Title: "Virulence and intermediate resistance to high-end antibiotic (teicoplanin) among coagulase-negative staphylococci sourced from retail market fish"

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Declarations

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Conflict of Interest

Authors declare no conflict of interests

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Author's contribution

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Abstract

This study reports the distribution of enterotoxigenic determinants among staphylococci and the susceptibility of staphylococci to various classes of antibiotics. We observed all the isolates as resistant to beta-lactam antibiotics and a few as resistant to non-beta-lactam antibiotics such as clindamycin (47.4%), erythromycin (44.7%), gentamicin (23.7%), norfloxacin (34.2%), tetracycline (26.3%), trimethoprim-sulfamethoxazole (15.8%) etc. The resistance of *S. sciuri* (n=1) and *S. haemolyticus* (n=1) to rifampicin and intermediate resistance of *S. gallinarum* (n=2) to teicoplanin, a high-end antibiotic, are also observed in this study. The multidrug-resistance (≥ 3 classes of antibiotics) was recorded in 23 (60.5%) isolates. The virulomes such as *sea*, *seb*, *seg* and *sei* were identified predominantly in *S. haemolyticus*. Surprisingly, certain isolates which were phenotypically confirmed as biofilm-producers by Congo red agar (CRA) test did not harbour biofilm-associated loci. This implies the protein-mediated mechanism of biofilm formation as an alternative to polysaccharide intercellular adhesin (PIA) in staphylococci. However, *icaAD* locus which encodes PIA was identified in 10 (26.3%) isolates and the *eno* locus, encoding elastin-binding protein which can accelerate the biofilm production, is identified in all the isolates. The possession of type V SCC*mec* elements by the *S. haemolyticus* (15.8%) raised the concern about the rapid dissemination of *mecA* gene to other species of staphylococci including the virulent *S. aureus*. In short, this study acknowledges the toxigenicity of coagulase-negative staphylococci (CoNS). Through this study, surveillance of antimicrobial resistance and transference of virulomes in staphylococci is warranted.

Keywords: Coagulase-negative staphylococci, virulence genes, *mecA* gene, type V SCC*mec* elements, enterotoxins.

1. INTRODUCTION

The coagulase–negative staphylococci (CoNS), versatile, heterogenous Gram-positive cocci, are characterized by lack of coagulase enzyme which helps to evade the host immune system. Besides, staphylococcal enterotoxin (SEs), exfoliative toxins, toxic shock syndrome toxins (TSST), and Panton-Valentine luecocidin (PVL) toxin can also determine the virulence of staphylococci. In general, staphylococci are salt-tolerant but, certain strain of staphylococci which has undergone autolysis even at the lower salt concentration has also been reported [1]. Also, high sodium chloride concentration can select the staphylococci harbouring *mecA* gene. Apart from this, given that the CoNS are less aggressive, they can cause severe clinical complications particularly in immunocompromised patients [2]. In Europe, detection of CoNS in food is not advised or rather coagulase-positive staphylococcal detection is recommended. Perhaps, this is because CoNS are commonly found in foods [3]. However, this theory was dismissed as a mere speculation when the CoNS recognized to play a major role in the epidemiology of foodborne diseases. The toxigenic potential of CoNS has been demonstrated by several studies that documented CoNS carrying extended virulomes [3-5]. Moreover, the pathogenicity is also determined by the ability of CoNS to produce biofilm which helps to colonize in foreign materials including medical devices [6]. The infections associated with indwelling medical devices by CoNS may also be attributed to the biofilm production. Additionally, biofilm offers the resistance to antibiotics and environmental stress. Moreover, CoNS carrying SEs which are often associated with foodborne diseases [4]. In addition to this, methicillin-resistant CoNS (MRCoNS), being the repository of *mecA* gene for the highly virulent *Staphylococcus aureus*, can contribute to the emergence of methicillin-resistant *S. aureus* (MRSA) [7]. The outstanding ability of staphylococci to transfer the methicillin-resistance determinant from one species to another has been elucidated by *in vitro* conjugation studies [8-9]. Recently, a study that investigated the MRSA colonization in HIV-infected patients revealed the role of MRCoNS as a protective factor of MRSA [10]. The study also observed a significant similarity in the resistance profiles of MRSA and MRCoNS that may reflect on the MRCoNS as a potential donor of resistance genes. At this point, the MRCoNS equipped with virulence factors gradually achieved research interests over MRSA. Furthermore, the occurrence of staphylococci has been reported in healthy individuals and animals, aquaculture, animal husbandry, food industry and many other settings, pointing at the host non-specificity [11-15]. Generally, CoNS is not an inhabitant of aquatic fishes and its occurrence represents the

