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The Correlation between Clumping Factor A Gene Expression in Biofilm Formation and Antibiotic Resistance Among *Staphylococcus aureus* Isolated from Urine Samples of Imam Khomeini Hospital, Tehran

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Abstract

Background & Objective: *Staphylococcus aureus* causes problems in hospitals and it has emerged as a serious agent acquired from the environment in recent years. One of the capabilities of *S. aureus* is the formation of biofilms, in which bacteria can exchange antibiotic-resistance genes among themselves and increase the virulence of other strains of this species (*S. aureus*). A surface protein attached to the cell wall in S. aureus clumping factor A is a virulence factor in various staphylococcal infections.
Materials & Methods: In this study, after the Urea Analysis (UA) test, the urea culture test was applied to the blood agar and Baird-Parker Agar culture media from the infectious urine samples in Imam Hospital, Tehran, to identify *S. aureus* isolates. Finally, a molecular method was used for the confirmation of identified isolates. The microliter plate method was performed to determine the biofilm formation ability. The disk diffusion method was also used for profiling the antibiotic resistance of the isolates.
Results: In the results of this study, 45 out of 160 urinary clinical samples were positive for *S. aureus*, among which 42 isolates expressed the *clfA* gene. Moreover, 39 isolates had the ability to form biofilms in vitro. Among these 42 isolates, the highest (88%) and the lowest (16%) rates of antibiotic resistance were observed against penicillin and cefoxitin, respectively. Data analysis with SPSS software and chi-square indicated a significant relationship between gene expression and biofilm production with antibiotic resistance ($P < 0.05$).
Conclusion: The resistance of *S. aureus* bacteria is increasing strongly due to the repeated use of antibiotics such as beta-lactams, especially in respiratory infections and pharyngitis. Moreover, biofilm formation and virulence factors, such as *clfA* and *clfB*, cause concerns to the World Health Organization for treatment, especially for people with sepsis or toxemia.

Keywords: *Staphylococcus aureus*, clumping factors, coagulase, biofilm, microtitre plate, disk diffusion, virulence factors

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Introduction

Staphylococcus is a pathogenic bacterium, and its important species is *S. aureus*. This bacterium is found on the surface of the skin and mucous membranes, and contact with soil, water, and food products leads to the contamination of humans (1).

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S. aureus is considered a short-term resident organism or a long-term colonizing organism that can cause a wide range of infections such as septicemia, sepsis, sepsis wound, septic arthritis, osteomyelitis, food poisoning, urinary tract infections (UTIs), and toxic shock syndrome (1, 2).

S. aureus is a Gram-positive bacterium that is carried by one-third of the general population and causes common infectious diseases. A known problem is the emergence of multidrug-

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resistant strains of *S. aureus*, which are of concern in the clinical setting because they are one of the most common causes of antibiotic-associated diarrhea in hospitalized patients (1, 3). One of the virulence factors of this bacterium is adhesin proteins, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) on the *S. aureus* surface, which mediate staphylococcal adherence to the extracellular matrix components of the host. These components are attached covalently to peptidoglycan by sortase enzymes. Furthermore, these components participate in biofilm formation in addition to the ica operon that produces the polysaccharide intercellular adhesion (4, 5).

Surface substances are reported to help the adhesion and colonization of bacteria, proteases, and toxins that inhibit phagocytosis by phagocytic agents, hemolysin, and other enzymes involved in the bacterial community and its invasion mentioned (6). Studies show that about 30-53% of *S. aureus* have become resistant to methicillin to these strains. Methicillin-resistant strains are called MRSA because they carry the *mecA gene* (7, 8), which encodes the penicillin-binding protein called PBP2a. The severity and spread of multidrug-resistant *S. aureus* are known as a worrying issue in the World Health Organization (9-12).

S. aureus is responsible for more than 65% of hospital infections and 80% of all bacterial infections that cause primary and secondary infections worldwide (13,14).

