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Bacteriology

Prevalence of pathogens and antimicrobial resistance of isolated *Staphylococcus* spp. in bovine mastitis milk in South Korea, 2018–2022

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ABSTRACT. *Staphylococcus* spp. are one of the most predominant isolates in milk samples of dairy cows with mastitis worldwide. The aims of this study were to investigate the prevalence of bacterial pathogens in bovine mastitis milk samples in South Korea and the antimicrobial resistance profiles of staphylococcal isolates. In total, 1,245 strains were isolated from 1,260 mastitis quarter milk samples (with somatic cell counts ≥ 200,000 cells/mL) from 66 dairy farms between 2018 and 2022. The bacterial genus with the highest prevalence in bovine mastitis milk samples was *Staphylococcus* spp. (33.9%), followed by *Streptococcus* spp. (11.5%). *S. aureus* and non-aureus staphylococci (NAS) accounted for 11.0% and 89.0% of staphylococcal isolates, respectively. *S. chromogenes* was the most prevalent species among the 22 NAS species detected. *S. aureus* showed the highest resistance rates to penicillin (25.0%) and ampicillin (20.8%), whereas NAS showed the highest resistance rates to penicillin (18.3%), tetracycline (11.4%) and erythromycin (10.1%). Sixteen multidrug-resistant (MDR) isolates were only isolated from NAS, and the most commonly detected antimicrobial resistance gene in the 16 MDR isolates was *mecA* (75.0%), followed by *tetK* (62.5%), *blaZ* (50.0%), *ermC* (50.0%), and *lnuA* (43.8%). In conclusion, NAS were the most common isolates from mastitis milk in South Korea and MDR isolates carried a variety of antibiotic resistance genes. Our study suggests that continuous monitoring of the distribution and antimicrobial resistance in *Staphylococcus* spp., particularly NAS, is needed to improve the effectiveness of management and treatment strategies in dairy farms.

KEYWORDS: antimicrobial resistance, bovine milk, mastitis pathogens, *Staphylococcus* spp.

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Bovine mastitis has a significant impact on both animal welfare and the economy due to its frequent occurrence, which results in reduced milk production and quality [10, 39, 44]. Annually, mastitis imposes significant costs on the global dairy industry, with a median total cost of €230 per intramammary infection [8]. Bovine mastitis can be caused by a wide variety of bacterial species [39]. Among them, staphylococci are frequently identified as predominant bacteria in dairy cows with mastitis worldwide [17, 25, 49]. In the context of routine mastitis diagnosis, staphylococci are typically classified into two categories: *S. aureus* and non-aureus staphylococci (NAS) [47]. As a contagious pathogen, *S. aureus* is transmitted from an infected cow to a healthy cow during milking [10, 44]. It can attach to epithelial cells and infiltrate interstitial tissues of the mammary gland, leading to deep infection in dairy cows [42]. NAS are emerging mastitis pathogens in many countries and can be both contagious and environmental pathogens [10]. They are associated with subclinical mastitis, which is a greater economic concern due to its higher frequency and ability to decrease milk production without notice [10]. In South Korea, *S. aureus* and NAS have been reported as the primary causative agents of bovine mastitis [23, 31].

The use of antibiotics in the treatment of mastitis is a crucial method to manage mastitis in dairy cows in the majority of countries worldwide, and there is concern over the potential development of antimicrobial resistance [10, 49]. The occurrence of antibiotic

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resistance is associated with low cure rates of bovine mastitis [22, 47]. The dissemination of bacteria resistant to antimicrobials in dairy cows can create a reservoir for genes conferring antimicrobial resistance, presenting a potential threat in terms of the transfer of these genes to both humans and animals [25]. Hence, it is crucial to monitor antimicrobial resistance in order to achieve the best outcomes from antimicrobial use and reduce the potential for the spread of antimicrobial-resistant bacteria [2, 49]. In South Korea, dairy cow mastitis is treated with antibiotics such as ampicillin, gentamicin, penicillin, tetracycline, and tylosin [22]. Moreover, the national mastitis control program in South Korea provides free laboratory diagnostics and antimicrobial susceptibility testing at regional laboratories and centers for veterinarians and farmers [36]. A recent study reported the occurrence of antimicrobial-resistant bacteria among major mastitis-causing pathogens, including methicillin-resistant staphylococci, in South Korea [23, 31].

Multidrug-resistant (MDR) pathogens, which are resistant to three or more classes of antibiotics, are a serious global public health concern [17]. Several previous studies reported multidrug resistance among strains isolated from bovine mastitis samples [5, 17, 49]. In particular, staphylococci have developed multidrug resistance on a global scale, making infections caused by them challenging to treat [7, 30].

Understanding the pathogen profile associated with mastitis is crucial for the successful implementation of control and prevention strategies for bovine mastitis [2, 16]. This is because mastitis is associated with a variety of bacterial species, the distribution of which is influenced by management practices and geographic factors, and because each bacterial species interacts differently with the host's microbiome and immune system, requiring different management strategies depending on the specific causative agent [3, 38, 39]. However, there is a lack of recent data on the prevalence of pathogens in cows with mastitis based on a national survey in South Korea. Most prevalence studies have focused only on specific pathogens [1, 22, 23, 36]. Moreover, monitoring antimicrobial resistance of the main mastitis-causing bacteria in dairy cows is important not only for making treatment decisions in the field but also from a public health standpoint [47, 49].

Therefore, the objective of this study was to address the lack of recent data on pathogens in cows with mastitis in South Korea, and to this end, we investigated the prevalence of bacterial pathogens in mastitis milk samples with somatic cell counts (SCCs) ≥ 200,000 cells/mL in South Korea, 2018–2022 and the antimicrobial resistance of *Staphylococcus* spp. isolated from the milk samples. In addition, we detected antimicrobial resistance genes in the MDR staphylococcal isolates found in this study.

