boards, sewage, floors, and tables. All swabs were kept at 4℃ and delivered to the laboratory within 24 h of sample collection to isolate staphylococci.

Isolation and species identification of staphylococci

For NAS isolation, swabs collected were inoculated into 3 mL of tryptic soy broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% sodium chloride (TSB-NaCl) and cultured at 37℃ for 18–24 h. Next, 10-μL aliquots of pre-enriched TSB-NaCl cultures were streaked onto Baird-Parker agar (Difco Laboratories) containing egg yolk supplements and potassium tellurite. After 24–48 h incubation at 37℃, presumptive staphylococcal colonies were picked from each agar plate and re-streaked on Baird-Parker agar for subsequent identification. Individual isolates were subcultured on tryptic soy agar (Difco Laboratories) at 37℃ for 18 h to extract genomic DNA using a Genmed DNA kit (Seoul, Korea) based on the manufacturer's protocols. NAS species were identified using 16S rRNA sequencing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonics, Bremen, Germany).

Detection of *mecA* and staphylococcal cassette chromosome *mec* elements typing

All staphylococcal isolates exhibiting cefoxitin resistance were screened for the presence of the *mecA* gene using polymerase chain reaction (PCR), as described previously (Geha et al., 1994). For *mecA*-positive isolates, SCC*mec* types were determined using multiplex PCR, which amplified the chromosomal cassette recombinase genes and *mec* regulatory elements (Kondo et al., 2007).

Antimicrobial susceptibility assays

To determine the antimicrobial susceptibility phenotype of each NAS isolate, the standard disc diffusion method was used according to the Clinical and Laboratory Standards Institute's (CLSI) and CLSI VET01S guidelines (CLSI, 2015; CLSI, 2022). Fourteen different antibiotic discs were utilized for the disc diffusion assay on Mueller-Hinton agar (Difco Laboratories): cefoxitin (FOX, 30 μg), penicillin (PEN, 10 μg), ampicillin (AMP, 10 μg), gentamicin (GEN, 50 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (CHL, 30 μg), erythromycin (ERY, 15 μg), fusidic acid (FUS, 50 μg), clindamycin (CLI, 2 μg), mupirocin (MUP, 200 μg), rifampicin (RIF, 5 μg), sulfamethoxazole-trimethoprim (SXT, 23.75–1.25 μg), tetracycline (TET, 30 μg), and quinupristin-dalfopristin (SYN, 15 μg).

Susceptibilities to vancomycin (VAN), linezolid (LZD), tigecycline (TGC), and teicoplanin (TEC) were examined using a standard Etest (bioMérieux, Craponne, France). Two reference strains, *Staphylococcus aureus* ATCC 29213 and *S. aureus* MW2, were included for the disc diffusion assay and Etest.

Detection of quinolone-resistance determining regions mutations

FQ-resistant NAS isolates frequently carry point mutations within the QRDRs of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*; Ito et al., 1994; Ng et al., 1996). Genomic DNA from each FQ-resistant isolate was subjected to PCR amplification using QRDR-specific primer sets (Supplementary Table S1). The QRDRs-specific primer sets were designed based on the following published sequences of reference genomes: *Staphylococcus agnetis* (NCTC7887, NCBI GenBank accession number UHAH01000002.1), *Staphylococcus chromogenes* (17A, NCBI GenBank accession

number NZ_CP031274.1), *S. arlettae* (P2, NCBI GenBank accession number AP019698.1), *S. lentus* (H29, NCBI GenBank accession number CP059679.1), *Staphylococcus nepalensis* (JS1, NCBI GenBank accession number NZ_CP017460.1), *S. simulans* (MR2, NCBI GenBank accession number NZ_CP016157.1). Then, the PCR products were sequenced at Bionics (Seoul, Korea). Differences in the amino acid sequences of the QRDRs were determined using the nucleotide sequences of the PCR results. Finally, multiple sequence alignments of *gyrA*, *gyrB*, *parC*, and *parE* genes were analyzed using the CLUSTALW server (www.genome.jp/tools-bin/clustalw).

Staphylococcal enterotoxin gene detection

Multiplex PCR was performed as previously described (Omoe et al., 2005) to examine the carriage of the toxic shock syndrome toxin-1 gene (*tst1*) and five classical SE genes (*sea*, *seb, sec, sed,* and *see*) in the NAS isolates. Five *S. aureus* reference strains (N315, MW2, COL, FRI472, and FRI913) were used as positive controls for the SE gene detection.

