Brief Report

The Emergence of Linezolid-Resistant *Staphylococcus Epidermidis* in the COVID-19 Hospitalized Intubated Patients in North Khorasan, Iran

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Abstract

The present study aimed to investigate secondary bacterial infections among patients infected with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Coagulase-negative Staphylococci can infect immunocompromised patients. Linezolid resistance among *Staphylococcus epidermidis* is one of the most critical issues.

In 2019, 185 SARS-CoV-2-positive patients who were admitted to North Khorasan Province Hospital (Bojnurd, Iran), were investigated. Patients having positive SARS-CoV-2 reverse transcriptase real-time polymerase chain reaction (RT-PCR) test results, who had a history of intubation, mechanical ventilation, and were hospitalized for more than 48 hours were included.

After microbiological evaluation of pulmonary samples, taken from intubated patients with clinical manifestation of pneumonia, co-infections were found in 11/185 patients (5.94%) with *S. epidermidis, Staphylococcus aureus,* and *Acinetobacter baumani,* respectively. Remarkably, seven out of nine *S. epidermidis* isolates were linezolid resistant. Selected isolates were characterized using antimicrobial resistance patterns and molecular methods, such as Staphylococcal cassette chromosome *mec* (SCC*mec*) typing, and gene detection for *ica,* methicillin resistance *(mecA),* vancomycin resistance *(vanA),* and chloramphenicol–florfenicol resistance *(cfr)* genes.

All of the isolates were resistant to methicillin, and seven isolates were resistant to linezolid. Nine out of 11 isolated belonged to the SCC*mec* I, while two belonged to the SCC*mec* IV. It should be noted that all patients had the underlying disease, and six patients had already passed away.

The increasing linezolid resistance in bacterial strains becomes a real threat to patients, and monitoring such infections, in conjunction with surveillance and infection prevention programs, is very critical for reducing the number of linezolid-resistant Staphylococcal strains.

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Keywords ● *Staphylococcus epidermidis* ● COVID-19 ● Drug resistance ● Microbial, linezolid ● Vancomycin

What's Known

- It is known that the *Staphylococcaceae* family, especially *Staphylococcus aureus* and *Staphylococcus epidermidis*, are sensitive to Linezolid.

What's New

- Linezolid resistance was found among *Staphylococcus epidermidis* strains isolated from respiratory samples of hospitalized SARS-CoV-2-positive patients.

Introduction

Microbial co-infections are one of the major causes of increased mortality and morbidity among patients with respiratory tract viral infections such as COVID-19. Its rate is variable between 2% to 65%. Respiratory viruses such as Influenza Virus, Adenovirus, Respiratory Syncytial Virus, Parainfluenza Virus, Human metapneumovirus, and Bocavirus are the most common viruses that account for viral co-infections. Moreover, bacteria such as *Staphylococcus*, and *Streptococcus* spp., as well as some Gram-negative bacteria, account for these co-infections.[1] Coagulase-negative Staphylococci (CoNS) colonize mucosal membranes and human skin. Therefore, they were typically classified as harmless commensals. However, they are increasingly isolated as related agents to hospital-acquired infections (HAIs), such as bloodstream, urinary tract, and pulmonary infections.[2] Among CoNS related to humans, *Staphylococcus epidermidis* is the most frequent species.[2] Antimicrobial resistance in *S. epidermidis* is increasingly reported in Europe.[3] Linezolid, a member of oxazolidinones, has been added to the treatment regimen of various skin and pulmonary communities, as well as hospital Gram-positive infections. Since 2000, however unfortunately, shortly after its entrance into clinical use, the first case of linezolid-resistant *Staphylococcus aureus* was reported in the United States in 2001. Since then, linezolid resistance in *S. epidermidis* (LRSE) has been increasingly reported in many countries, such as France, Germany, Ireland, Italy, Greece, and Portugal.[3] Resistance to linezolid can be acquired by mutations during long-term linezolid therapy or horizontal gene transfer. The G-T mutation in the 23S rRNA gene is the primary cause of the linezolid resistance. It should be noted that other mutations in the Ribosomal protein L (*rpl*) gene family are frequently found in linezolid resistant *S. epidermidis* (LRSE).[4]