MATERIALS AND METHODS

Sampling and somatic cell count analysis

A total of 2,082 quarter milk samples were collected by local dairy farmers from individual quarters of 1,368 lactating cows that were suspected to have clinical or subclinical mastitis from 2018 to 2022 according to the procedure of the National Mastitis Council [21]. In total, 66 dairy farms located in South Korea were involved in this study (Gyeongsang, farms=45, quarter milk samples=962; Gyeonggi, farms=13, quarter milk samples=720; Chungcheong, farms=6, quarter milk samples=216; and Jeolla, farms=2, quarter milk samples=184). The samples were examined at the Mastitis Diagnostic Laboratory in the Animal and Plant Quarantine Agency. The SCCs of all quarter milk samples were tested using a Fossmatic System 400 (Foss Electric, Hillerød, Denmark), according to the manufacturer's instructions. Milk samples with SCCs higher than 200,000 cells/mL were considered indicative of mastitis and used for bacteriological examination [1, 31].

Bacterial isolation and identification

A total of 1,260 mastitis quarter milk samples with SCCs higher than 200,000 cells/mL were tested for the presence of pathogens according to the procedure of the National Mastitis Council [21]. Briefly, 0.01 mL of each milk sample was inoculated on blood agar (Komed, Gyeonggi, South Korea) and incubated at 37°C for 24–48 hr. No growth after 48 hr of incubation was classified as 'no growth'. In cases where two mastitis pathogens were present in one milk sample, each pathogen was isolated and reported as a separate isolate. Milk samples that grew three or more types of colonies were considered contaminated during collection and excluded.

After incubation, colonies of potential mastitis-causing pathogens were isolated. After confirming the pure culture, isolates were identified using MALDI-TOF MS (Biomerieux, Marcy L'Etoile, France), as previously described [23]. The VITEK MS V3.2 library was used, which contains 603 strains and 457 species/subspecies. Briefly, the appropriate number of cells from a pure single colony was directly smeared onto the MALDI target plate. Thereafter, 1 µL of VITEK MS-CHCA matrix was immediately added and each spot was allowed to dry completely. The target slide was run in the VITEK MS instrument according to the instructions provided in the manufacturer's manual. The isolates were stored at −80°C for further analysis.

Antimicrobial resistance testing

Based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, staphylococcal isolates were investigated for antimicrobial susceptibility by the broth microdilution method using Sensititre mastitis plates (CMV1AMAF; Trek Diagnostics, Cleveland, OH, USA) [1]. These plates test ten antimicrobials at the following concentrations (µg/mL): ampicillin (0.12–8), cephalothin (2–16), ceftiofur (1–4), penicillin (0.12–8), penicillin-novobiocin combination (1/2–8/16), pirlimycin (0.5–4), sulfadimethoxine (32–256), erythromycin (0.25–4), oxacillin + 2% NaCl (2–4), and tetracycline (1–8). Plates were read using the Sensititre™ ARIS 2X system (TREK Diagnostic Systems Inc., Westlake, OH, USA). The susceptibility results were interpreted in accordance with the interpretive criteria established by CLSI M100, CLSI VET08, and Saini *et al*. (2012) [12, 13, 41]. *S. aureus* ATCC 25913 was used as the quality control strain. Multidrug resistance was defined as acquired resistance to three or more classes of antibiotics.

Detection of antimicrobial resistance genes

Antimicrobial resistance genes of MDR staphylococcal isolates were analyzed. DNA was extracted through the boiling method, as described previously [6]. MDR staphylococcal isolates were analyzed by PCR to detect genes conferring resistance to lincosamides (*lnuA* and *lnuB*), macrolides (*ermA*, *ermB*, and *ermC*), penicillins (*blaZ* and *mecA*), and tetracyclines (*tetK*, *tetL*, *tetM*, and *tetO*), as described previously [9, 24, 27–29, 37] (Supplementary Table 1). The antimicrobial resistance genes were selected by considering the proportion of isolates that exhibited phenotypic resistance to the tested antimicrobials.

Statistical analysis

Statistical analysis using Pearson's χ^2 test and Fisher's exact test with the Bonferroni correction was performed in Statistical Package for the Social Sciences version 25 (SPSS; IBM, Seoul, South Korea). Significant differences were considered at $P<0.05$.

RESULTS

Prevalence of mastitis-causing pathogens

To determine the prevalence of bacteria causing mastitis in dairy cows, 1,245 pathogens were isolated from 1,260 mastitis quarter milk samples with SCCs higher than 200,000 cells/mL in South Korea. The prevalence of bacterial genera in mastitis quarter milk samples is presented in Table 1. The proportion of *Staphylococcus* spp. (33.9%, 427/1,260) was highest, followed by *Streptococcus* spp. (11.5%, 144/1,260), *Acinetobacter* spp. (5.9%, 74/1,260), *Pseudomonas* spp. (5.5%, 69/1,260), *Enterococcus* spp. (4.9%, 62/1,260), *Escherichia* spp. (4.6%, 58/1,260), *Lactococcus* spp. (3.5%, 44/1,260), and *Corynebacterium* spp. (3.1%, 39/1,260) ($P<0.05$). Some other bacteria were also detected at lower frequencies (<3.0%), such as *Enterobacter* spp., *Aerococcus* spp., *Macrococcus* spp., *Bacillus* spp., *Klebsiella* spp., *Serratia* spp., and *Raoultella* spp. Isolates from 47 mastitis milk samples were not identified. No growth was observed in 129 (10.2%) of the 1,260 mastitis milk samples.