Results

Species profiles of the non-*aureus* staphylococci isolates from poultry slaughterhouses

During the study period, 100 NAS isolates of 10 different species were obtained from 270 swab samples (37.04%, 100/270) collected from six different poultry slaughterhouses (Fig. 1A and B). Out of the 100 NAS isolates, 91 NAS isolates were obtained from chicken carcasses, and nine were isolated from the slaughterhouse environments. Based on their ability to produce coagulase, these NAS isolates were divided into coagulase-variable (CoVS; n=19 isolates) and CoNS (n=81; Table 1). While the 19 CoVS isolates comprised only two species of NAS, *S. agnetis* (n=17) and *S. chromogenes* (n=2), the 81 CoNS isolates had eight different *Staphylococcus* species: *S. simulans* (n=49), *S. lentus* (n=11), *S. arlettae* (n=8), *S. xylosus* (n=4), *Staphylococcus sciuri* (n=4), *Staphylococcus warneri* (n=3), *S. epidermidis* (n=1), and *Staphylococcus urealyticus* (n=1). The two most prevalent NAS species isolated from the chicken carcasses were *S. simulans* (49.5%, 45/91 isolates) and

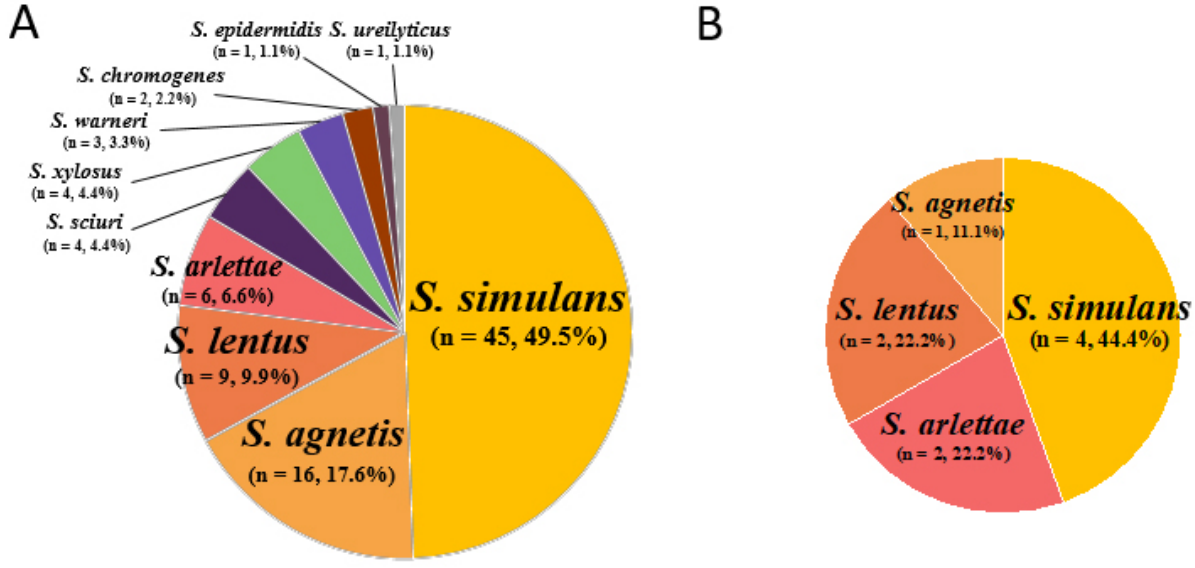

Fig. 1. Species distribution of non-*aureus* staphylococci (NAS) isolated from chicken carcasses (A) and slaughterhouse environments (B) in Korea. In total, 100 NAS isolates from 10 different species were identified in poultry slaughterhouses in Korea.