One of the capabilities of this bacterium is biofilm formation. These bacteria can exchange antibiotic-resistance genes among themselves and increase the virulence factor of other strains of this species (*S. aureus*) among hospitalized patients. It is considered a dangerous issue in treatment failure, especially in intensive care units (15).

The surface adhesive polysaccharide encoded by the ADBC ica operon gene is significantly involved in biofilm formation. In addition, the surface-related adhesive proteins may have an essential function in the binding of *S. aureus* bacteria. Clumping factor A and Clumping factor B are coded by *clfA* and *clfB* genes (13, 16). The surface protein attached to the cell wall of *S. aureus* is the clumping factor A (ClfA), which is a virulence factor in various staphylococcal infections (16).

ClfA increases bacterial adhesion to the blood plasma protein fibrinogen (fg) through molecular forces that have not yet been studied (13).

Clumping factor A (ClfA) is the main staphylococcal fibrinogen-binding protein (fg) and is responsible for the accumulation of *S. aureus* in blood plasma, which is greatly increased in arthritis and endocarditis. MSCRAMMs play a key role in the initiation of endovascular, bone, joint, and prosthetic-device infections. These structures can bind to molecules, such as collagen (mostly via Cna), fibronectin (via FnbAB), and fibrinogen (with ClfA, B, and Fib), thus evade the immune system, and then can develop infections (13, 4).

One of the unique yet unknown features of clfA (protein) is its ability to adhere to Fg. The connection between clfA and Fg is strengthened in cases where the bacterium is subjected to mechanical and physical stress, and the bond between clfA and immobile Fg is very weak and loose. The clfA binds to biomaterials covered with plasma protein and strengthens thereby allowing bacteria to colonize and form a biofilm (17).

Urinary tract infections caused by *S. aureus* are related to biofilm production and toxic bacteria. Many studies have investigated biofilm production genes and infections. An important research topic is the importance of related genes in the formation of biofilms, the creation of colonies, and the exchange of antibiotic-resistance genes in bacteria (18).

Materials and Methods

In Imam Khomeini Hospital of Tehran, among the people who were sent to the laboratory for urinalysis (UA) and urine culture (UC) tests by an infectious specialist based on clinical

symptoms (frequency of urination, pain during urination, urinary retention) from January 2019 to June 2019. The urine sample was prepared in a sterile container. Firstly, these UA urine samples were examined, and UC and culture were performed for samples with WBC and Epithelial cells and the presence of bacteria in more amounts. They were cultured on blood agar medium (containing 5% diffused sheep blood) and mechanical medium. Those bacteria that had grown on blood agar culture medium and produced whitish yellow colonies were cultured in Brad barker's differential culture medium. Colonies that were black in color with a clear halo (like a fish eye) were identified as *S. aureus*. After preparing slides and staining and checking whether they are gram positive and cocci, catalase test was applied to these colonies. If they were positive, they were introduced as *S. aureus*. Biochemical coagulase test was used to check the species of *S. aureus*. In this bacterium, the enzyme Coagulase is secreted and fibrinogen converts the soluble fibrin in the plasma into insoluble fibrin.

In the coagulase test, citrated rabbit serum was used and in cases where agglutination occurred, it was isolated as coagulase positive and aureus species. To definitively confirm *S. aureus*, the molecular PCR method was used to check the *23SrRNA* gene using appropriate primers (Table 1).