Distribution of Staphylococcus spp.

A total of 435 *Staphylococcus* spp. were isolated from four regions of South Korea. Among them, 48 (11.0%) isolates were *S. aureus* and 387 (89.0%) isolates were NAS, indicating there was a higher prevalence of NAS. The distribution of *Staphylococcus* species in mastitis milk samples is shown in Table 2. *S. chromogenes* (36.3%, 158/435) was most frequently isolated ($P<0.05$) and

Table 1. Prevalence of bacterial genera in bovine mastitis milk samples with somatic cell counts ≥200,000 cells/mL in South Korea, 2018–2022

Genus	No. of pathogen-positive quarter milk samples (%[3])					
	2018	2019	2020	2021	2022	Total
Mastitis quarter milk samples [1]	149	199	538	193	181	1,260
Gram-positive bacteria						
Staphylococcus spp.	29 (19.5) $^{b}_{A}$	87 (43.7) $^{a}_{A}$	183 (34.0) $^{a}_{A}$	69 (35.8) $^{a}_{A}$	59 (32.6) $^{ab}_{A}$	427 (33.9) $_{A}$
Streptococcus spp.	15 (10.1) $_{AB}$	21 (10.6) $_{BC}$	67 (12.5) $_{B}$	23 (11.9) $_{B}$	18 (9.9) $_{BC}$	144 (11.4) $_{B}$
Enterococcus spp.	6 (4.0) $^{ab}_{B}$	4 (2.0) $^{b}_{BC}$	26 (4.8) $^{ab}_{BC}$	10 (5.2) $^{ab}_{BCD}$	16 (8.8) $^{a}_{BC}$	62 (4.9) $_{BC}$
Lactococcus spp.	2 (1.3) $^{ab}_{B}$	0 (0) $^{b}_{C}$	22 (4.1) $^{a}_{BC}$	9 (4.7) $^{a}_{BCD}$	11 (6.1) $^{a}_{BC}$	44 (3.5) $_{BC}$
Corynebacterium spp.	17 (11.4) $^{a}_{AB}$	10 (5.0) $^{ab}_{BC}$	9 (1.7) $^{b}_{BC}$	2 (1.0) $^{b}_{CD}$	1 (0.6) $^{b}_{C}$	39 (3.1) $_{BC}$
Aerococcus spp.	4 (2.7) $^{ab}_{B}$	11 (5.5) $^{a}_{BC}$	14 (2.6) $^{ab}_{BC}$	6 (3.1) $^{ab}_{BCD}$	0 (0) $^{b}_{C}$	35 (2.8) $_{BC}$
Macrococcus spp.	1 (0.7) $_{B}$	0 (0) $_{C}$	18 (3.3) $_{BC}$	2 (1.0) $_{CD}$	7 (3.9) $_{BC}$	28 (2.2) $_{BC}$
Bacillus spp.	2 (1.3) $_{B}$	1 (0.5) $_{BC}$	18 (3.3) $_{BC}$	1 (0.5) $_{CD}$	3 (1.7) $_{BC}$	25 (2.0) $_{BC}$
Others [2]	4 (2.7) $^{ab}_{B}$	2 (1.0) $^{b}_{BC}$	7 (1.3) $^{b}_{C}$	6 (3.1) $^{ab}_{BCD}$	14 (7.7) $^{a}_{BC}$	33 (2.6) $_{BC}$
Gram-negative bacteria						
Acinetobacter spp.	14 (9.4) $^{ab}_{AB}$	4 (2.0) $^{c}_{BC}$	27 (5.0) $^{bc}_{BC}$	6 (3.1) $^{bc}_{BCD}$	23 (12.7) $^{a}_{B}$	74 (5.9) $_{BC}$
Pseudomonas spp.	15 (10.1) $^{a}_{AB}$	1 (0.5) $^{b}_{BC}$	16 (3.0) $^{b}_{BC}$	21 (10.9) $^{a}_{BC}$	16 (8.8) $^{a}_{BC}$	69 (5.5) $_{BC}$
Escherichia spp.	18 (12.1) $^{a}_{AB}$	18 (9.0) $^{ab}_{BC}$	14 (2.6) $^{c}_{BC}$	2 (1.0) $^{c}_{CD}$	6 (3.3) $^{bc}_{BC}$	58 (4.6) $_{BC}$
Enterobacter spp.	8 (5.4) $_{B}$	3 (1.5) $_{BC}$	22 (4.1) $_{BC}$	1 (0.5) $_{CD}$	3 (1.7) $_{BC}$	37 (2.9) $_{BC}$
Klebsiella spp.	3 (2.0) $_{B}$	4 (2.0) $_{BC}$	10 (1.9) $_{BC}$	0 (0) $_{D}$	2 (1.1) $_{C}$	19 (1.5) $_{BC}$
Serratia spp.	1 (0.7) $^{ab}_{B}$	9 (4.5) $^{a}_{BC}$	2 (0.4) $^{b}_{C}$	2 (1.0) $^{ab}_{CD}$	1 (0.6) $^{ab}_{C}$	15 (1.2) $_{C}$
Raoultella spp.	1 (0.7) $_{B}$	1 (0.5) $_{BC}$	9 (1.7) $_{BC}$	0 (0) $_{D}$	2 (1.1) $_{C}$	13 (1.0) $_{C}$
Others [2]	5 (3.4) $_{B}$	13 (6.5) $_{BC}$	23 (4.3) $_{BC}$	16 (8.3) $_{BCD}$	8 (4.4) $_{BC}$	65 (5.2) $_{BC}$
Unidentified	2 (1.3)	18 (9.0)	18 (3.3)	6 (3.1)	3 (1.7)	47 (3.7)
Total isolates	149	212	508	182	194	1,245
No growth	44	32	35	11	7	129

Values with different superscript letters ($^{a–c}$) represent significant differences in the number of quarter milk samples for each pathogen, while values with different subscript letters ($_{A–C}$) represent significant differences in the number of quarter milk samples among pathogens ($P<0.05$). [1] Quarter milk samples containing more than 200,000 cells/mL (somatic cell count). [2] Other pathogens were isolated in less than 1% of samples. [3] Percentages were calculated according to the number of mastitis quarter milk samples in each year.