post-harvest contamination [12]. Undoubtedly, food fish contamination can occur at any stages of harvesting and processing which is majorly driven by the improper methods of handling and poor personal hygiene [16]. In the light of this, a surveillance study was conducted at the retail markets of Assam, India with molecular characterization and pathogenesis of MRCoNS as the major objectives.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

A total of 38 MRCoNS isolated from 173 fish samples sold at the retails markets in Assam, India were used in this study. To enrich the CoNS cultures, 25 grams of each fish sample was aseptically transferred to 225 mL of tryptic soy broth (TSB) supplemented with 10% sodium chloride and 1% sodium pyruvate and then seeded on mannitol salt agar (MSA) plate. For MRCoNS monitoring, the suspected CoNS colonies (n=5/sample) from MSA plates with the colony characteristics (pink colour), were further spot inoculated (10 µL) on Mueller-Hinton Agar (MHA) plates containing 6 µg/mL oxacillin [17].

2.2 Automated bacterial identification and antimicrobial susceptibility testing

The automated BD Phoenix M50™ (BD Diagnostics, USA) system was used to identify the isolates and to detect their resistance pattern to 20 antibiotics representing 13 classes [18]. Briefly, identification broth (ID broth) was inoculated with pure staphylococcal colonies until 0.5 McFarland value is achieved. Then, 25 µL of turbidity-adjusted-bacterial-suspension was transferred to antimicrobial susceptibility testing (AST) broth to which added a full drop of AST indicator. The BD phoenix PMIC/ID-70 is a combo panel that can be used for the bacterial identification as well as the AST. The ID broth and AST broth containing cultures were poured through fill port to identification and AST sides of the combo panel respectively. The panel, after sealing the fill port, is loaded to the BD instrument which maintains a 35°C temperature. The BDXpert™ system facilitates the interpretation of the MIC values and predicts resistance status of the isolates. Using this instrument, the resistance to antibiotics such as ampicillin (AMP), cefazolin (CFZ), cefoxitin (FOX) clindamycin (CLI), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), levofloxacin (LVX), linezolid (LZD), moxifloxacin (MXF), nitrofurantoin (NIT), norfloxacin (NOR), oxacillin (OXA), penicillin (PEN), quinupristin-dalfopristin (Q-D), rifampicin (RIF), teicoplanin (TEC), tetracycline (TET), trimethoprim-sulfamethoxazole (SXT) and

vancomycin (VAN) were tested. The isolates were interpreted as resistant, intermediate and sensitive based on the MIC value [17].

2.3 Molecular detection of genes associated with virulence and biofilm production

The list of primers used in this study is given in supplementary material 1. The DNA was isolated from overnight bacterial cultures using DNeasy Blood & Tissue Kits (Qiagen) according to user's manual. Two microgram of the DNA was used for each 25 µL PCR reaction mixture containing 1X REDTaq® ReadyMix™ (Sigma) and the primers of required concentration. Three multiplex PCRs were performed for the detection of SEs: *sea-sed*, *seg-sei*, *eta*, *etb* and *tsst-1* [19]. The isolates which retrospectively identified as biofilm-producers (black colonies on Congo red agar (CRA) plates), were genotypically assessed for the biofilm-associated genes. The method developed by Bose et al, 2009 was employed for the PCR detection of *clfA*, *fib* and *fnbpA* [20]. Similarly, uniplex PCRs were performed for the detection of *icaA*, *icaD*, *icaB*, *icaC* genes housed in the *icaADBC* operon as well as the *clfB, fnbpB, epbs, eno and sdrCDE* genes [21-24].

