IJKD KIDNEY DISEASES

Virulence Factors of *Staphylococcus Aureus* Hemolysin *HLA* and *HLB* Isolated from Catheters of Dialysis Patients Referred to Nikan Hospital in Tehran During the Spring and Summer of 2021

Mehrdad Jafari Fesharaki,[1] Sara Alipanahi,[2]
Nazila Arbabsoleimani,[3] Fatemeh Pourrezagholi,[4] Zeinab Piravar[2]

[1]Department of Cardiology, School of medicine, Shahid Beheshti University of medical sciences, Tehran Iran
[2]Department of Biology, Faculty of Sciences, Central Tehran Branch, Islamic Azad University, Tehran, Iran.
[3]Department of Microbiology, Faculty of Sciences, Damghan Branch, Islamic Azad University, Tehran, Iran.
[4]Chronic Kidney Disease Research Center, Shahid Labbafinejad Medical Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Keywords. staphylococcus aureus, biofilms, dialysis catheters, hemolysins

Original Paper

Introduction. *Staphylococcus aureus* (*S. aureus*) is one of the most frequent causes of infection around the world. Insertion of intravascular catheter and formation of biofilms by methicillin-resistant *Staphylococcus aureus* (MRSA) have contributed to the increased risk of infection, and morbidity and mortality rates. Biofilms formation on intravascular catheters and other medical devices are of major postoperative concerns because biofilms are often the source of persistent and difficult-to-treat bacterial infections. This study aimed to evaluate different genetic patterns of this bacterium in samples collected from dialysis patients of Nikan hospital.

Methods. In this descriptive cross-sectional study 30 samples from the removed catheters of patients suspected to have *S. aureus* infection and admitted to the dialysis ward of Nikan hospital were collected and phenotypic evaluations were done to confirm the type of the infectious species. Evaluation of antibiotic resistance of bacterial samples using Kirby-Bauer method was done. Biofilm production of the samples was assessed by the 96-plate microtiter method. The existence of two genes *hla* and *hlb* were evaluated using Multiplex PCR.

Results. The biofilm production test showed that 60% of the samples were able to produce strong biofilms. Multiplex PCR results revealed that both *hla* and *hlb* genes were expressed in 93% of the samples, while, *hlb* gene alone was expressed in 53% of cases.

Conclusion. The results of this study provide significant insight into the virulence gene makeup of catheter-colonizing *S. aureus* strains, and will assist in developing a more targeted treatment approach for persistent *S. aureus* biofilm contamination of medical devices.

IJKD 2022;16:348-54
www.ijkd.org
DOI: 10.52547/ijkd.7146

INTRODUCTION

Central venous catheters are commonly used as vascular access for dialysis in end-stage kidney disease patients, but infectious complications arising from them remain a major clinical problem. In particular, the high mortality rate and cost of general care with catheter infection and hospitalization caused by staphylococcus bacteria

necessitate more research in this field.[1] The most common cause of catheter-associated infection is Staphylococcus species; *Staphylococcus aureus* is a gram-positive voluntary anaerobic coccus that accounts for 40 to 81% of cases of infections, while gram-negative bacteria, such as enterococci, make up the remainder. In a study conducted on patients with the first episode of sepsis within a year of hemodialysis, *S. aureus* was the most common cause of infection, with no other bacteria exceeding 10%.[2] The prevalence of bacteremia due to intravascular devices is significantly increasing. Half of all cases of primary bacteremia in the intensive care units are caused by intravascular catheters. Both local and systemic infections can occur following contamination of intravascular devices.[3] Dialysis catheters are often associated with early and delayed complications.[4] Most infections associated with a central venous catheter occur at the catheter exit site, which also depends on the duration of catheter use. *S. aureus* may be present in the nose or skin as the normal flora and forms yellow colonies due to the production of a carotenoid gold pigment called "Staphyloxanthin".[3] *S. aureus* has several pathogenic factors including enzymes (deoxyribonuclease (DNase) that breaks down DNA) and toxins, each of which causes a specific disease.[6] TSST-1 toxin is involved in toxic shock syndrome and scaling toxin, which is a protease that breaks the skin barrier and causes staphylococcal scalded skin syndrome.[7] It also produces alpha, beta and gamma toxins, which break down the membranes of many cells in the body. Hemolysins are major contributors to *S. aureus* virulence which contribute to colonization of both alpha and beta types of bacteria.[8] Alpha hemolysin is essentially a porous toxin and is encoded by the *hla* gene. This particular gene encodes alpha-toxin with a molecular weight of 22 kD, located on a chromosome or plasmid which is capable to lyse human monocytes, lymphocytes, red blood cells, platelets and endothelial cells.[9] The cytotoxicity of alpha-hemolysin is applied through the formation of transmembrane pores.[10] Beta-hemolysin, coded by *hlb* gene, specifically cleaves cell membrane sphingomyelin with phospholipase C-like activity. Beta-hemolysin destroys epithelial cells, increases *S. aureus* adhesion and increases bacterial proliferation.[11] This study aimed to evaluate the phenotypic properties and antibiogram and study the different genotypes of the bacterium in samples collected from dialysis patients at Nikan hospital in Tehran between April and September 2021. According to scientific sources and databases, no study has been conducted on the virulence factors of *S. aureus* in patients with kidney failure on routine dialysis via a catheter, in Iran. Therefore, this study can hopefully provide more information regarding the selection of appropriate antibiotics and facilitating their recovery.