Table 2. Distribution of *Staphylococcus* species in bovine mastitis milk samples with somatic cell counts ≥200,000 cells/mL in South Korea, 2018–2022

Species		No. of isolates (% [1])					
		2018	2019	2020	2021	2022	Total
S. aureus		0 (0) $^{b}_{B}$	13 (14.4) $^{ab}_{B}$	13 (7.0) $^{b}_{BC}$	19 (27.5) $^{a}_{A}$	3 (5.1) $^{b}_{B}$	48 (11.0) $_{B}$
Non-aureus staphylococci	*S. chromogenes*	17 (54.8) $_{A}$	43 (47.8) $_{A}$	57 (30.6) $_{A}$	20 (29.0) $_{A}$	21 (35.6) $_{A}$	158 (36.3) $_{A}$
	S. haemolyticus	3 (9.7) $_{B}$	7 (7.8) $_{BC}$	19 (10.2) $_{BC}$	7 (10.1) $_{AB}$	5 (8.5) $_{B}$	41 (9.4) $_{BC}$
	S. sciuri	2 (6.5) $_{B}$	3 (3.3) $_{BC}$	22 (11.8) $_{B}$	2 (2.9) $_{B}$	3 (5.1) $_{B}$	32 (7.4) $_{BC}$
	S. xylosus	4 (12.9) $_{B}$	6 (6.7) $_{BC}$	15 (8.1) $_{BC}$	2 (2.9) $_{B}$	5 (8.5) $_{B}$	32 (7.4) $_{BC}$
	S. epidermidis	1 (3.2) $_{B}$	10 (11.1) $_{BC}$	9 (4.8) $_{BC}$	3 (4.3) $_{B}$	8 (13.6) $_{AB}$	31 (7.1) $_{BC}$
	S. simulans	1 (3.2) $_{B}$	5 (5.6) $_{BC}$	12 (6.5) $_{BC}$	9 (13.0) $_{AB}$	4 (6.8) $_{B}$	31 (7.1) $_{BC}$
	S. saprophyticus	1 (3.2) $_{B}$	0 (0) $_{C}$	14 (7.5) $_{BC}$	0 (0) $_{B}$	1 (1.7) $_{B}$	16 (3.7) $_{BC}$
	S. equorum	1 (3.2) $_{B}$	2 (2.2) $_{BC}$	4 (2.2) $_{BC}$	1 (1.4) $_{B}$	1 (1.7) $_{B}$	9 (2.1) $_{BC}$
	S. cohnii	0 (0) $_{B}$	0 (0) $_{C}$	6 (3.2) $_{BC}$	0 (0) $_{B}$	0 (0) $_{B}$	6 (1.4) $_{BC}$
	S. delphini	0 (0) $_{B}$	0 (0) $_{C}$	1 (0.5) $_{BC}$	4 (5.8) $_{B}$	1 (1.7) $_{B}$	6 (1.4) $_{BC}$
	S. hominis	0 (0) $_{B}$	1 (1.1) $_{C}$	1 (0.5) $_{BC}$	1 (1.4) $_{B}$	3 (5.1) $_{B}$	6 (1.4) $_{BC}$
	S. succinus	0 (0) $_{B}$	0 (0) $_{C}$	4 (2.2) $_{BC}$	0 (0) $_{B}$	0 (0) $_{B}$	4 (0.9) $_{BC}$
	S. hyicus	1 (3.2) $_{B}$	0 (0) $_{C}$	1 (0.5) $_{BC}$	0 (0) $_{B}$	1 (1.7) $_{B}$	3 (0.7) $_{BC}$
	S. kloosii	0 (0) $_{B}$	0 (0) $_{C}$	1 (0.5) $_{BC}$	0 (0) $_{B}$	1 (1.7) $_{B}$	2 (0.5) $_{BC}$
	S. muscae	0 (0) $_{B}$	0 (0) $_{C}$	2 (1.1) $_{BC}$	0 (0) $_{B}$	0 (0) $_{B}$	2 (0.5) $_{BC}$
	S. nepalensis	0 (0) $_{B}$	0 (0) $_{C}$	2 (1.1) $_{BC}$	0 (0) $_{B}$	0 (0) $_{B}$	2 (0.5) $_{BC}$
	S. capitis	0 (0) $_{B}$	0 (0) $_{C}$	0 (0) $_{C}$	1 (1.4) $_{B}$	0 (0) $_{B}$	1 (0.2) $_{C}$
	S. carnosus	0 (0) $_{B}$	0 (0) $_{C}$	1 (0.5) $_{BC}$	0 (0) $_{B}$	0 (0) $_{B}$	1 (0.2) $_{C}$
	S. infantanus	0 (0) $_{B}$	0 (0) $_{C}$	0 (0) $_{C}$	0 (0) $_{B}$	1 (1.7) $_{B}$	1 (0.2) $_{C}$
	S. lentus	0 (0) $_{B}$	0 (0) $_{C}$	1 (0.5) $_{BC}$	0 (0) $_{B}$	0 (0) $_{B}$	1 (0.2) $_{C}$
	S. pasteuri	0 (0) $_{B}$	0 (0) $_{C}$	1 (0.5) $_{BC}$	0 (0) $_{B}$	0 (0) $_{B}$	1 (0.2) $_{C}$
	S. warneri	0 (0) $_{B}$	0 (0) $_{C}$	0 (0) $_{C}$	0 (0) $_{B}$	1 (1.7) $_{B}$	1 (0.2) $_{C}$
	Total	31 (100) ^{a}	77 (85.6) ^{ab}	173 (93.0) ^{a}	50 (72.5) ^{b}	56 (94.9) ^{a}	387 (89.0)
Total		31 (100)	90 (100)	186 (100)	69 (100)	59 (100)	435 (100)