2.4 PCR detection of methicillin-resistance determinant and SCC*mec* typing

A uniplex PCR was employed for the detection of *mecA* gene; methicillin-resistance determinant [25]. Besides, multiplex PCR was performed for the SCC*mec* typing [26]. SCC*mec* types of the studied isolates were defined based on the PCR amplification of *mec* gene complex class and *ccr* gene allotypes.

3. RESULTS

3.1 Diversity of staphylococcal species

In this study, we report only MRCoNS (n=38) excluding the methicillin-sensitive staphylococcal isolates. Among MRCoNS, *S. haemolyticus* (21/38, 55.3%) were the predominant species followed by *S. sciuri* (9/38, 23.7%), *S. gallinarum* (4/38, 10.5%), *S. lentus* (2/38, 5.3%), *S. saprophyticus* (1/38, 2.6%) and *S. xylosus* (1/38, 2.6%). These isolates were recovered from the fresh water food fishes such as *Hypopthalmichthys molitrix* (silver carp), *Labeo rohita* (rohu), *Pygocentrus nattereri* (red belly piranha), *L. catla* (catla) etc. and some indigenous varieties such as *Mystus tengara* (singara), *Heteropneustes fossilis* (singhi), *Anabas testudineus* (kowoi), *Sperata seenghala* (aree) etc. which are very common at the retails markets of Assam, India.

3.2 Susceptibility patterns of MRCoNS

Percentage of the isolates resistant to different classes of antibiotics is given in Fig 1. All the isolates (100%) studied were completely resistant to ampicillin, cefoxitin, cefazolin, oxacillin and penicillin (beta-lactam antibiotics). Similarly, 18 isolates (47.4%) exhibited resistance to clindamycin whereas 6 isolates (15.6%) showed intermediate resistance. The complete resistance and intermediate resistance to gentamicin was recorded in 9 (23.7%) and 7 (18.4%) isolates respectively. Besides, 17 isolates (44.7%) were resistant to erythromycin. The minimum inhibitory concentration (MIC) of levofloxacin for 13 isolates (34.2%) comprising *S. haemolyticus* (n=12) and *S. sciuri* (n=1) was ≥2 µg/mL and noted as intermediate resistant and a complete resistance to norfloxacin was observed in same panel of isolates. The isolates of *S. haemolyticus* (n=1) and *S. sciuri* (n=1) were noteworthy for their resistance to rifampicin. Moreover, tetracycline-resistance and –intermediate resistance was noted in 10 (26.3%) and 4 (10.5%) isolates. All the isolates studied were susceptible to high-end antibiotics such as vancomycin and linezolid with an exception of *S. gallinarum* (n=2) exhibited teicoplanin-intermediate resistance. Among 38 isolates studied, 23 (60.5%) exhibited resistance to mainly three or more than three classes of antibiotics and thus recognized as multidrug-resistant (MDR) isolates.

3.3 Genes associated with virulence and biofilm production

The genes associated with virulence and biofilm production are outlined in table 1. A large panel of CoNS were found not to harbour the genes encoding enterotoxins. Nevertheless, *S. haemolyticus* (n=6) and *S. sciuri* (n=1) isolates harboured *seb*, *seg* and *sei* genes whereas the one *S. haemolyticus* carried *sea* instead of *seb* along with *seg* and *sei*. On the positive note, none of the isolates carried the genes encoding exfoliative toxins and toxic shock syndrome toxin-1. Similarly, occupancy of the genes associated with biofilm-production was limited to a few isolates. Among 38 CoNS, 4 *S. haemolyticus* (10.5%) carried *icaAD* complex, 10 (26.3%) carried *ebps* gene, 5 (13.2%) carried *clfB* gene, 2 (5.3%) carried *fnbpA* gene and 1 (2.6%) carried *fib* gene. Irrespective of the species diversity, all the isolates harboured *eno* locus. All the isolates carried neither *icaBC* complex nor *sdrCDE* genes.