MATERIALS AND METHODS

Sample Collection

This descriptive cross-sectional study included catheters removed from 30 patients, suspected to catheter infection with *S. aureus,* and admitted to the regular ward of Nikan hospital between April and September 2021. Patients with fever and chills or signs of catheter exit site infection were enrolled. Written consent was received from the patients, and the study was approved by the ethics committee of Shahid Beheshti University of Medical sciences in Tehran.

Phenotypic Evaluation of *Staphylococcus Aureus*

S. aureus was purified on Müller-Hinton agar. The mean age of the enrolled patients was 46 years. For identification of *S. aureus,* routine biochemical tests such as gram staining, catalase, coagulase, Mannitol fermentation and DNases were performed.

Evaluation of Antibiotic Resistance of Bacterial Samples Using Kirby-Bauer Method

After 24 hours of incubation in culture medium, a single pure colony was isolated for antibiogram testing. First, we placed the bacterium on a solid medium on Tryptic Soy Broth (TSB) medium and kept it at 37 °C for 15 minutes to allow the bacterium to enter the logarithmic phase. It was necessary to ascertain the number of bacteria to reach 1.5×10^8 mL/CFU in the tube. Then the prepared bacterial suspension was cultured on a plate of Müller-Hinton culture medium using sterile swabs. The medium was kept at 37 °C for 10 minutes. The discs were placed on the perimeter and the discs distance from each other and from the side of the plate were set 3 cm and 1.5 cm, respectively. The plates were incubated for 24 hours at 37 °C.

Then, different disks were measured for the aura of bacterial growth. *S. aureus* ATCC 25923 was used as positive control.

Production of Biofilm by 96-plate Microtiter Method

In order to evaluate biofilm production, enriched *S. aureus* colonies were cultured on TSB medium in blood agar. We used Red Congo agar medium (Merck, Germany Co.) containing 40 g/L of sucrose to assess biofilm formation by the phenotypic method. We considered the black colonies in isolates as strong biofilms, dark red colonies as medium biofilms and light red colonies as negative biofilm strains. Enriched turbidity samples equivalent to half McFarland were prepared for the purpose of microplate titration and 200 µL of each suspension was transferred to microplate wells of 96 polystyrene houses and heated for 37 hours at 37 °C. Violet crystal dye was used for the well staining for 15 minutes. The dye in each well was then washed with ordinary water. Later, 100 microliters of 10% isopropyl alcohol combined with 70% ethanol were added to each well to release the dye from the walls of biofilm-producing bacteria. Finally, the dye released in each well at 492 and 630 nm was examined using a sample ELISA device (Elisa Reader stat fax 2100). Negative control in this method was TSB medium containing 1% glucose. The light absorption of each isolate was examined three times to ensure accuracy.

DNA Extraction

S. aureus DNA was extracted using lysostaphin digestion. The pellet was resuspended with 350 µL of lysis buffer (Tris-HCL 0.01 M, EDTA 0.01 M). The sample was incubated at 37 °C overnight. Equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1 by volume) were added, and nucleic acid was precipitated by ethanol, using the standard protocol.