Values with different superscript letters ($^{a–c}$) represent significant differences in the number of isolates for each *Staphylococcus* spp., while values with different subscript letters ($_{A–C}$) represent significant differences in the number of isolates among *Staphylococcus* spp. ($P<0.05$). [1] Percentages were calculated according to the total number of staphylococcal isolates in each year.

S. aureus (11.0%, 48/435) was the second most prevalent, followed by *S. haemolyticus* (9.4%, 41/435), *S. sciuri* (7.4%, 32/435), *S. xylosus* (7.4%, 32/435), *S. epidermidis* (7.1%, 31/435), *S. simulans* (7.1%, 31/435), and *S. saprophyticus* (3.7%, 16/435). Some other species were identified at lower frequencies (<3.0%), such as *S. equorum*, *S. cohnii*, *S. delphini*, *S. hominis*, *S. succinus*, *S. hyicus*, *S. kloosii*, *S. muscae*, *S. nepalensis*, *S. capitis*, *S. carnosus*, *S. infantanus*, *S. lentus*, *S. pasteuri*, and *S. warneri*. The isolation rate of NAS was consistently high (72.5–100%) throughout the study period and was lowest (72.5%) in 2021 ($P<0.05$). The isolation rate of *S. aureus* was highest in 2021 (27.5%) and lower in 2018 (0%), 2022 (5.1%), and 2020 (7.0%) ($P<0.05$).

Antimicrobial resistance and multidrug resistance of Staphylococcus spp.

To investigate the antimicrobial resistance of staphylococci isolated from milk samples of cows with mastitis, 435 staphylococcal isolates were tested with 10 antimicrobials of seven antimicrobial classes (Table 3). The highest percentages of *S. aureus* isolates were resistant to penicillin (25.0%, 12/48) and ampicillin (20.8%, 10/48). On the other hand, they exhibited low resistance to sulphadimethoxine (6.3%, 3/48), ceftiofur (2.1%, 1/48), cephalothin (2.1%, 1/48), and oxacillin + 2% NaCl (2.1%, 1/48), and 100% susceptibility to pirlimycin, erythromycin, penicillin/novobiocin, and tetracycline. NAS isolates showed the highest resistance rate to penicillin (18.3%, 71/378), followed by tetracycline (11.4%, 44/378), erythromycin (10.1%, 39/378), ampicillin (9.3%, 36/378), and sulphadimethoxine (9.3%, 36/378) ($P<0.05$). By contrast, the rates of resistance to cephalothin, penicillin/novobiocin, ceftiofur, and pirlimycin were low (0.5–4.9%, 2/378–19/378). NAS showed higher rates of resistance to erythromycin and tetracycline, and a lower rate of resistance to ampicillin, than *S. aureus* ($P<0.05$).

The rates of antimicrobial resistance varied between NAS species. *S. saprophyticus* showed higher resistance rates to tetracycline (68.8%, 11/16) and penicillin (62.5%, 10/16) than to the other antibiotics tested. *S. epidermidis* showed higher resistance rates to erythromycin (45.2%, 14/31) and penicillin (45.2%, 14/31) than to other antibiotics tested. *S. haemolyticus* showed higher resistance rates to penicillin (51.2%, 21/41) and sulphadimethoxine (29.3%, 12/41) than to the other antibiotics tested.

The distribution of MDR isolates among *Staphylococcus* spp. is shown in Table 4. In total, 16 MDR *Staphylococcus* isolates were detected. These all belonged to the NAS group, and there were no MDR *S. aureus* isolates. The proportion of MDR isolates was 37.5% (6/16) for *S. saprophyticus*, 16.7% (1/6) for *S. hominis*, 12.9% (4/31) for *S. epidermidis*, 11.1% (1/9) for *S. equorum*, 4.9% (2/41) for *S. haemolyticus*, 3.1% (1/32) for *S. sciuri*, and 0.6% (1/158) for *S. chromogenes*.

Table 3. Antimicrobial resistance of *Staphylococcus* spp. isolates from mastitis quarter milk samples with somatic cell counts ≥200,000 cells/mL in South Korea, 2018–2022