3.4 Assigning SCC*mec* type

All the 38 MRCoNS isolates equally carried *mecA* gene as evidenced by the PCR amplification of the locus. The SCC*mec* V, comprising *ccr1* allotype and *mec* gene class C2 was identified in 6 isolates (15.8%) whereas the 2 isolates, though carried *ccr1* gene, the *mec*

gene complex was completely absent and thus are non-typeable by the method developed by Milherico et al (2007). The remaining 30 isolates tested negative for any of the *ccr* gene allotypes and *mec* gene complex other than *mecA* gene internal control.

4. DISCUSSION

For many years, CoNS were considered as less aggressive and hence received little attention from the researchers. However, this concept of considering the CoNS as non-virulent strains was amended when their clinical significance has been started to recognize. Despite this, the pathogenicity and antimicrobial resistance of CoNS in fishery environment is still sparse. In this context, this study characterized 38 non-duplicate MRCoNS sourced from retail market fishes in Assam, India. Among the CoNS, *S. epidermidis* is the most frequently isolates species followed by *S. haemolyticus* [27]. In contrast, the present study recorded the high prevalence of *S. haemolyticus* and a complete absence of *S. epidermidis* in the samples monitored was really unexpected. This may be due to the fact that, certain staphylococcal species are sensitive to salt concentration [1]. However, the species such as *S. lentus* belonging *S. sciuri* group, is not a common inhabitant of healthy microbiota of humans and thus its appearance represents the contamination driven by animal contacts. Besides, they are isolated majorly from animal sources and thus speculated to be a safe to humans [28]. Notwithstanding this, *S. lentus*, being the carriers of resistomes and virulomes, can share the resistance- and virulence-genes with other infectious staphylococcal species.

In this study, all the isolates exhibited resistance to beta-lactam antibiotics which is attributed to the presence of *mecA* gene. This gene encodes a structurally altered penicillin-binding protein namely PBD2a having low affinity to beta-lactam antibiotics [29]. Furthermore, non-beta-lactam antibiotic resistance was also recorded in a few isolates with a major resistance to clindamycin, erythromycin and gentamicin. Notably, MIC of levofloxacin for staphylococci ≥4 μg/mL is generally considered as resistant while the range between 2 and 4 μg/mL is considered as intermediate resistance [17]. Here, BDXpert™ system identified the MIC is greater than two but not four. Because to this dilemma, we could only say non-conclusively that the isolates were intermediate resistant to levofloxacin and further tests are warranted to predict the actual resistance status. Given that the rifampicin along with trimethoprim-sulfamethoxazole can synergistically be used in anti-MRSA therapy, the rifampicin-resistance encountered in this study is indeed a major concern. Also, intermediate resistance to teicoplanin; a high-end glycopeptide antibiotic with a spectrum of

activity as same as that of vancomycin observed in *S. gallinarum* intensified the magnitude of the problem. In spite of being intermediate-resistant, staphylococcal isolates are extremely capable to develop rapid resistance to several antibiotics and thus continuous surveillance by testing of isolates repeatedly is advised. Furthermore, Multidrug-resistance is majorly observed in *S. haemolyticus*; an important carriers of resistance genes, whereas the other species such as *S. saprophyticus*, *S. lentus* and *S. sciuri* also exhibited multidrug-resistance which is not common.