Table 1. Profiles of NAS and SCC*mec* types of methicillin-resistant NAS strains isolated from poultry slaughterhouses

NAS (n=isolates)	*mecA* positive (%)	*mec* gene	*ccr* gene	SCC*mec* type
CoVS (19)				
Staphylococcus agnetis (17)	-	-	-	-
Staphylococcus chromogenes (2)	-	-	-	-
CoNS (81)				
Staphylococcus arlettae (8)	-	-	-	-
Staphylococcus epidermidis (1)	1 (100)	B	A2B2	SCC*mec* IV
Staphylococcus lentus (11)	6 (54.5)	Multi	-	NT
		Multi	-	
		Multi	-	
		Multi	-	
		Multi	-	
		Multi	Multi	
Staphylococcus simulans (49)	8 (16.3)	B	A1B6	NT
		B	A1B1	SCC*mec* I
		B	-	NT
		B	A1B1	SCC*mec* I
		B	-	NT
		-	A1B6	NT
		E	-	NT
		A	A1B1	NT
Staphylococcus sciuri (4)	-	-	-	-
Staphylococcus urealyticus (1)	-	-	-	-
Staphylococcus warneri (3)	-	-	-	-
Staphylococcus xylosus (4)	-	-	-	-

NAS, non-*aureus* staphylococci; SCC*mec*, staphylococcal cassette chromosome *mec*; ccr, chromosomal cassette recombinase; CoVS, coagulase-variable staphylococci; CoNS, coagulase-negative staphylococci; NT, non-typeable.

S. agnetis (17.6%, 17/91 isolates; Fig. 1A). Similarly, the nine NAS isolates from the facility environments were *S. simulans* (n=4 isolates), *S. arlettae* (n=2), *S. lentus* (n=2), and *S. agnetis* (n=1; Fig. 1B).

Detection of *mecA* and staphylococcal cassette chromosome *mec* types of methicillin-resistant non-*aureus* staphylococci

PCR analysis revealed that none of the CoVS isolates were positive for *mecA* gene (Table 1). Among the 81 CoNS isolates, 15 (18.5%) were positive for *mecA*: *S. simulans* (n=8), *S. lentus* (n=6), and *S. epidermidis* (n=1). Furthermore, SCC*mec* element typing of the 15 *mecA*-positive NAS isolates revealed that eight *S. simulans* isolates had either non-typeable SCC*mec* (n=6) or SCC*mec* I (n=2). In addition, all six methicillin-resistant *S. lentus* strains contained non-typeable SCC*mec* elements. One methicillin-resistant *S. epidermis* isolate from a chicken carcass sample possessed SCC*mec* IV.

Antimicrobial resistance profiles of the non-*aureus* staphylococci isolates

Compared with the other antibiotics, the CoVS isolates exhibited higher resistance to FUS (84.2%, 16/19 isolates), PEN (84.2%, 16/19 isolates), AMP (84.2%, 16/19), GEN (78.9%, 15/19), and CIP (63.2%, 12/19; Table 2 and Fig. 2). Although a higher resistance level to these antibiotics was also observed in CoNS isolates, CoNS exhibited lower resistance rates to the five antibiotics compared with CoVS isolates. In addition, CoVS isolates exhibited higher levels of multidrug resistance (MDR) phenotypes (resistant to three or more classes/subclasses of antimicrobial agents) than CoNS isolates (84.2% vs. 63.0%, respectively). When the AMR of the dominant CoVS species (*S. agnetis*) and the two major species of CoNS (*S. simulans* and *S. lentus*) were compared, *S. agnetis* (82.4%) and *S. lentus* (90.9%) exhibited significantly higher degrees of MDR than *S. simulans* (57.1%; Table 2). Fig. 3 illustrates the heterogeneity of the AMR profiles within the three major species of NAS isolates, *S. simulans*, *S. agnetis*, and *S. lentus*. *S. agnetis* isolates tended to show high resistance to AMP, GEN, PEN, CIP, and FUS (Fig. 3A). Interestingly, *S. lentus,* and *S. simulans* isolates exhibited rather distinct AMR profiles (Fig. 3B and C). However, both *S. lentus* and *S. simulans* displayed comparatively high levels of resistance to CHL, CIP,

Table 2. Antimicrobial resistance profiles of NAS strains isolated from poultry slaughterhouses