Table 1. Primers and conditions and lengths of PCR products

Row	gene	primer	Primer sequence 3<.....5	Size (bp)
1	*23SrRNA*	23SrRNA-1 23SrRNA-2	ACGGAGTTACAAAGGACGAC AGCTCAGCCTTAACGAGTAC	1250
2	*ClfA*	ClfA-1 ClfA-2	ATTGGCGTGGCTTCAGTGCT CGTTTCTTCCGTAGTTGCATTTG	292

In DNA extraction, bacteria were cultured in nutrient broth culture and after reaching the appropriate turbidity (after 24 hours of incubation at 37˚C), 100 microliters of the created turbidity were removed and mixed with 400 microliters of sterile distilled water in a tube. and for 10 minutes, the tube was placed in boiling water at 100˚C. After that, we centrifuged the tubes at 1000 rpm for 5 minutes at room temperature to remove sediments and the supernatant containing the pure extract DNA was extracted; In the PCR of *clfA* gene as virulence factor, as mentioned in the article of Sareh Beigom et al, the specific primer was used according to Table 1 (4). To perform PCR, the reaction in a volume of 25 microliters contains 1 µL and 2 µL of a mixture of primer pairs (20 mL/µmol), (20 µL/mmol) dNTPs Liter 10, (3 µL) Buffer PCR 10X (KCl 500Mm), Tris 100mM pH, (HCl: 4.8), 1X Triton-100, $Mgcl_2$ 15mM, 0.5Taq

mL/µmol (DNA Polymerase 5) and 15.5µL of sterile distilled water was done. Then the thermal steps of PCR were transferred by a thermocycler device with a suitable program. For the *clfA* gene we used a specific primer with 32 cycles in a thermocycler as follows: denaturation at 95˚C for 1 minute, annealing at 50˚C for 1 minute, extension at 72˚C for 1 minute and final extension at 72˚C for 2 minutes and thermocycler program for *23SrRNA* gene as 37 cycles, denaturation at 94°C for 40 seconds, annealing step at 64˚C for 1 minute, extension at 72˚C for 75 seconds were observed after electrophoresis of 1-5. Agarose gel and ethidium bromide staining under UV rays, and bands of *23SrRNA* gene with size 1250 and *clfA* gene with size 292 were seen (14, 18).

To evaluate the production of biofilm, the colonies of *S. aureus* enriched on TSB medium (Trypticase Soy Broth) containing 1% glucose were cultured and incubated for 4 hours at 37˚C. After 24 hours, the microplates were gently shaken three times with the sterile PBS solution and washed and then the plate was placed upside down on a filter paper to dry. Then, methanol was used for fixation and 100 microliters of methanol were added to all the wells and left at room temperature for 15 minutes. After that, removing the alcohol and drying the plate in room conditions, 100 microliters of 1% crystal violet dye were added to all the wells of the plate and placed in room conditions for 20 minutes, then the plates were gently washed with tap water until the excess dye should be removed. Then the bound dye was released with 150 microliters of 33% acetic acid and ready to be read with a wavelength of 492 nm by ELISA reader (14). All measurements were repeated three times and medium alone was used as a negative control. A standard deviation higher than the optical absorbance OD of negative control was considered as the cut-off. The ability to form biofilm was calculated from the following formulas as described in the Table 2.

Table 2. Evaluation of biofilm formation rate

Row	evaluate	Colorimetric intensity (calorimetry)
1	Absence of biofilm formation	OD<ODC
2	weak	ODC<OD<2xODC
3	medium	2xODC<OD<4xODC
4	Strong	4xODC

In order to check the antibiotic resistance of *S. aureus*, first some of the Staphylococcus vareus colony was removed from Brad Barker's culture medium with fish eye-like colonies and dissolved in sterile physiological serum. Swap was cultivated on Muller Hinton agar culture medium. After cultivation, the antibiotic discs (Antibodnet Tab-Iran) including cefoxitin (31 micrograms), oxacillin (9 micrograms), erythromycin (19 micrograms), penicillin (11 micrograms), ciprofloxacin (9 micrograms), tetracycline (31 micrograms), amikacin (31 micrograms), cefazolin (31 micrograms), gentamicin (11 micrograms), were used by Bauer-Kirby disk diffusion method (3, 19, 20).