The pathogenicity of the isolates studied is determined by the presence of enterotoxins such as *sea*, *seb*, *seg* and *sei*. The involvement of these enterotoxins in the foodborne illnesses has already been studied [30]. Among the 20 enterotoxins reported, *sea* and *seb* are the well-studied enterotoxins otherwise called as superantigens due to their innate ability to bind the class II major histocompatibility (MHC) protein resulting in the increased T-cell production [31]. Whereas the other enterotoxins such as *seg*-*seu*, even though involved in foodborne infections, have not received enough attention and thus, they are poorly studied toxins. Despite this, there is no doubt about the toxigenicity of these enterotoxins as evidenced by the prior studies [32]. Undoubtedly, horizontal transfer of toxic traits among staphylococci are not uncommon because the genes encoding SEs are predominantly housed in the mobile elements such as plasmids, pathogenic islands etc. [33]. On a side note, a previous study from our lab reported MRSA recovered from the same geographical regions carrying similar SEs such as *seb*, *seg* and *sei*, reflecting the interspecies transmission of virulence factors among staphylococci [14].

Biofilm production by staphylococci is also assumed to be a pathogenic determinant [34]. The ability to produce biofilm facilitates staphylococci to colonize in hosts, mostly humans resulting in the chronic infections. Nevertheless, the polysaccharide intercellular adhesin (PIA), encoded by *icaADBC* along with *icaR* promoter is a prerequisite for the biofilm production [35]. Among *ica* operon, genetic expression of *icaA* and *icaD* are sufficient to produce biofilm and the interplay between *icaA* and *icaD* helps to accelerate the activity of N-acetylglucosaminyltransferase that finally resulting in the biofilm production [36]. On a side note, CRA is a standard method which facilitates the direct identification of biofilm-production by staphylococci. Those isolates producing biofilm may appear as black colonies on CRA plates whereas non-biofilm producers will remain red colour colonies. In this study, all the isolates were phenotypically confirmed as biofilm producers by CRA test but when subjected to genotypic characterization, only a few isolates (26.3%) are observed to carry

icaADBC locus. However, PIA-independent biofilm has also been uncovered in *S. aureus* where protein-mediated biofilm recognized as an effective alternative to PIA [37]. All the isolates carried *eno* locus encoding laminin binding protein which can significantly escalate the biofilm production in *S. aureus* while the other associated genes such as *clfB* encoding clumping factor B, *fib* encoding fibrinogen-binding protein precursor, *fnbpA* encoding fibronectin-binding protein A and *ebps* encoding elastin-binding protein are unevenly distributed.

SCC*mec* typing is a fundamental typing method to study the epidemiology of methicillin-resistant staphylococci. Despite the advancement in the epidemiological classification of staphylococci, the origin of SCC*mec* is still blurred. However, *S. sciuri* is speculated to be the effective donor of SCC*mec* elements, otherwise any CoNS carrying *mecA* gene [38]. Despite the several SCC*mec* types, non-typeable SCCmec elements are widely distributed in CoNS which implies the potential of CoNS being the reservoir highly diverse SCC*mec* elements [39]. Besides, type V SCCmec elements are the most dominant in methicillin-resistant *S. haemolyticus* which can contribute to the high prevalence of community-associated methicillin-resistant *S. aureus* (CA-MRSA). Prior studies have substantiated the transference of SCC*mec* elements from CoNS, especially *S. epidermidis* to virulent *S. aureus*. Importantly, given that the size of type V SCC*mec* elements are smaller (28 kb) than other types which are prevailing in hospital-associated (HA) and livestock-associated (LA) MRSA, the transference of these elements become easier, resulting in the rapid dissemination of *mecA* gene [40]. On a positive note, type V SCC*mec* elements do not carry any resistance genes other than *mecA*.

5. CONCLUSION

The present study was proposed to investigate distribution of virulomes among coagulase-negative staphylococci and their resistance profile. Besides beta-lactam antibiotic resistance, a few isolates were noteworthy for their resistance to rifampicin and intermediate resistance to teicoplanin. The toxigenicity of the isolates was acknowledged by the virulence factors as well as their ability to produce biofilm. Those isolates of *S. haemolyticus* carrying type V SCC*mec* elements are expected to contribute to the prevalence of CA-MRSA through the horizontal gene transfer of *mecA* locus. In comparison to a previously reported study from our lab which recorded the occurrence of SEs such as *seb*, *seg* and *sei* in MRSA, this study also observed the presence of the same panel of SEs in MRCoNS. Nonetheless, further

studies are required to understand transmission of these toxin genes among different staphylococcal species.