Antimicrobial	Breakpoint [1] (μg/mL)	No. of resistant isolates (% [5])									
			Non-aureus staphylococci								
		S. aureus (n=48)	*S. chromogenes* (n=158)	*S. haemolyticus* (n=41)	*S. sciuri* (n=32)	*S. xylosus* (n=32)	*S. epidermidis* (n=31)	*S. simulans* (n=31)	*S. saprophyticus* (n=16)	Others [6](n=46)	Total (n=387)
Cephems											
Cephalothin	≥ 32 [2]	**1 (2.1)** $_{B}$	0 (0) $_{C}$	1 (2.4) $_{CD}$	0 (0) $_{B}$	0 (0) $_{B}$	0 (0) $_{B}$	1 (3.2)	0 (0) $_{B}$	0 (0) $_{B}$	**2 (0.5)** $_{E}$
Ceftiofur	≥ 8 [4]	**1 (2.1)** $_{B}$	1 (0.6) $_{BC}$	2 (4.9) $_{BCD}$	5 (15.6) $_{AB}$	0 (0) $_{B}$	0 (0) $_{B}$	0 (0)	6 (37.5) $_{AB}$	0 (0) $_{B}$	**14 (3.6)** $_{DE}$
Sulfonamides											
Sulphadimethoxine	≥ 512 [3]	**3 (6.3)** $_{AB}$	9 (5.7) $_{AB}$	12 (29.3) $_{AB}$	0 (0) $_{B}$	0 (0) $_{B}$	5 (16.1) $_{AB}$	3 (9.7)	0 (0) $_{B}$	7 (15.2) $_{AB}$	**36 (9.3)** $_{BC}$
Lincosamides											
Pirlimycin	≥ 4 [4]	**0 (0)** $_{B}$	3 (1.9) $_{ABC}$	0 (0) $_{D}$	2 (6.3) $_{B}$	2 (6.3) $_{AB}$	1 (3.2) $_{B}$	1 (3.2)	4 (25.0) $_{AB}$	6 (13.0) $_{AB}$	**19 (4.9)** $_{CD}$
Macrolides											
Erythromycin	≥ 8 [3]	**0 (0)** $^{b}_{B}$	1 (0.6) $_{BC}$	1 (2.4) $_{CD}$	1 (3.1) $_{B}$	6 (18.8) $_{AB}$	14 (45.2) $_{A}$	0 (0)	4 (25.0) $_{AB}$	12 (26.1) $_{A}$	**39 (10.1)** $^{a}_{BC}$
Penicillins											
Ampicillin	≥ 0.5 [2]	**10 (20.8)** $^{a}_{A}$	8 (5.1) $_{ABC}$	8 (19.5) $_{BC}$	5 (15.6) $_{AB}$	0 (0) $_{B}$	6 (19.4) $_{AB}$	0 (0)	6 (37.5) $_{AB}$	3 (6.5) $_{AB}$	**36 (9.3)** $^{b}_{BC}$
Penicillin	≥ 0.25 [3]	**12 (25.0)** $_{A}$	11 (7.0) $_{A}$	21 (51.2) $_{A}$	6 (18.8) $_{AB}$	0 (0) $_{B}$	14 (45.2) $_{A}$	1 (3.2)	10 (62.5) $_{A}$	8 (17.4) $_{AB}$	**71 (18.3)** $_{A}$
Oxacillin + 2% NaCl	*S. aureus* ≥ 4 [3]	**1 (2.1)** $_{B}$	–	–	–	–	–	–	–	–	–
Penicillins/ Aminocoumarin											
Penicillin/Novobiocin	≥ 4/8 [4]	**0 (0)** $_{B}$	1 (0.6) $_{BC}$	0 (0) $_{D}$	0 (0) $_{B}$	0 (0) $_{B}$	0 (0) $_{B}$	0 (0)	4 (25.0) $_{AB}$	0 (0) $_{B}$	**5 (1.3)** $_{DE}$
Tetracylines											
Tetracycline	≥16 [3]	**0 (0)** $^{b}_{B}$	1 (0.6) $_{BC}$	1 (2.4) $_{CD}$	12 (37.5) $_{A}$	7 (21.9) $_{A}$	4 (12.9) $_{AB}$	0 (0)	11 (68.8) $_{A}$	8 (17.4) $_{AB}$	**44 (11.4)** $^{a}_{AB}$

Values with different superscript letters ($^{a–b}$) represent significant differences in resistance rates of *S. aureus* and non-aureus staphylococci, while values with different subscript letters ($_{A–E}$) represent significant differences in resistance rates to each antimicrobial (P<0.05). [1] The minimum inhibitory concentration at which a strain is considered susceptible based on the CLSI guideline. [2] Resistance breakpoints based on Saini *et al*. (2012). [3] Resistance breakpoints based on Clinical and Laboratory Standards Institute M100 (CLSI, 2020). [4] Resistance breakpoints based on Clinical and Laboratory Standards Institute VET08 (CLSI, 2018). [5] Percentages were calculated according to the number of staphylococcal isolates of each species. [6] Other *Staphylococcus* spp. comprised fewer than 10 strains.

Table 4. Phenotypic and genotypic resistance profiles of multidrug-resistant *Staphylococcus* isolates (n=16) from mastitis quarter milk samples with somatic cell counts ≥200,000 cells/mL in South Korea, 2018–2022