Statistical Analysis

In this study, each sample was considered as a variable and in each variable, three factors (one expression of *clfA* gene with biofilm formation and the other antibiotic resistance with expression of *clfA* gene) were investigated. *clfA* gene expression and antibiotic resistance of each sample were checked in chi-square (primary evaluation) and then the same sample was checked in terms of *clfA* gene expression and antibiotic resistance (secondary evaluation). It was observed between gene expression and antibiotic resistance. When the primary and secondary evaluations were significant, it was declared as a significant relationship.

Results

Out of 160 clinical samples with positive urine culture in Imam Hospital, 70 samples (43.75%) were Staphylococcus, which were grown in blood agar and Brad Parker, and 50 samples (72%) were *S. aureus* and positive for coagulase. Among these, 45 (90%) samples were determined to be *S. aureus* through the examination of the *23S rRNA* gene having a band with a length of 1250 bp using the molecular PCR method (Figure 1).

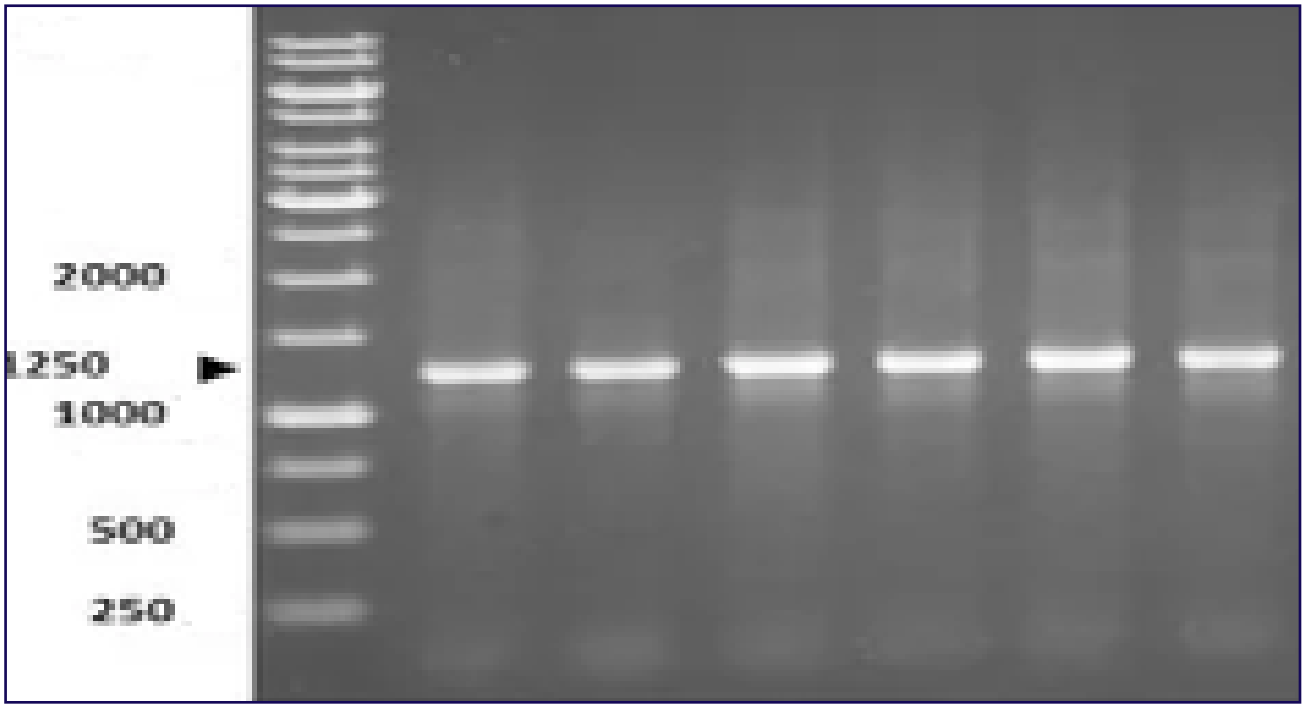


Figure1. Tested samples with a product length of 1250 in gene expression and amplification of *23SrRNA* gene

Out of 45 isolates (94%), 42 samples expressed the *clfA* gene with a band length of 292 bp (Figure 2).