References

[1] Ochiai T. Salt-sensitive growth of Staphylococcus aureus: stimulation of salt-induced autolysis by multiple environmental factors. *Microbiol Immunol.* 1999;43(7):705-709.

[2] Bemer-Melchior P, Drugeon H.B. Choice of the NaCl Concentration for optimizing the detection of methicillin resistance in Staphylococcus using the gel diffusion method. *Pathologie-biologie*. 2001;49(3):216-221.

[3] Peters G, Schumacher-Perdreau F, Pulverer G. [Infections caused by coagulase-negative Staphylococci in immunocompromised patients]. *Immun Infekt*. 1986;14(5):165-169. http://www.ncbi.nlm.nih.gov/pubmed/3770797

[4] Chajęcka-Wierzchowska W, Gajewska J, Wiśniewski P, Zadernowska A. Enterotoxigenic potential of coagulase-negative staphylococci from ready-to-eat food. *Pathogens*. 2020;9(9):1-12. doi:10.3390/pathogens9090734

[5] Udo EE, Al-Bustan MA, Jacob LE, Chugh TD. Enterotoxin production by coagulase-negative staphylococci in restaurant workers from Kuwait City may be a potential cause of food poisoning. *J Med Microbiol*. 1999;48(9):819-823. doi:10.1099/00222615-48-9-819

[6] Podkowik M, Park JY, Seo KS, Bystroń J, Bania J. Enterotoxigenic potential of coagulase-negative staphylococci. *Int J Food Microbiol*. 2013;163(1):34-40. doi:10.1016/j.ijfoodmicro.2013.02.005

[7] Seng R, Kitti T, Thummeepak R, et al. Biofilm formation of methicillin-resistant coagulase negative staphylococci (MR-CoNS) isolated from community and hospital environments. *PLoS One*. 2017;12(8). doi:10.1371/journal.pone.0184172

[8] Osman K, Alvarez-Ordóñez A, Ruiz L, et al. Antimicrobial resistance and virulence characterization of Staphylococcus aureus and coagulase-negative staphylococci from imported beef meat. *Ann Clin Microbiol Antimicrob*. 2017;16(1). doi:10.1186/s12941-017-0210-4

[9] Ray MD, Boundy S, Archer GL. Transfer of the methicillin resistance genomic island among staphylococci by conjugation. *Mol Microbiol*. 2016;100(4):675-685. doi:10.1111/mmi.13340

[10] Bitrus AA, Zunita Z, Bejo SK, Othman S, Nadzir NAA. In vitro transfer of methicillin resistance determinants mecA from methicillin resistant Staphylococcus aureus (MRSA)

to methicillin susceptible Staphylococcus aureus (MSSA). *BMC Microbiol*. 2017;17(1). doi:10.1186/s12866-017-0994-6

[11] Li Y, Lin J, Li L, et al. Methicillin-Resistant Coagulase-Negative Staphylococci Carriage is a Protective Factor of Methicillin-Resistant Staphylococcus Aureus Nasal Colonization in HIV-Infected Patients: A Cross-Sectional Study. *Can J Infect Dis Med Microbiol*. 2021;2021. doi:10.1155/2021/5717413

[12] Hammad AM, Watanabe W, Fujii T, Shimamoto T. Occurrence and characteristics of methicillin-resistant and -susceptible Staphylococcus aureus and methicillin-resistant coagulase-negative staphylococci from Japanese retail ready-to-eat raw fish. *Int J Food Microbiol*. 2012;156(3):286-289. doi:10.1016/j.ijfoodmicro.2012.03.022

[13] Kumar LRG, Kasim AK, Lekshmi M, Nayak BB, Kumar S. Incidence of Methicillin-Resistant Staphylococci in Fresh Seafood. *Adv Microbiol*. 2016;06(06):399-406. doi:10.4236/aim.2016.66039