NAS (n=isolates)	Number of antimicrobial resistance (%)														
	AMP	FOX	PEN	CHL	CIP	CLI	ERY	FUS	GEN	MUP	RIF	SXT	SYN	TET	MDR
CoVS (19)															
Staphylococcus agnetis (17)	14 (83.3)	0	14 (83.3)	2 (16.7)	10 (61.1)	6 (38.9)	6 (38.9)	15 (88.2)	14 (77.8)	0	0	0	0	4 (27.8)	14 (82.4)
Staphylococcus chromogenes (2)	2 (100)	0	2 (100)	1 (50)	2 (100)	2 (100)	2 (100)	1 (50)	1 (50)	0	0	0	0	1 (50)	2 (100)
CoVS total	16 (84.2)	0	16 (84.2)	3 (15.8)	12 (63.2)	8 (42.1)	8 (42.1)	16 (84.2)	15 (78.9)	0	0	0	0	5 (26.3)	16 (84.2)
CoNS (81)															
Staphylococcus arlettae (8)	8 (100)	0	8 (100)	7 (85.7)	7 (85.7)	6 (75)	8 (100)	8 (100)	0	0	0	0	0	3 (37.5)	8 (100)
Staphylococcus epidermidis (1)	1 (100)	1 (100)	1 (100)	0	0	0	1 (100)	1 (100)	0	0	0	0	0	0	1 (100)
Staphylococcus lentus (11)	6 (54.5)	4 (36.4)	6 (54.5)	7 (63.6)	7 (63.6)	7 (63.6)	7 (63.6)	8 (72.7)	0	0	0	5 (45.5)	0	2 (18.2)	10 (90.9)
Staphylococcus sciuri (4)	2 (50)	0	2 (50)	1 (25)	0	0	0	4 (100)	0	0	0	0	0	0	0
Staphylococcus simulans (49)	5 (10.2)	2 (4.1)	4 (8.2)	26 (53.1)	8 (16.3)	37 (75.5)	38 (77.6)	3 (6.1)	0	0	0	10 (20.4)	0	4 (8.2)	28 (57.1)
Staphylococcus urealyticus (1)	0	0	0	1 (100)	0	1 (100)	1 (100)	1 (100)	0	0	0	0	0	0	1 (100)
Staphylococcus warneri (3)	0	0	0	0	0	3 (100)	3 (100)	0	0	0	0	0	3 (100)	0	3 (100)
Staphylococcus xylosus (4)	3 (75)	0	4 (100)	0	0	0	0	4 (100)	0	0	0	0	0	0	0
CoNS total	25 (30.9)	7 (8.6)	25 (30.9)	42 (51.9)	23 (28.4)	54 (66.7)	58 (71.6)	29 (35.8)	0	0	0	15 (18.5)	3 (3.7)	9 (11.1)	51 (63)
Total (100)	41 (41)	7 (7)	41 (41)	45 (45)	35 (35)	62 (62)	66 (66)	45 (45)	15 (15)	0	0	15 (15)	3 (3)	14 (14)	67 (67)

NAS, non-*aureus* staphylococci; AMP, ampicillin; FOX, cefoxitin; PEN, penicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FUS, fusidic acid; GEN, gentamycin; MUP, mupirocin; RIF, rifampin; SXT, trimethoprim-sulfamethoxazole; SYN, quinupristin-dalfopristin; TET, tetracycline; MDR, multi-drug resistance; CoVS, coagulase-variable staphylococci; CoNS, coagulase-negative staphylococci.

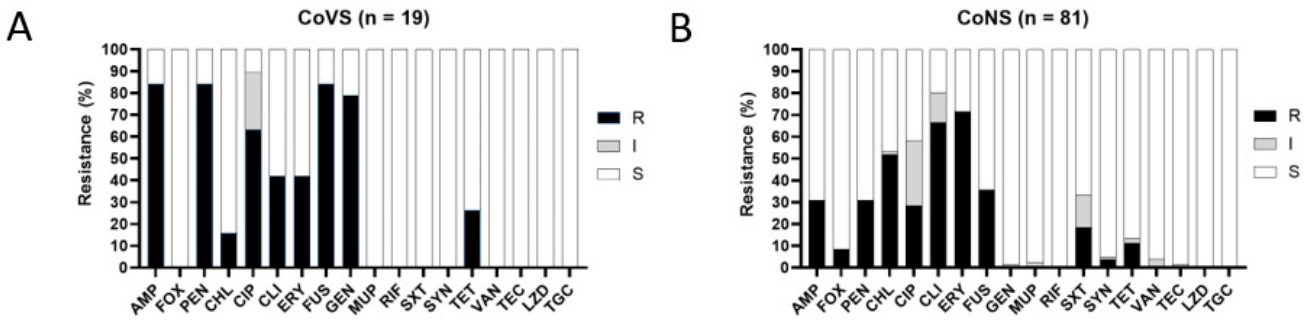

Fig. 2. Antimicrobial resistance patterns of the non-*aureus* staphylococci (NAS) isolates collected from the slaughterhouses in Korea. Antimicrobial resistance phenotypes of coagulase-variable staphylococci (CoVS; A) and coagulase-negative staphylococci (CoNS; B) isolates. R, resistant; I, intermediate; S, susceptible; AMP, ampicillin; FOX, cefoxitin; PEN, penicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FUS, fusidic acid; GEN, gentamicin; MUP, mupirocin; RIF, rifampicin; SXT, trimethoprim-sulfamethoxazole; SYN, quinupristin-dalfopristin; TET, tetracycline; VAN, vancomycin; TEC, teicoplanin; LZD, linezolid; TGC, tigecycline.