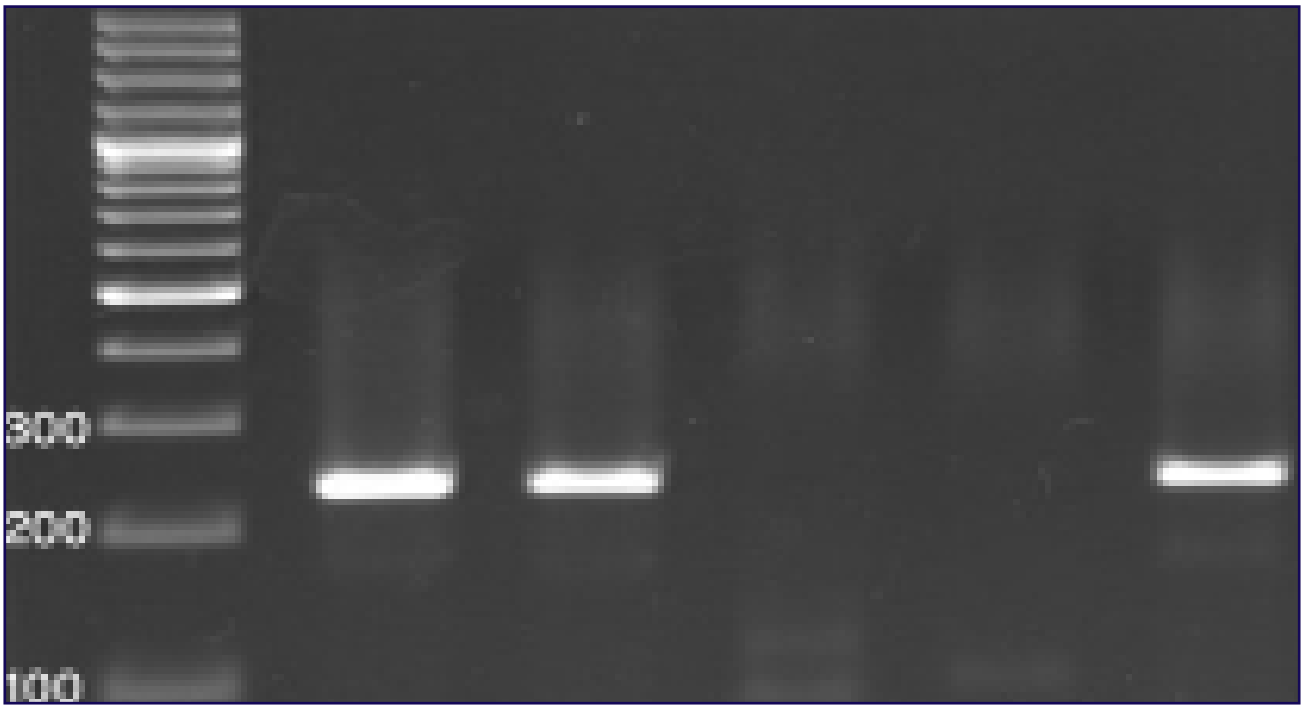


Figure 2. Tested samples with a length of 292 in the expression and amplification of the *clfA* gene

Out of 42 isolates, three (7%) isolates did not form a biofilm, six (14%) isolates formed a weak biofilm, 27 (65%) isolates produced a moderate biofilm, and six (14%) isolates created a strong biofilm (a total of 39 isolates (93 %) had biofilm) (Table 3).

Table 3. The amount of biofilm formation in 42 samples isolated by microtitreplate method

Number	No formation of biofilm	Weak biofilm formation	Moderate biofilm formation	Strong biofilm formation
42 sample	7% (3 sample)	14% (6 sample)	65% (27sample)	14% (6 sample)

Of these 42 isolates, the most antibiotic resistance was against penicillin and the most sensitive against cefoxitin (Table 4).