[14] Sivaraman GK, Muneeb KH, Sudha S, Shome B, Cole J, Holmes M. Prevalence of virulent and biofilm forming ST88-IV-t2526 methicillin-resistant Staphylococcus aureus clones circulating in local retail fish markets in Assam, India. *Food Control*. 2021;127. doi:10.1016/j.foodcont.2021.108098

[15] Chenouf NS, Mama OM, Messaï CR, et al. Detection of methicillin-resistant coagulase-negative staphylococci and PVL/mecA genes in cefoxitin-susceptible Staphylococcus aureus (t044/ST80) from unpasteurized milk sold in stores in Djelfa, Algeria. *J Dairy Sci*. 2021;104(3):2684-2692. doi:10.3168/jds.2020-19270

[16] Wekell MM, Manger R, Colburn K, Adams A, Hill W. Microbiological quality of seafoods: viruses, bacteria and parasites. *Seafoods Chem Process Technol Qual*. Published online 1994:196-219. doi:10.1007/978-1-4615-2181-5_11

[17] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 30th edition, CLSI document M100. CLSI, (2020), Wayne, PA

[18] Hong JS, Kim D, Kang DY, et al. Evaluation of the BD Phoenix M50 automated microbiology system for antimicrobial susceptibility testing with clinical isolates in Korea. *Microb Drug Resist*. 2019;25(8):1142-1148. doi:10.1089/mdr.2018.0370

[19] Jarraud S, Cozon G, Vandenesch F, Bes M, Etienne J, Lina G. Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J Clin Microbiol*. 1999;37(8):2446-2449. doi:10.1128/jcm.37.8.2446-2449.1999

[20] Bose S, Khodke M, Basak S, Mallick SK. Detection of biofilm producing staphylococci: need of the hour. *J Clin Diagnos Res*. 2009;*3*(6):1915-1920

[21] Kiem S, Oh WS, Peck KR, et al. Phase variation of biofilm formation in Staphylococcus aureus by IS256 insertion and its impact on the capacity adhering to polyurethane surface. *J Korean Med Sci*. 2004;19(6):779-782. doi:10.3346/jkms.2004.19.6.779

[22] Tang J, Chen J, Li H, Zeng P, Li J. Characterization of adhesin genes, staphylococcal nuclease, hemolysis, and biofilm formation among staphylococcus aureus strains isolated from different sources. *Foodborne Pathog Dis*. 2013;10(9):757-763. doi:10.1089/fpd.2012.1474

[23] Notcovich S, DeNicolo G, Flint SH, et al. Biofilm-forming potential of Staphylococcus aureus isolated from bovine mastitis in New Zealand. *Vet Sci*. 2018;5(1). doi:10.3390/vetsci5010008

[24] Chen Q, Xie S, Lou X, et al. Biofilm formation and prevalence of adhesion genes among Staphylococcus aureus isolates from different food sources. *Microbiologyopen*. 2020;9(1). doi:10.1002/mbo3.946

[25] Lee JH. Methicillin (Oxacillin)-Resistant Staphylococcus aureus Strains Isolated from Major Food Animals and their Potential Transmission to Humans. *Appl Environ Microbiol*. 2003;69(11):6489-6494. doi:10.1128/AEM.69.11.6489-6494.2003

[26] Milheiriço C, Oliveira DC, de Lencastre H. Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome mec type IV in methicillin-resistant Staphylococcus aureus: "SCCmec IV multiplex." *J Antimicrob Chemother*. 2007;60(1):42-48. doi:10.1093/jac/dkm112

[27] Barros EM, Ceotto H, Bastos MCF, Dos Santos KRN, Giambiagi-deMarval M. Staphylococcus haemolyticus as an important hospital pathogen and carrier of methicillin resistance genes. *J Clin Microbiol*. 2012;50(1):166-168. doi:10.1128/JCM.05563-11

[28] Pedroso SHSP, Sandes SHC, Luiz KCM, et al. Biofilm and toxin profile: A phenotypic and genotypic characterization of coagulase-negative staphylococci isolated from human bloodstream infections. *Microb Pathog*. 2016;100:312-318. doi:10.1016/j.micpath.2016.10.005