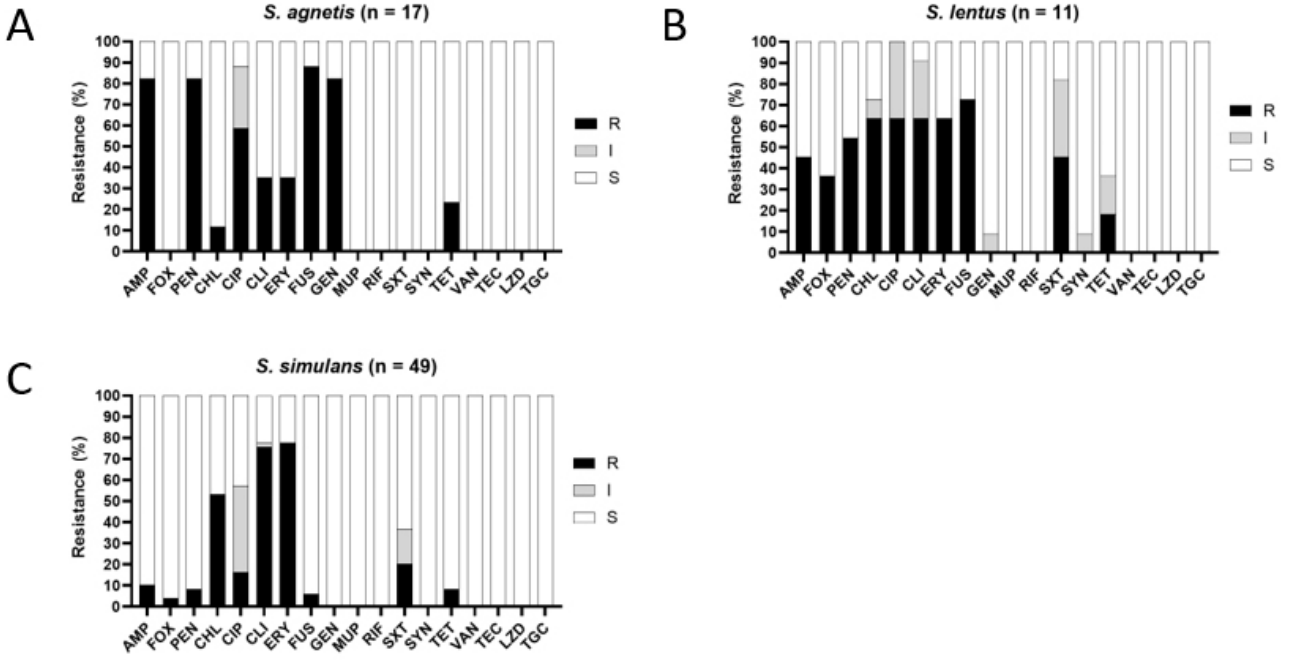

Fig. 3. Antimicrobial resistance patterns of the major non-*aureus* staphylococci (NAS) collected from poultry slaughterhouses. Antimicrobial resistance profiles of *Staphylococcus agnetis* (A), *Staphylococcus lentus* (B), and *Staphylococcus simulans* (C) isolates. R, resistant; I, intermediate; S, susceptible; AMP, ampicillin; FOX, cefoxitin; PEN, penicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FUS, fusidic acid; GEN, gentamicin; MUP, mupirocin; RIF, rifampicin; SXT, trimethoprim-sulfamethoxazole; SYN, quinupristin-dalfopristin; TET, tetracycline; VAN, vancomycin; TEC, teicoplanin; LZD, linezolid; TGC, tigecycline.

CLI, and ERY. Notably, *S. agnetis* and *S. lentus* isolates exhibited higher levels of CIP resistance (61.1% and 63.6%, respectively) than *S. simulans* isolates. Antimicrobial resistance profiles of each strain are shown in Supplementary Table S2.