The chloramphenicol–florfenicol resistance (*cfr)* is the primary transmissible gene that encodes ribosomal methyl transferase, which confers linezolid resistance in Staphylococci. Linezolid resistance is also influenced by oxazolidinone and phenicol transferable *A (optrA)* and phenicol-oxazolidinone-tetracycline resistance A (*poxtA)* genes of ribosomal protection protein genes. Of these genes, only *cfr* has been reported in LRSE.[4] Resistance to linezolid and methicillin are commonly combined. Therefore, Staphylococcal cassette chromosome *mec* (SCC*mec*) is critical in these isolates. The *cfr* gene harboring plasmids can be transferred from primary resistant bacteria such as *S. aureus* to sensitive receiver bacteria such as *S. epidermidis*. It should be noted that the *cfr* gene is inserted into various plasmids by mobile genetic elements such as insertion sequences or transposons related to SCC*mec* elements.[4] Regarding the importance of secondary viral and bacterial infections in increasing mortality and length of hospitalization in COVID-19 patients, we previously evaluated and reported viral co-infections in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) patients,[5] and this study evaluated bacterial co-infections in these patients. Thus, the emergence of LRSE in the nasal tract of COVID-19 hospitalized patients in North Khorasan (Iran) was reported.

Materials and Methods

Sample Collection

In this cross-sectional study, 185 patients who were hospitalized in the intensive care unit (ICU) with SARS-CoV-2 in North Khorasan Province Hospitals, from February 19, 2021, to August 23, 2021, were evaluated. Samples were collected in accordance with the guidelines established by Islamic Azad University Ethics Committee of Damghan, Iran, (code: IR.IAU.DAMGHAN.REC.1402.002) and North Khorasan University of Medical Sciences (code: IR.NKUMS.REC.1399.020).

The inclusion criteria were hospitalization, a positive reverse transcriptase real-time PCR (RT-PCR) result for SARS-CoV-2, intubation, and mechanical ventilation for more than 48 hours in ICUs.

Microbiological Identification

In the first step, respiratory samples were evaluated using conventional microbiological methods. The nasal swab cultures of these patients were also evaluated. After primary culture and growth of Gram-positive cocci in all samples, biochemical tests including catalase, coagulase, susceptibility to polymyxin B and novobiocin, acetoin production, ornithine decarboxylase test, pyrrolidinyl aminopeptidase (PYR) test, and fermentation of mannitol, glucose, sucrose, maltose, mannose, and trehalose were performed on all isolates.

Antimicrobial Susceptibility Test

The antibiogram test was performed using cefoxitin (CEF), penicillin (PEN), tetracycline (TET), erythromycin (ERY), levofloxacin (LEV), moxifloxacin (MOX), clindamycin (CLN),

gentamicin (GEN), trimethoprim/ sulfamethoxazole (COT), minocycline (MIN), rifampicin (RIF), linezolid (LIN), and vancomycin (VAN) antimicrobial disks using Kirby Bauer method (MAST DISKSTM, UK), based on CLSI guidelines (Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. CLSI supplement M100).[6] Methicillin resistance was initially identified using cefoxitin disks (30 µg). The E-test (Biomeriux, Marcy l'Etoile, France) method was used to determine the minimum inhibitory concentration (MIC) values of linezolid and vancomycin. The *S. aureus* ATCC 29213 was utilized as a control.

Genomic DNA Extraction

Genomic DNAs of Coagulase Negative Staphylococci (CoNS) isolates were extracted using the Genetbio Genomic DNA Extraction Kit (KR-2000, South Korea). According to the manufacturer's protocol for *Staphylococcus* spp., the lysostaphin at a final concentration of 20 µg/mL was added to the lysis buffer.