Existence of Two Genes *Hla* and *Hlb* Using Multiplex PCR

This method amplifies several genes via PCR reaction. It examines the genes that encode toxins and antibiotic resistance. In *Staphylococcus aureus* isolates, Multiplex PCR was used to identify *hla* and *hlb* genes. For isolation in PCR, one pair of primers was used for each gene to identify resistance factor genes (Table 1).

Table 1. Primers Used in the Present Study

Primer		Sequence
hla 306bp	F	5′ – GACTCACACGGAAACTTAGG - 3′
	R	5′ – ACACAGGTTAGGAGAAGGAG - 3′
hlb 350bp	F	5′ – GACGAAAATCAAGCGGAA - 3′
	R	5′ – TCTAAATACTCTGGCGCAC - 3′

Data was analyzed using SPSS software. $P < .05$ was considered statistically significant.

RESULTS

Phenotypic Analysis

Figure 1 shows the characteristics of 30 isolated samples from dialysis catheters along with biochemical tests. All isolated samples were positive for *S. aureus*.

Antibiotic Resistance of *Staphylococcus Aureus* Samples Using Kirby- Bauer Method

The sensitivity of the isolated samples to vancomycin, novobiocin and nitrofurantoin and resistance to tetracycline, clindamycin and cefazolin were higher compared to other antibiotics. In the collected samples, 70% were sensitive to cefazolin ($P < .01$), 90% to novobiocin ($P < .01$), 100% to vancomycin ($P > .05$), 95% to nitrofurantoin ($P < 0.05$), 50% to tetracycline ($P > 0.05$) and 60% to clindamycin ($P < .05$). Resistance to various antibiotics was 30% for cefazolin, 10% for novobiocin, 5% for nitrofurantoin, 50% for tetracycline and 40% for clindamycin. Resistance to vancomycin was not observed in any of the samples (Table 2).

Evaluation of *Staphylococcus Aureus* Sample Biofilm Production by 96-plate Microtiter Method

Biofilm production tests, performed on isolated and identified samples of *Staphylococcus aureus*, revealed that 60% of the samples were able to produce strong biofilms, 33.33% were able to produce moderate biofilms while, 6.67% were not able to produce biofilms (Figure 2).

Multiplex PCR

Both *hla* and *hlb* genes were expressed in 93% of isolated samples from patients' catheters, while in 53% of cases, *hlb* gene was expressed alone (Figure 3).