Mutations in the quinolone-resistance determining regions of fluoroquinolone-resistant non-*aureus* staphylococci isolates

Among the 100 NAS isolates, 12 CoVS (10 *S. agnetis* and two *S. chromogenes*) and 22 CoNS (eight *S. simulans*, seven *S.*

arlettae, and seven *S. lentus*) isolates exhibited resistance to CIP (Table 3). Sequencing analysis of QRDRs within *gyrA*, *gyrB*, *parC*, and *parE* revealed that mutations at codon 84 in *gyrA* (S84L and D84N) and codon 80 in *parC* (S80L, T80I, T80R, S80F, and S80Y) were most frequently associated with the CIP-resistant phenotype of the NAS isolates (Table 3). Unlike point mutations in *gyrA* and *parC*, a high degree of mutation heterogeneity was observed in *gyrB* and *parE* within and between different NAS species (Table 3). Nevertheless, none of the FQ-resistant *S. lentus* (n=7) isolates carried point mutations in *gyrB*. Similarly, none of the FQ-resistant *S. agnetis* isolates harbored point mutations in *parE*.

Table 3. Point mutations within QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes associated with quinolone resistance in the study strains

NAS species		No. of FQ-resistant isolates (%)	Mutations in QRDRs				Total	Zone of inhibition (mm)
			gyrA	*gyrB*	*parC*	*parE*		
CoVS	*Staphylococcus agnetis* (17)	10 (58.8)	S84L	-	S80L	-	6	12.8±1.2
			S84L	D562Y	S80L	-	2	13.5±2.1
			S84L	F522V, K550R, D562Y	S80L	-	1	13
			S84L	F522V, E560Q, D562Y, E566Q, E597D	S80L	-	1	13
	Staphylococcus chromogenes (2)	2 (100)	S84L	I359V, G375A, I379V, T514K	H77L, G78A, S80L	T348P, D356E, S357A, S360A, D565T	1	6
			S84L, I131L	H511I, T514N	S80L, D84Y	K568N	1	13
CoNS	*Staphylococcus arlettae* (8)	7 (87.5)	S84L	-	T80I	-	3	6
			-	-	T80I	-	1	6
			S84L	-	T80I	A531Q	1	6
			S84L		T80I	A360S	1	6
			S84L	K554N, R561Q, K565Q, K567T	T80I	K375T, V382G, K383E, R395G, R406Q, K407E, G413S, A418P	1	6
	Staphylococcus lentus (11)	7 (63.6)	S84L, T172A	-	S80L	-	2	6
			S84L, T172A	-	S80L	Y497T	3	6
			S84L, A162S, T172A	-	-	Y497T	1	10
			S84L, A162S, T172A	-	S80L	Y497T	1	9
	Staphylococcus simulans (49)	8 (16.3)	S84L, A173S	-	F74Y, S80F	-	1	14
			S84L, A173S	-	S80Y, D84N	-	1	6
			S84L, A173S	A512R	S80Y, D84N	-	1	6
			S84L, A173S	A512P	S80F, D84N	-	1	6
			S84L, A173S	-	S80Y, D84N	L377I	1	6
			S84L, A173S	-	S80F, D84N	N498K, R499S	1	6
			S84L, A132S, A173S	E489G, S494T	S80I	V359I, F365Y, E467D	2	6

QRDRs, quinolone resistance determining regions; NAS, non-*aureus* staphylococci; FQ, fluoroquinolone; CoVS, coagulase-variable staphylococci; CoNS, coagulase-negative staphylococci.

Detection of staphylococcal enterotoxin and toxic shock syndrome toxin-1 genes in non-*aureus* staphylococci isolates

Among the 100 NAS isolates, only two CoNS isolates (one *S. simulans* and one *S. xylosus*) from chicken carcasses were positive for TSST-1 gene. None of the 19 CoVS isolates were positive for the five classical SE genes and *tst1*.

Discussion

Many recent studies have revealed that NAS are important reservoirs of AMR and enterotoxin genes, which can be transmitted to other pathogenic bacteria, including more pathogenic strains of *S. aureus*. In our previous research, we suggested that NAS in retail chicken meat can play an important role in the transmission of AMR and enterotoxin genes (Lee et al., 2020). However, not many studies have investigated the species profiles of antimicrobial-resistant NAS and their significance in food-related public health.