PCR Detection of Autolysin (atlE), Methicillin Resistance (mecA), Vancomycin Resistance (vanA), and Chloramphenicol–florfenicol Resistance (cfr) Genes

To identify the *S. epidermidis* isolates at the species level, phenotypic methods as well as the PCR for the *atlE* gene were used. Moreover, PCR tests were performed to evaluate the presence of *altE*, *mecA, vanA, and cfr* genes as previously described (table 1).[7]

SCC*mec Typing*

SCC*mec* typing was performed as previously described.[8]

Statistical Analysis

The data was imported to a Microsoft Excel spreadsheet for analysis. Statistical analysis was performed using SPSS software version 16 (IBM, USA).

Results

From February 19 to August 23, 2021, 185 hospitalized intubated patients were evaluated in North Khorasan Province (Iran). In total, 11 patients had signs of bacterial pneumonia, such as persistent or progressive infiltration in chest radiography, body temperature more than 38 °C or less than 36 °C, WBC counts more than 10^4 or less than 5×10^3 cells/mL, tracheal secretions, or decreased of O_2 saturation. It should be noted that all 11 patients had a history of cough, sore throat, and distress on physical examination. Besides, they had neutropenia, elevated estimated sedimentation rate (ESR), and C-reactive protein (CRP) in laboratory blood analysis. The *S. epidermidis* were isolated from nine samples, and *S. aureus* and *Acinetobacter baumani* were isolated from the remaining two samples. All *S. epidermidis* isolates were resistant to methicillin, and seven isolates were resistant to linezolid (table 2). The most frequent SCC*mec* types were I (77.8%) and IV (22.2%), respectively. It should be highlighted

Table 1: Primer sequences for *S. epidermidis* detection and antimicrobial resistance genes

Gene	Primer Sequence	Product Size (bp)
altE	5′-CAACTGCTCAACCGAGAACA-3′	682
	5′-TTTGTAGATGTTGTGCCCCA-3′	
mecA	5′-ATGTATGTGCGATTGTATTGC-3′	584
	5′-AGAAGATGGTATGTGGAAGTTAG-3′	
cfr	5′-ACCATATAATTGACCACAAGCAGC-3′	746
	5′-TGAAGTATAAAGCAGGTTGGGAGTCA-3′	
vanA	5′-ATCAAGCGGTCAATCAGTTC-3′	713
	5′-GGCAAGTCAGGTGAAGATG-3′	

atlE: Autolysin; *mecA*: Methicillin resistance Afig; *cfr:* Chloramphenicol–florfenicol resistance; *vanA:* vancomycin resistance

Table 2: Results of antibiotic susceptibility test

	PEN n (%)	ERY n (%)	CEF n (%)	TET n (%)	CLN n (%)	LEV n (%)	MOX n (%)	COT n (%)	GEN n (%)	RIF n (%)	MIN n (%)	VAN n (%)	LIN n (%)
Resistant	8 (88.9)	5 (55.5)	5 (55.5)	4 (44.4)	4 (44.4)	5 (55.5)	4 (44.4)	4 (44.4)	4 (44.4)	5 (55.4)	2 (22.2)	0	7 (77.7)
Intermediate	0	3 (33.3)	0	2 (22.3)	1 (11.2)	1 (11.2)	1 (11.2)	2 (22.3)	2 (22.3)	2 (22.3)	0	0	0
Sensitive	1 (11.2)	1 (11.2)	4 (44.4)	3 (33.3)	4 (44.4)	3 (33.3)	4 (44.4)	3 (33.3)	3 (33.3)	2 (22.3)	7 (77.8)	9 (100)	2 (22.3)

PEN: Penicillin; ERY: Erythromycin; CEF: Cefoxitin; TET: Tetracycline; CLN: Clindamycin; LEV: Levofloxacin; MOX: Moxifloxacin; COT: Trimethoprim/sulfamethoxazole; GEN: Gentamicin; RIF: Rifampicin; MIN: Minocycline; VAN: Vancomycin; LIN: Linezolid