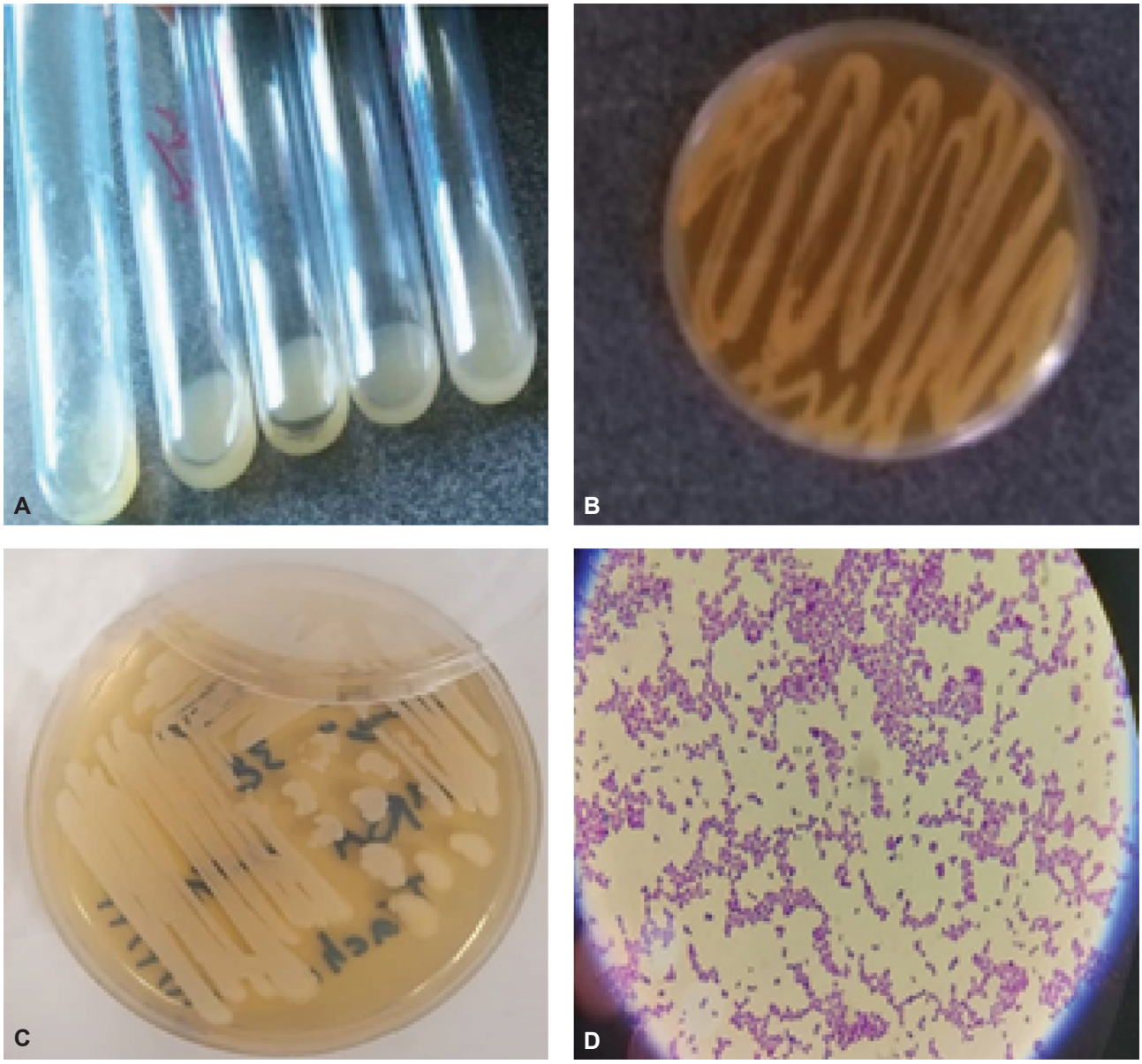

Figure 1. Phenotypic study of *Staphylococcus aureus*. A) Coagulase test, which shows that the rabbit plasma clots due to the presence of bacteria, B) Mannitol fermentation test, which shows the presence of golden colonies indicating the presence of bacteria, C) DNase test, which is the result of bacterial ribonuclease enzyme, D) gram-staining. Purple shoulder color is gram-positive bacteria, 10× microscope scale.

Table 2. Distribution of Antibiotic Resistance in *Staphylococcus Aureus* from Collected Samples (The highest susceptibility of Samples was to vancomycin, novobiocin, and nitrofurantoin. the most antibiotic resistance was related to tetracycline

Antibiotics	Sensitive %	Resistant %	*P*
Cephazolin	70	30	.01**
Novobiocin	90	10	.01**
Vancomycin	100	-	.76
Nitrofurantoin	95	5	.05*
Tetracycline	50	50	.77
Clindamycin	60	40	.05*

ns: non-significant (**P < .05, **P < .01)*).

DISCUSSION

Over the past decade, the emergence and spread of antibiotic-resistant micro-organisms has become a major concern and has put the patients' health at risk.[12] Basically, bacterial resistance to antibiotic can occur in two ways, including development of single colonies of resistant clonal lineages and horizontal transfer of resistance genes.[13]

Staphylococcus is one of the most common bacteria that can cause skin, soft tissue and invasive infections, either in hospital setting or