In the present study, we investigated NAS species distribution and their AMR profiles in chicken carcasses, facility environments, and poultry slaughterhouse workers in Korea. Similar to the findings of our previous studies on NAS profiles in broiler farms (Park et al., 2023) and retail chicken meat (Lee et al., 2020), the prevalence of CoNS (81.0%, 81/100) was considerably higher than that of CoVS (19.0%, 19/100; Table 1). A higher prevalence of CoNS compared with CoVS has also been reported in both healthy broilers and broilers with signs of illness from other countries. In the present study, *S. simulans* (49.0%, 49/100) and *S. agnetis* (17.0%, 17/100) were the most frequently identified NAS species in poultry slaughterhouses; this is consistent with the previously reported high prevalence of *S. agnetis*, *S. simulans*, *S. haemolyticus*, and *S. xylosus* in broiler farms (Park et al., 2023). Sample origin did not affect the species distribution of NAS isolates in chicken carcasses and facility environments in poultry slaughterhouses (Figs. 1A and B). The four major NAS species, *S. simulans*, *S. agnetis*, *S. lentus*, and *S. arlettae*, were commonly identified in both chicken carcasses and facility environments. These results suggest that broiler-associated NAS species (Park et al., 2023), both CoVS and CoNS species are potential sources of contaminants in chicken carcasses and facility environments in poultry slaughterhouses.

Approximately 20% of all the antibiotics sold in the livestock industry, particularly FQs, β-lactams, and TETs, are consumed in poultry farming in Korea (Lim et al., 2014). Thus, recent studies on livestock-associated antimicrobial-resistant NAS, particularly methicillin-resistant NAS isolates, have raised significant concerns regarding the transmission of resistance genes to more pathogenic bacteria in the food production system. In a previous study in Switzerland (Huber et al., 2011), researchers reported that 52.8% (38/72) of *mecA*-positive NAS isolates, i.e., *S. lentus* (n=30), *S. sciuri* (n=6), and *S. epidermidis* (n=2), were obtained from chicken carcasses. In the present study, *mecA*-positive NAS isolates (15.0%, 15/100) were present at relatively low levels in poultry slaughterhouses. As summarized in Table 1, eight *mecA*-positive but FOX-susceptible NAS isolates (two *S. lentus* and six *S. simulans*) carrying non-typeable SCC*mec* elements were identified. Recent studies revealed the *mecA*-positive but FOX-susceptible phenotype of staphylococci (Goering et al., 2019; Ho et al., 2020). This discrepancy could be owing to the altered expression of *mecA* because of dysregulation in *mecR1* (encoding the inducer protein MecR1), *mecI* (encoding the repressor protein MecI), or *bla* regulatory system (*blaR1-blaI*; Liu et al., 2016). In addition, a single-nucleotide insertion within *mecA*, resulting in the premature termination of *mecA* expression, has been identified in *S. aureus* strains (Kime et al., 2019).

Largely correlating with the sales amounts of antibiotics in the poultry industry in Korea, relatively high levels of resistance to β-lactams, GEN, TET, and CIP were identified in NAS isolates, particularly in CoVS isolates. This suggests that

antibiotic selective pressure affect the prevalence of antimicrobial-resistant NAS isolates in poultry slaughterhouses (Table 2). Furthermore, the high prevalence of FUS resistance in some of NAS isolates, particularly *S. agnetis*, *S. arlettae*, *S. lentus*, and *S. xylosus*, may be because of intrinsic FUS resistance owing to the frequent carriage of *fusD* and *fusF* (Hung et al., 2015). Notably, the high prevalence of NAS isolates with MDR phenotypes to the antibiotics extensively consumed in poultry farms suggests that antibiotic selective pressure facilitates the proliferation of antimicrobial-resistant NAS in broiler farms, and thus, its transmission to chicken carcasses in slaughterhouses.