Species	No. of multidrug-resistant isolates [1] (%)	Strain	Isolation year	Resistance phenotype	Resistance genotype
S. aureus	0 (0) ^{c}	–	–	–	–
NAS *S. saprophyticus*	6 (37.5) ^{a}	M-20-BK-BYH3	2020	AMP/ PEN + PN + XNL + ERY + PIRL + TET	*mecA* + *ermC* + *lnuB* + *tetK*
		M-20-ER-BCH3	2020	AMP/ PEN + PN + XNL + ERY + PIRL + TET	*mecA* + *ermC* + *tetK*
		M-20-EV-BJS2	2020	AMP/ PEN + PN + XNL + TET	*mecA* + *lnuA* + *tetK*
		M-20-DZ-BCH6	2020	AMP/ PEN + XNL + ERY + PIRL + TET	*ermC* + *lnuB* + *tetK*
		M-20-FZ-BDH5	2020	AMP/ PEN + XNL + TET	*mecA* + *lnuA* + *tetK*
		M-22-R-CH01	2022	XNL + ERY + PEN + PN + PIRL	*blaZ*/*mecA* + *ermC*
S. hominis	1 (16.7) ^{b}	M-22-AU-GC04	2022	ERY + PEN + SDM + TET	*blaZ*/*mecA* + *lnuA* + *tetK*
S. epidermidis	4 (12.9) ^{b}	M-20-C-BKYH4	2020	AMP/PEN + ERY + TET	*blaZ* + *ermC* + *lnuA* + *tetK*
		M-19-W-BUN43	2019	AMP/PEN + ERY + TET`	*blaZ* + *ermB*/*ermC* + *tetK*
		M-22-BC-SYS17	2022	ERY + PEN + SDM	*blaZ*/*mecA*
		M-22-AL-GC02	2022	ERY + PEN + SDM	*blaZ*/*mecA* + *lnuA* + *tetK*
S. equorum	1 (11.1) ^{bc}	M-18-J-BYM-12-1	2018	PEN + SDM + PIRL + TET	*mecA* + *lnuA* + *tetK*
S. haemolyticus	2 (4.9) ^{bc}	M-20-EE-BJY3	2020	AMP/OXA/PEN + CEP/XNL + ERY + SDM	*blaZ*/*mecA* + *ermC* + *tetL*
		M-21-C-BCH1	2021	PEN + XNL + SDM	*blaZ*
S. sciuri	1 (3.1) ^{bc}	M-20-I-BSY2-1	2020	ERY + PIRL + TET	*mecA* + *ermC* + *lnuA*
S. chromogenes	1 (0.6) ^{c}	M-20-AV-BSY13	2020	AMP/ PEN + ERY + SDM + PIRL + TET	*mecA*

Values with different superscript letters ($^{a–c}$) represent significant differences in the multidrug resistance rate among *Staphylococcus* spp. (P<0.05). NAS, non-aureus staphylococci; AMP, ampicillin; CEP, cephalothin; ERY, erythromycin; PN, penicillin/novobiocin; PEN, penicillin; PIRL, pirlimycin; SDM, sulphadimethoxine; TET, tetracycline; XNL, ceftiofur. [1] Multidrug-resistant to three or more antimicrobial classes.

Phenotypic and genotypic antimicrobial resistance of MDR isolates

To determine the genotypic antimicrobial resistance of MDR staphylococci, the 16 MDR isolates were analyzed for the presence of 11 antimicrobial resistance genes of four antibiotic classes. *mecA*, which was carried by 75% (12/16) of the 16 MDR isolates, was most frequently detected, followed by *tetK* (62.5%, 10/16), *blaZ* (50.0%, 8/16), *ermC* (50.0%, 8/16), *lnuA* (43.8%, 7/16), *lnuB* (12.5%, 2/16), *ermB* (6.3%, 1/16), and *tetL* (6.3%, 1/16). None of the MDR isolates were positive for *ermA*, *tetM*, and *tetO*. The most frequent gene combination pattern was *mecA* + *lnuA* + *tetK* (18.8%, 3/16).

The phenotypic and genotypic resistance profiles of the 16 MDR isolates are shown in Table 4. The proportion of isolates with phenotypic resistance did not correspond to the proportion of isolates carrying the tested resistance genes. Of the 15 penicillin-resistant isolates, *blaZ* and *mecA* were identified in 8 and 11, respectively. Five of these isolates contained both genes, while one penicillin-resistant isolate was negative for both *blaZ* and *mecA*. One penicillin-susceptible isolate was positive for *mecA*. Among the 12 erythromycin-resistant isolates, *ermB* and *ermC* were detected in one and eight, respectively. Four erythromycin-resistant isolates were negative for *ermA*, *ermB* and *ermC*. Of the 11 tetracycline-resistant isolates, nine carried *tetK*. Two tetracycline-resistant isolates were negative for *tetK*, *tetL*, *tetM*, and *tetO*, while two tetracycline-susceptible isolates were positive for *tetK* and *tetL*, respectively. Among the seven pirlimycin-resistant isolates, *lnuA* and *lnuB* were present in two and two isolates, respectively. Three pirlimycin-resistant isolates were negative for *lnuA* and *lnuB*, while five pirlimycin-susceptible isolates were positive for *lnuA*.

DISCUSSION

In this study, the most frequent bacterial isolates detected in mastitis quarter milk samples were *Staphylococcus* spp. Our result agree with those of previous reports from China, Germany, and Sweden [15, 43, 45]. Meanwhile, among the staphylococcal isolates, *S. aureus* accounted for only 11.0%, while NAS accounted for 89.0%, with *S. chromogenes* being the most prevalent staphylococcal isolate in this study. These results are similar to those reported in Missouri, USA [20]. However, our results differ from those of studies conducted in China, Sweden, and India, where *S. aureus* was found to be the predominant staphylococcal isolate in mastitis milk [5, 15, 45]. This difference may be because the distribution of mastitis-causing bacteria is influenced by mastitis management and topographical factors [3, 39]. A national mastitis control program has been implemented since the late 1990s in South Korea. This program aims to control bovine mastitis and mitigate the potential issue of antimicrobial resistance [36]. While traditional mastitis control methods have successfully decreased the occurrence of contagious mastitis-causing pathogens including *S. aureus*, they are ineffective in controlling environmental pathogens [40]. *S. chromogenes* primarily infects the cow's udder before and around the first calving, and exhibits a higher virulence tendency than other NAS, leading to an increased inflammatory capacity and a prolonged duration of intramammary infection [11, 34]. Hence, it is essential to establish a strategy to prevent mastitis caused by NAS, particularly *S. chromogenes*, in dairy farms.