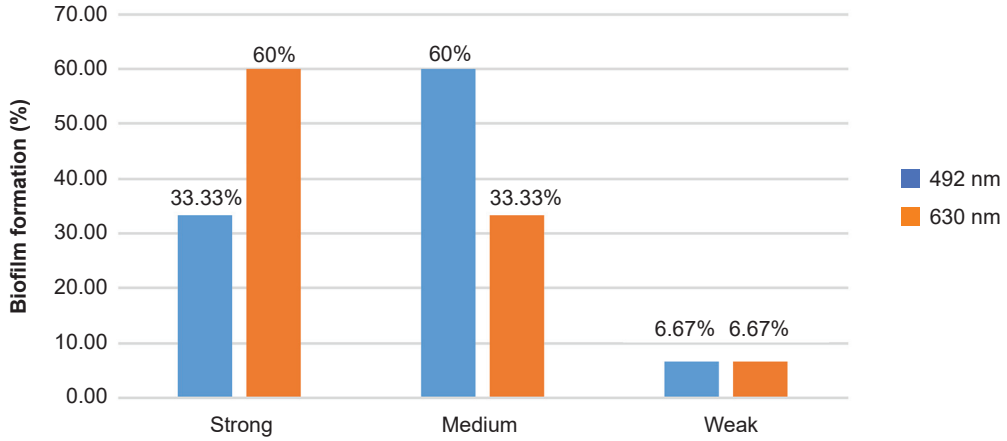

Figure 2. Biofilm Formation in the Collected Samples

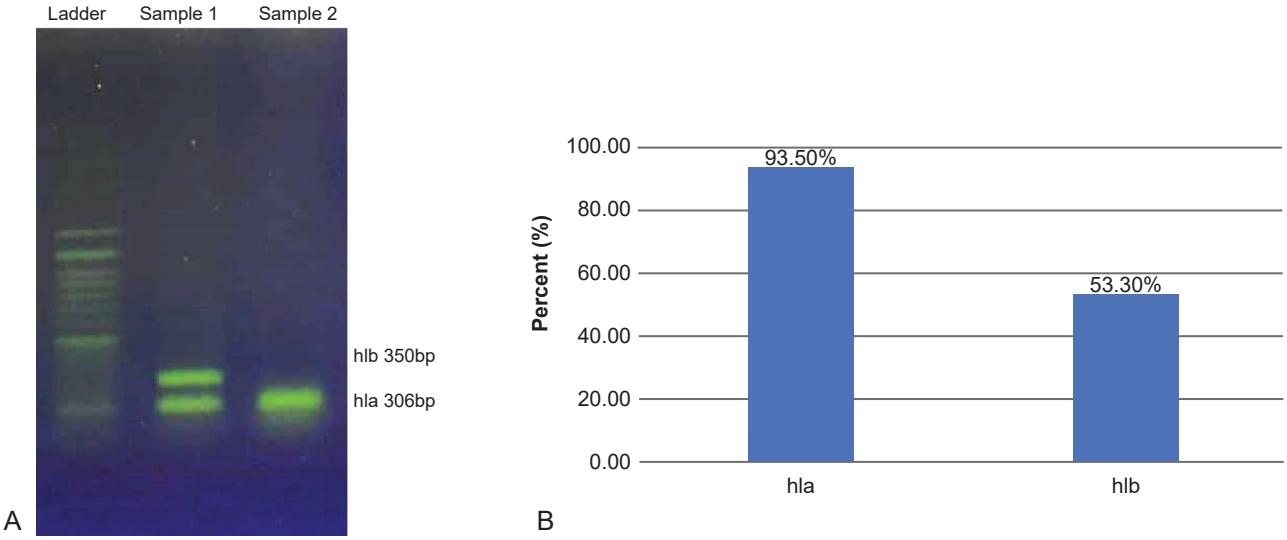

Figure 3. Detection of *S. Aureus* Virulence Factor of hla and hlb in Two Samples for Instance (A) In sample1, both *hla* and *hlb* genes were expressed, whereas in sample 2 only *hla* was expressed. B) distribution of virulence factors *hla* and *hlb* in all patient studied in this article].

in the community.[14] Nowadays, the development of antibiotic resistance in staphylococcal strains is a major challenge for the medical community. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important cause for nosocomial infections worldwide.[15] Moreover, MRSA infections are common in patients with intravascular catheters, inserted for dialysis or other medical issues.[16] The present study was carried out on *Staphylococcus aureus* isolates from catheters of 30 dialysis patients at Nikan hospital in Tehran during 6 months. It evaluated the resistance to common antibiotics in the treatment of catheter associated infections and the development of bacterial biofilms. Seventy percent of the collected samples were sensitive to cefazolin, 90% to novobiocin, 100% to vancomycin, 95% to nitrofurantoin, 50% to tetracycline, and 60% to clindamycin. Resistance to various antibiotics among the collected samples was 30% to cefazolin, 10% to novobiocin, 5% to nitrofurantoin, 50% to tetracycline and 40% to clindamycin. Resistance to vancomycin was not observed in any of the samples.