The World Health Organization has categorized FQs as the highest-priority critically important antibiotics (Scott et al., 2019). However, FQ-resistant *S. aureus* and NAS strains have been frequently isolated in livestock farms, particularly poultry farms, in Korea. In this study, 34 FQ-resistant NAS isolates (12 CoVS and 22 CoNS) were identified to harbor multiple point mutations in the QRDRs of DNA gyrase and topoisomerase IV, except for one *S. arlettae* isolate carrying a single point mutation in *parC* (Table 3). Consistent with the findings of previous studies of FQ-resistant staphylococci (Takahashi et al., 1998; Wang et al., 2013), all 34 FQ-resistant NAS isolates possessed point mutations in *gyrA* (codon 84) or *parC* (codon 80). In contrast to the mutations in *gyrA* and *parC*, which are frequently concentrated in codons 84 and 80, respectively, point mutations in *gyrB* and *parE* were identified at various locations in QRDRs. Moreover, previous studies on FQ-resistant staphylococci (Nakaminami et al., 2014; Yamada et al., 2008) revealed a high degree of heterogeneity in the point mutations in *gyrB* and *parE*. Although major mutations, including S84L at *gyrA*, S80L at *parC*, and D84N at *parC*, were previously known to be associated with FQ resistance (Li et al., 1998; Takahashi et al., 1998; Vila et al., 1997), the precise role of other minor mutations in FQ resistance should be elucidated in the future research.

SEs produced by coagulase-positive *S. aureus* are a major cause of SFP (Argudín et al., 2010; Fisher et al., 2018). However, many recent studies have indicated that NAS isolates, including those from livestock and food products, carry SE genes and are potentially enterotoxigenic (Mekhloufi et al., 2021; Podkowik et al., 2013). In this study, our results demonstrate that CoVS isolates from poultry slaughterhouses do not harbor the classical SE nor *tst-1* genes. Although previous studies have revealed the highest prevalence of *sec* and *tst1* in CoNS strains (Hájek, 1978; Valle et al., 1990), only two *tst1*-positive (one *S. simulans* and one *S. xylosus*) NAS isolates were detected in the present study. Since SEA and TSST-1 were first detected in CoNS strains isolated from patients with toxic shock syndromes (Crass and Bergdoll, 1986), potential enterotoxigenic or pathogenic NAS strains carrying SE genes have been detected in humans (Achek et al., 2018; Banaszkiewicz et al., 2022), livestock (Ruiz-Ripa et al., 2020; Ünal and Çinar, 2012), and ready-to-eat foods (Chajęcka-Wierzchowska et al., 2020; Crass and Bergdoll, 1986; Cunha et al., 2006; Rall et al., 2010; Zell et al., 2008). Despite the proposed instability of SE genes in CoNS isolates (Banaszkiewicz et al., 2022), the carriage of SE and TSST-1 genes in NAS isolates from poultry slaughterhouses should be monitored as a significant threat to food safety.

Conclusion

In summary, this is the first report on the species diversity, AMR patterns, and genetic characteristics of methicillin- and FQ-resistant NAS isolates obtained from poultry slaughterhouses in Korea. Our findings suggest that (i) the overall species distribution of NAS isolates in poultry slaughterhouses is similar to that in broiler farms (Park et al., 2023) in Korea; (ii) MDR phenotypes with relatively high resistance levels of resistance to PEN, AMP, GEN, CIP, and TET are observed in NAS isolates; (iii) FQ-resistance in NAS isolates is caused by point mutations occurring at specific locations (*gyrA* and *parC*) or rather various locations (*gyrB* and *parE*) of QRDRs; and (iv) the carriage of *tst1* only in CoNS isolates, indicating the

extremely low prevalence of the TSST-1 and SE genes in NAS isolates. In addition, this study underscores a potential role of poultry slaughterhouses in transmission of antimicrobial-resistant NAS strains to human food chains.

To combat AMR in poultry industry, a multisectoral approach, including poultry farms, slaughterhouses, and human workers, is necessary. Improper use of antibiotics and indiscriminate prescription of antibiotics should be avoided to prevent occurrence and amplification of antimicrobial-resistant bacteria in poultry farms. Furthermore, importance of hygiene measures in slaughterhouses should be emphasized along with a multisectoral surveillance networks to better detect and mitigate spread of AMR.

Supplementary Materials

Supplementary materials are only available online from: https://doi.org/10.5851/kosfa.2024.e58.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Author Contributions

Conceptualization: Lee KJ, Yang SJ. Data curation: Lim JH, Yang SJ. Formal analysis: Lim JH, Park JH, Lee GY, Lee JB. Methodology: Lim JH, Park JH, Lee GY. Investigation: Lim JH, Park JH, Yang SJ. Writing - original draft: Lim JH, Lee JB, Lee KJ, Yang SJ. Writing - review & editing: Lim JH, Park JH, Lee GY, Lee JB, Lee KJ, Yang SJ.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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