Table 3: Complete details of *Staphylococcus epidermidis*-positive patients

Patient	Sex	Resistance gene	SCC*mec* type	*atlE* gene	Blood culture	Nasal swab for *S. epidermidis*	Underlying disease	Survived/ died
1	Female	*cfr, mecA*	*I*	+	Positive/*S. epidermidis*	Positive	Diabetes	Died
2	Female	*mecA*	*IV*	+	Positive/*S. epidermidis*	Positive	Diabetes/ hypertension	Survived
3	Male	*cfr, mecA*	*I*	+	Negative	Positive	Diabetes	Died
4	Male	*cfr, mecA*	*I*	+	Positive/*S. epidermidis*	Positive	Diabetes	Died
5	Male	*cfr, mecA*	*I*	+	Negative	Positive	Diabetes	Survived
6	Female	*mecA*	*IV*	+	Negative	Positive	Hypertension	Survived
7	Male	*mecA*, *cfr*	*I*	+	Positive/*S. epidermidis*	Positive	Diabetes	Died
8	Female	*cfr, mecA*	*I*	+	Negative	Positive	Heart disease	Died
9	Male	*cfr, mecA*	*I*	+	Positive/*S. epidermidis*	Positive	Diabetes	Died

SCC*mec* type: Staphylococcal cassette chromosome *mec* type, *atlE*: Autolysin; *mecA*: Methicillin resistance Afig; *Cfr:* Chloramphenicol–florfenicol resistance

that all linezolid-resistant isolates were resistant to cefoxitin and belonged to SCC*mec* type I. All isolates were positive for the *ica* gene. The complete details of nine *S. epidermidis*-positive patients are presented in table 3.

Discussion

In this study, 11/185 (5.94%) bacterial co-infections in hospitalized patients were found. Nine of these cases were positive for *S. epidermidis*. All nine isolates were resistant to methicillin, and seven of them were resistant to linezolid. All patients had the same underlying disease, and six patients had already passed away.

There are two types of bacterial infections associated with COVID-19, including: concurrent infections with the virus and subsequent infections. Co-infection was detected in 3.5% of patients, and secondary infection was reported in 14.3% of patients with COVID-19.[9] Bacterial infections are an important factor in increased morbidity and mortality in viral respiratory infections and require precise and timely diagnosis and treatment. Sharifipour and others reported that the most common bacteria in co-infections in COVID-19 patients were *A. baumani* and *S. aureus*.[1] In other research on respiratory viral infections, the most commonly found bacterial co-pathogens were *S. aureus* and *Streptococcus pneumoniae.*[10] Unlike previous research, in the present study, *S. epidermidis*, *S. aureus*, and *A. baumani* were found to be the most frequent bacteria, respectively, in COVID-19 intubated patients who were hospitalized in the ICU.[1]

The members of Staphylococcaceae are among the most frequent colonizing bacteria in the skin and mucus membranes, which act as opportunistic pathogens. Of these families, *S. aureus* is the most pathogenic bacteria, and other members such as *S. epidermidis* and *S. saprophyticus* have a lower pathogenicity. *S. aureus* is a significant colonizing bacterium in the nasal tract, while *s. epidermidis* is a significant skin colonizing bacteria, related to external medical devices, in the human body, such as catheters, tubes, and so on.[2] Although *S. epidermidis* was isolated from lung and blood samples in the examined patients, it should be noted that these bacteria typically colonize the upper respiratory tract and are resistant to a variety of antibacterial agents. Linezolid is an effective treatment for multidrug-resistant Gram-positive bacteria, and despite being widely used for nearly 20 years, it still has outstanding activity against Staphylococci.[11] Linezolid resistance among *S. epidermidis* remains uncommon worldwide, but increasing resistance in European countries such as Greece, Spain, Portugal, Italy, France, and Ireland was reported. Kosecka and others reported an isolation of 11 LRSA strains from blood cultures in Poland between 2015 to 2017. All isolates belonged to SCC*mec* types II and III and were also *cfr* positive.[12] The isolates of the present study belonged to SCC*mec* types I and IV. Another distinction was that these strains were isolated from respiratory samples, whereas the previous strains were isolated from the blood cultures of children.[12] In 2021, Ruiz-Ripa and colleagues published a report on the molecular characteristics of linezolid-resistant *S. aureus* and *S. epidermidis*. They found that the Linezolid-resistance gene (*cfr*) in *S. aureus* was located on a plasmid, while linezolid resistance in *S. epidermidis* was related to 23srRNA gene mutations.[13] Therefore, it could be concluded