According to the result of our study, the highest sensitivity and the lowest antibiotic resistance were observed with vancomycin. In a study by Khanal *et al.* in 2012 in Nepal, the frequency of MDR-MRSA strains of *S. aureus* was reported to be 50%, with the highest resistance to ampicillin and erythromycin, and the lowest resistance to

sparfloxacin, gentamicin, and vancomycin.[17]

In the next step, the isolated samples were evaluated for biofilm production. As mentioned before, biofilm formation is important in the pathogenicity and helps the bacterium to escape the host immune system and adverse environmental conditions. In this study, biofilm production was measured at 493 and 630 nm. Among thirty samples, twenty-nine samples produced biofilms at 493 nm, out of which twenty samples produced strong biofilm strains, nine samples produced moderate biofilm strains, and no biofilm formation was observed in one sample. In addition, out of thirty samples, twenty-nine samples produced biofilms at 630 nm, of which twenty-four produced strong biofilm strains, five produced moderate biofilm strains., and one sample did not produce any biofilm. In 2016, Nourbakhsh *et al.* reported that biofilm formation was one of the mechanisms by which *S. aureus* binds to different surfaces and increases antibiotic resistance.[18] It was reported that 73.5% of isolates produced strong, 33.5% produced moderate, and 15.4% produced weak biofilms. As a result of its ability for biofilms production, *S. aureus* which is colonized on surfaces, can be an important source of infection. It is also known for producing an enamel layer, as compared to other bacteria.[19] Bacteria that are unable to produce an enamel layer, form a weaker biofilm. Biofilm plays a pivotal role in the development of multiple drug resistance among patients, by producing resistance to antibiotics and interfering with the treatment process.[20]

We also assessed the frequency of expression of *hla* and *hlb* genes by Multiplex PCR. Among a total of 30 samples, 93.5% were reported to express the *hla* gene, while 53.3% expressed the *hlb* gene, alone. A study by Coelho *et al.* in Brazil on 50 *S. aureus* bacteria collected from 15 farms showed that all isolates carried the *coa* gene with three different sizes of PCR.[21] The *SpaA* gene product for positive samples had a PCR product of 315 open pairs. Among the isolates, 26% and 19% were positive for *hla* and *hlb* genes, respectively. The expression of 22 different profiles among the samples indicated a wide variation in *S. aureus* strains that caused mastitis in this region. Portaghi *et al.* evaluated the pattern of *S. aureus* isolated from the mastitis cases in Alborz province, in 2017.[22] In this study, the genes coding the bulking factor (*clfA*), coagulase (*coa*), region X encoding protein A (*SpaA-X* region), *hemolysin A (hla), hemolysin B (hlb) and subregulatory* genes *(agrlll*) were evaluated. Isolated staphylococci were genetically analyzed and 17 different profiles were found in the studied samples, which demonstrated high diversity of *Staphylococcus aureus* strains in this region of Iran.

CONCLUSION

The results of the antibiogram of *S. aureus* isolated from the catheter of our dialysis patients revealed that this microorganism is mostly sensitive to vancomycin and resistant to tetracycline. In addition, virulence factor of *hla* expression in these cases are more than *hlb* expression.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

This study was financially supported by Shahid Behashti Medical Science University and Tehran Central Branch, Islamic Azad University.

REFERENCES

1. Yu J, Rao L, Zhan L, et al. The small molecule ZY-214-4 may reduce the virulence of Staphylococcus aureus by inhibiting pigment production. BMC Microbiol. 2021; 21(1):67.
2. Chu C, Wong MY, Tseng YH, et al. Vascular access infection by Staphylococcus aureus from removed dialysis accesses. Microbiologyopen. 2019;8(8):e00800.
3. Ibberson CB, Parlet CP, Kwiecinski J, et al. Hyaluronan Modulation Impacts Staphylococcus aureus Biofilm Infection. Infect Immun. 2016; 84(6):1917-29.
4. Luzum M, Sebolt J, Chopra V. Catheter-Associated Urinary Tract Infection, Clostridioides difficile Colitis, Central Line-Associated Bloodstream Infection, and Methicillin-Resistant Staphylococcus aureus. Med Clin North Am. 2020; 104(4):663-79.
5. Pranno N, La Monaca G, Polimeni A, et al. Antibacterial Activity against Staphylococcus Aureus of Titanium Surfaces Coated with Graphene Nanoplatelets to Prevent Peri-Implant Diseases. An In-Vitro Pilot Study. Int J Environ Res Public Health. 2020; 17(5).
6. Cheung GYC, Bae JS, Otto M. Pathogenicity and virulence of Staphylococcus aureus. Virulence. 2021;12(1):547-69.
7. Archer NK, Mazaitis MJ, Costerton JW, et al. Staphylococcus aureus biofilms: properties, regulation, and roles in human disease. Virulence. 2011; 2(5):445-59.
8. Schlievert PM. Effect of non-absorbent intravaginal menstrual/contraceptive products on Staphylococcus