[29] King DT, Sobhanifar S, Strynadka NCJ. The mechanisms of resistance to β-lactam antibiotics. *Handb Antimicrob Resist*. Published online 2017:177-201. doi:10.1007/978-1-4939-0694-9_10

[30] Helak I, Daczkowska-Kozon EG, Dłubała AA. Short communication: Enterotoxigenic potential of coagulase-negative staphylococci isolated from bovine milk in Poland. *J Dairy Sci*. 2020;103(4):3076-3081. doi:10.3168/jds.2019-17574

[31] Pinchuk I V., Beswick EJ, Reyes VE. Staphylococcal Enterotoxins. *Toxins (Basel)*. 2010;2(8):2177-2197. doi:10.3390/toxins2082177

[32] Chen TR, Chiou CS, Tsen HY. Use of novel PCR primers specific to the genes of staphylococcal enterotoxin G, H, I for the survey of Staphylococcus aureus strains isolated from food-poisoning cases and food samples in Taiwan. *Int J Food Microbiol*. 2004;92(2):189-197. doi:10.1016/j.ijfoodmicro.2003.10.002

[33] Lindsay JA, Ruzin A, Ross HF, Kurepina N, Novick RP. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in Staphylococcus aureus. *Mol Microbiol*. 1998;29(2):527-543. doi:10.1046/j.1365-2958.1998.00947.x

[34] Vestby LK, Grønseth T, Simm R, Nesse LL. Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics*. 2020;9(2). doi:10.3390/antibiotics9020059

[35] Arciola CR, Campoccia D, Ravaioli S, Montanaro L. Polysaccharide intercellular adhesin in biofilm: Structural and regulatory aspects. *Front Cell Infect Microbiol*. 2015;5(FEB). doi:10.3389/fcimb.2015.00007

[36] Liberto MC, Matera G, Quirino A, et al. Phenotypic and genotypic evaluation of slime production by conventional and molecular microbiological techniques. *Microbiol Res*. 2009;164(5):522-528. doi:10.1016/j.micres.2007.04.004

[37] Boles BR, Thoende M, Roth AJ, Horswill AR. Identification of genes involved in polysaccharide- independent Staphylococcus aureus biofilm formation. *PLoS One*. 2010;5(4). doi:10.1371/journal.pone.0010146

[38] Ghaznavi-Rad E, Fard-Mousavi N, Shahsavari A, Japoni-Nejad A, Van Belkum A. Distribution of staphylococcal cassette chromosome mec types among methicillin-resistant coagulase negative staphylococci in central Iran. *Iran J Microbiol*. 2018;10(1):7-13.

[39] Bouchami O, Ben Hassen A, De Lencastre H, Miragaia M. High prevalence of mec complex C and ccrC is independent of SCCmec type V in Staphylococcus haemolyticus. *Eur J Clin Microbiol Infect Dis*. 2012;31(4):605-614. doi:10.1007/s10096-011-1354-3

[40] Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K. Novel type V staphylococcal cassette chromosome mec driven by a novel cassette chromosome recombinase, ccrC. *Antimicrob Agents Chemother*. 2004;48(7):2637-2651. doi:10.1128/AAC.48.7.2637-2651.2004

Legends to the figures

Fig 1 Percentage of isolates which are resistant, intermediate-resistant and sensitive to 13 classes of antibiotics. AMP-ampicillin; CFZ-cefazolin, FOX-cefoxitin, CLI-clindamycin, DAP-daptomycin, ERY-erythromycin; GEN-gentamicin, LVX-levofloxacin; LZD-linezolid; MXF-moxifloxacin; NIT-nitrofurantoin; NOR-norfloxacin; OXA-oxacillin; PEN-penicillin; Q-D-quinupristin-dalfoprisitin; RIF-rifampicin; TEC-teicoplanin; TET-tetracycline; SXT-trimethoprim-sulfamethoxazole; VAN-vancomycin