that this resistance is transferable between *S. aureus* and not between *S. epidermidis* strains, and it occurred as a result of mutations in each *S. epidermidis* strain. Therefore, It was expected that *S. aureus* exhibited a higher prevalence of linezolid resistance than *S. epidermidis*.[13] Linezolid resistance is rare in Iran. Most previously found cases of resistance were related to *S. aureus,* and there was no report of resistance to these antibiotics in *S. epidermidis*.[14] Based on the findings of the present study, the most effective antibiotics against *S. epidermidis* were vancomycin and minocycline, and the least effective antibiotics were penicillin and linezolid (table 2). Since the number of examined samples was too small, the results could not be generalized to all strains. Minocycline and vancomycin were also highly effective against *S. aureus*. Based on the average rate of resistance to various antibiotics (about 50%), it seems that antimicrobial susceptibility tests should be performed on each sample, and decisions should be made based on the results of each sample. The high prevalence of linezolid resistance in the present *S. epidermidis* isolates could be attributed to the exact origin of the strains. However, MLST should be performed on the studied isolates to prove this claim. Another important finding of the present study was the presence of biofilm formation-related genes (*ica*) in all isolates. *S. epidermidis* is typically related to medical devices, and all the patients of the present study were intubated and under mechanical ventilation. The presence of this bacteria in the nasal and pulmonary tract of patients might be related to these devices, and it seemed that we need to pay more attention to possible infections with biofilm-forming bacteria. However, to prove this relationship, sampling of the aforementioned devices and molecular typing of potential isolated strains should be performed. All isolates were resistant to methicillin, and the studied isolates were belonged to SCC*mec* I and IV. In methicillin-resistant Staphylococci, SCC*mec* types I- III were related to deep and severe infections, and IV-V were related to mild and superficial infections.[15] In the present study, four of six dead patients had simultaneous septicemia, and the isolates were belonged to SCC*mec* I, while two SCC*mec* IV strains were isolated from surviving patients. It might be related to the severity of the infection and its connection with the SCC*mec* type. The growth of *S. epidermidis* in specimens is typically considered a contagion unless its role is clinically proven. In the present research, the simultaneous presence of *S. epidermidis* in the nose, pulmonary tract, and bloodstream, as well as hematology, serology, and clinical findings of the patients ruled out the possibility of contamination. All of the patients in this study had underlying diseases such as diabetes, hypertension, and cardiovascular diseases. It should be noted that due to the different pathogenesis and target regions, the treatment of bacterial co-infections might not directly affect COVID-19 treatment. However, due to the wide range of COVID-19 complications, it might result in more extended hospitalization of patients and more complicated COVID-19 symptoms, demonstrating synergism in their effects. The only limitation of the present study was its small number of examined samples. To prove the relationship between underlying disease and the risk of co-infection, it is essential to evaluate more patients.

Conclusion

The findings indicated that more attention should be paid to bacterial co-infections, especially, antibiotic-resistant bacteria. Based on the findings, the most effective antibiotics against *S. epidermidis* were vancomycin and minocycline. However, the number of the examined samples was too small for such a conclusion. Due to the effect of COVID-19 and related treatments, microorganisms, especially common skin and mucus membrane bacteria, might cause co-infections. The emergence of antibiotic-resistant strains is a warning issue and should be considered in selecting experimental treatment regimens. To overcome increasing resistance, it is essential to start or continue the surveillance, control, and update antimicrobial usage guidelines against linezolid-resistant Staphylococci.

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Authors' Contribution

R.S: Performing laboratory tests, data analysis, drafting, and revision; H.Af: Study design, Data analysis, reviewing the manuscript; H.GM: Study concept and design, data analysis, and drafting and revision, A.A: Study concept and design, performing laboratory tests, data analysis, drafting and revision; All authors have read and approved the final manuscript and

agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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