aureus and production of the superantigen TSST-1. Eur J Clin Microbiol Infect Dis. 2020; 39(1):31-8.

9. Zhang X, Hu X, Rao X. Apoptosis induced by Staphylococcus aureus toxins. Microbiol Res. 2017; 205:19-24.
10. Putra I, Rabiee B, Anwar KN, et al. Staphylococcus aureus alpha-hemolysin impairs corneal epithelial wound healing and promotes intracellular bacterial invasion. Exp Eye Res. 2019; 181:263-70.
11. Feng J, Sun D, Wang L, et al. Biochanin A as an alpha-hemolysin inhibitor for combating methicillin-resistant Staphylococcus aureus infection. World J Microbiol Biotechnol. 2021; 38(1):6.
12. Ezugworie FN, Igbokwe VC, Onwosi CO. Proliferation of antibiotic-resistant microorganisms and associated genes during composting: An overview of the potential impacts on public health, management and future. Sci Total Environ. 2021; 784:147191.
13. Mackulak T, Cverenkarova K, Vojs Stanova A, et al. Hospital Wastewater-Source of Specific Micropollutants, Antibiotic-Resistant Microorganisms, Viruses, and Their Elimination. Antibiotics (Basel). 2021;10(9).
14. Cheung GYC, Bae JS, Otto M. Pathogenicity and virulence of Staphylococcus aureus. Virulence. 2021;12(1):547-69.
15. Lakhundi S, Zhang K. Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. Clin Microbiol Rev. 2018;31(4).
16. Kale P, Dhawan B. The changing face of community-acquired methicillin-resistant Staphylococcus aureus. Indian J Med Microbiol. 2016;34(3):275-85.
17. Shrestha LB, Baral R, Poudel P, Khanal B. Clinical, etiological and antimicrobial susceptibility profile of pediatric urinary tract infections in a tertiary care hospital of Nepal. BMC pediatrics. 2019 Dec;19(1):1-8.
18. Nourbakhsh F, Namvar AE. Detection of genes involved in biofilm formation in Staphylococcus aureus isolates. GMS Hygiene and infection control. 2016;11.
19. Lister JL, Horswill AR. Staphylococcus aureus biofilms: recent developments in biofilm dispersal. Front Cell Infect Microbiol. 2014; 4:178.
20. Pranno N, La Monaca G, Polimeni A, Sarto MS, Uccelletti D, Bruni E, et al. Antibacterial Activity against Staphylococcus Aureus of Titanium Surfaces Coated with Graphene Nanoplatelets to Prevent Peri-Implant Diseases. An In-Vitro Pilot Study. Int J Environ Res Public Health. 2020;17(5).
21. Coelho SM, Pereira IA, Soares LC, et al. Short communication: profile of virulence factors of Staphylococcus aureus isolated from subclinical bovine mastitis in the state of Rio de Janeiro, Brazil. J Dairy Sci. 2011; 94(7):3305-10.
22. Portaghi, H. Profile of virulence genes of Staphylococcus aureus isolated from subclinical and clinical bovine mastitis in the Alborz province. Comparative pathobiology. 2017 Jun 22; 14 (No. 2): 2165-72.

Correspondence to:
Zeinab Piravar
Department of Biology, Faculty of Sciences, Central Tehran Branch, Islamic Azad University, Ashrafi Isfahani Highway, Imam Hassan Blvd., Postal Code: 19558 47881, Tehran, Iran
Orchid ID: 0000-0001-8949-362
Tel: (+98) 9127015446
E-mail: saba.piravar@gmail.com

Received July 2022
Revised August 2022
Accepted October 2022