In South Korea, the amount of antibiotics sold for cattle has been high, reaching about 90 tons per year, since 2018, although it is lower than the amount of antibiotics sold for pigs and chickens [4]. The primary method to treat mastitis in dairy cows is administration of antibiotics, making mastitis the leading cause of antibiotic administration in dairy cows [26, 44]. Recent antimicrobial susceptibility data assist veterinarians in selecting the appropriate antibiotic to treat mastitis, which is critical because mastitis is primarily treated empirically [14]. In our study, *S. aureus* exhibited the highest resistance rates to ampicillin and penicillin, but showed low resistance rates to the other tested antibiotics. These results are similar to those of previous studies in North America, nine EU countries, and South Korea [14, 31, 46]. Although caution should be exercised when comparing antibiotic resistance rates with those reported in previous studies due to differences in sampling schedules, methodologies, and antibiotic interpretation criteria [14], resistance rates to ampicillin and penicillin were lower in our study (20.8% and 25.0%, respectively) than in previous research conducted in South Korea. Moon *et al*. (2007) reported resistance rates to ampicillin and penicillin of 72.4% and 73.3%, respectively, for *S. aureus* isolated from 1997 to 2004 [33]. Nam *et al*. (2011) reported a penicillin resistance rate of 66.4% for *S. aureus* isolated from 2003 to 2009 [35]. Mechesso *et al*. (2021) reported resistance rates to ampicillin and penicillin of 51.8% and 54.8%, respectively, for *S. aureus* isolated from 2014 to 2018 [31]. The lower ampicillin and penicillin resistance rates in our study than in other studies could be due to the decreasing rate of bovine mastitis in dairy farms, which would reduce the need for antimicrobials and thus decrease antimicrobial resistance rates [23]. Differences in antibiotic susceptibility testing methods might also explain the discrepancy. Previous studies used the disc diffusion method, whereas the current study used the MIC method. The disc diffusion method may overestimate antimicrobial resistance in *S. aureus* [32].

In this study, the high resistance rates of NAS to penicillin, tetracycline, and erythromycin may be because penicillins, tetracyclines, and macrolides are the classes of antibiotics most commonly sold to Korean livestock farms [4]. Penicillins, tetracyclines, and macrolides are frequently used to prevent and treat diseases in dairy cows [23, 49]. A previous study reported similar antibiotic resistance rates in South Korea [23]. Collectively, these results show that the rates of antibiotic resistance among NAS and *S. aureus* are similar to or lower than those reported previously in South Korea. Therefore, continued vigilance and monitoring are needed to ensure that this remains the case.

Monitoring antimicrobial resistance on farms is crucial because resistance genes can be transferred between bacteria of diverse taxonomic and ecological groups through mobile genetic elements, such as phages, plasmids, naked DNA, and transposons [26, 38]. In our study, beta-lactam resistance genes (*mecA* and *blaZ*) were most commonly detected in MDR staphylococcal isolates. Similar results were reported in Egypt and Kenya [2, 30]. *tetK*, which is found in staphylococcal bacteria that cause bovine mastitis, is frequently identified and is responsible for producing efflux proteins associated with the cell membrane [48]. The primary cause of macrolide-lincosamide resistance in staphylococci is dimethylation of an adenine residue in 23S rRNA [25, 48]. Among the *erm* genes,

ermC is commonly found in bovine mastitis caused by NAS [25, 49]. Likewise, *tetK* and *ermC* were prevalent in MDR isolates in our study. The high detection rates of these antibiotic resistance genes in MDR isolates are predicted to correlate with high resistance to tetracyclines, ampicillin, penicillin, and erythromycin. Many MDR staphylococci have been isolated from milk samples and humans working in dairy farms, and there is a risk of transmitting them to humans through the food chain and livestock [7]. To investigate the public health implications of mastitis-causing bacteria with multiple antimicrobial resistance genes, future studies need to examine whether mobile genetic elements containing these antimicrobial resistance genes can be transferred to human-derived bacteria.

On the other hand, some of the examined antimicrobial resistance genes (*mecA*, *tetK*, *tetL*, and *lnuA*) were present in some of the phenotypically susceptible isolates in this study. Additionally, antimicrobial resistance genes were not detected in some phenotypically resistant isolates. Similar results were reported in previous studies from China, Poland, and Kenya [18, 25, 30]. The absence of resistance genes in isolates with phenotypic resistance may be because the isolates have non-specific resistance to these agents or because not all resistance genes associated with this phenotype were tested [50]. The observed discrepancy between genotype and phenotype could potentially be attributed to the lack of activation of resistance genes in specific isolates [19, 49]. Furthermore, aside from the commonly observed resistance mechanisms, an efflux pump may serve as the primary mechanism underlying resistance [18, 49]. The mechanisms underlying antibacterial resistance are highly intricate [19]. Therefore, further studies, such as whole-genome sequencing, are needed to understand fully the genetic basis of antibacterial resistance [30].

Our study revealed that various bacteria are associated with mastitis, with *Staphylococcus* spp., especially NAS, the most predominant in dairy cows in South Korea. *S. aureus* isolates from milk with mastitis had low levels of resistance to antibiotics commonly used to treat mastitis, except penicillins. NAS showed the highest resistance rates to penicillin, erythromycin, and tetracycline. MDR *Staphylococcus* isolates carried a variety of antibiotic resistance genes. *Staphylococcus* is of great concern in bovine mastitis infections; therefore, the results of the present study could provide a valuable resource for selecting appropriate antibiotics to effectively treat staphylococcal infections. Continuous monitoring of the distribution and antimicrobial resistance of the main pathogens in mastitis milk is necessary to improve the effectiveness of treatment strategies in dairy farms.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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