Table 4. The level of resistance and sensitivity of 42 isolated from urinary tract infections

antibiotic	sensetive	Semi-sensetive	resistant
cefazoline	79% (23 sample)	12% (5 sample)	9% (4 sample)
cefoxitine	84% (35 sample)	12% (5 sample)	4% (2 sample)
oxacillin	22% (9 sample)	22% (9 sample)	56% (24 sample)
erythromycin	30% (7 sample)	30% (7 sample)	40% (28 sample)
penicillin	0% 0 sample)	12% (5 sample)	88% (37 sample)
Ceprofloxacin	76% (32 sample)	10% (4 sample)	14% (6 sample)
tetracyclin	7% (3 sample)	29% (12 sample)	64% (27 sample)
amikacine	62% (26 sample)	24% (10 sample)	14% (6 sample)
gentamicin	19% (8 sample)	15% (7 sample)	66% (27 sample)

The results obtained after the statistical analysis by SPSS showed the expression of the *clfA* gene in the samples isolated from urinary infections with the biofilm formation in vitro and the antibiotic resistance in each sample that underwent each test. Thus, it can be claimed that gene expression had significant relationships with biofilm formation, strain virulence, and antibiotic resistance in most cases (P-value = 0.001).

Discussion

S. aureus is an important pathogen that causes significant morbidity and mortality. The origin of methicillin-resistant *S. aureus* includes hospital infections and infected patients colonized with this bacterium. Transmission from one patient to another causes the spread and resistance of *S. aureus* and this is considered a serious threat to the whole world (3). One of the virulence factors of *S. aureus* bacteria is the production of toxins and extracellular factors, such as biofilm, which can adhere to and resist phagocytosis (15, 19).

S. aureus has the capability of biofilm formation and the expression of adhesion factors to survive in the environment. Biofilm formation and the transfer of antibiotic-resistance genes between different strains lead to resistance. It also spreads from cell phagocytosis by biofilm formation, which causes *S. aureus* to become a chronic infection in the clinical setting. In this study, the biofilm was formed by 93% out of 42 samples that expressed the *clfA* gene, indicating that this *clfA*, as a surface protein of the *S. aureus* bacterial wall, causes biofilm formation in

the bacterial community. Biofilm is a compound formed by microorganisms with surface junctions enclosed outside their cells. This surface protein aggregation factor causes escape from phagocytosis and the host's immune system during and after biofilm production.

At biofilm formation, antibiotic-resistance gene fragments are transferred between the plasmids of different strains, thereby increasing antibiotic resistance intensity. The production of biofilm by *clfA* also raises the issue of quorum sensing in *S. aureus* bacteria, which accepts a community and solidarity for better bacterial activity (20, 21).

In a study on the phenotype and genotype of biofilm-forming genes in *S. aureus* isolated from clinical cases and food and examined by a microtitre plate and multiplex PCR methods, the *clfA* gene was reported to be 93.33% abundant in clinical samples (Sara Begi Madani et al., 2016). The authors also observed a significant relationship between this gene and biofilm production (phenotype). All the isolates could produce biofilms, even a weak amount, and the frequency of the *clfA* gene in clinical samples was higher than in food samples (14).

The low abundance of *clfA* and other adhesive factors in food items indicates that these adhesive factors *(clfA)* are highly expressed for binding to the mucus and skin for the colony. Moreover, biofilm production is important especially in mucous tissues, as in our study, in which the isolates were isolated from the urinary mucosa, and the *clfA* gene was examined with a frequency of 94%, and 93% of these isolates produced biofilms.

In another study, 299 infectious clinical samples were isolated from the ICU department of a hospital, among which 105 samples were *S. aureus*, 90.5% of which were MRSA and 91.5% showed multiple antibiotic resistance (MDR) (14).

Noorbakhsh et al. (2004) studied the ability to form biofilm by molecular and phenotypic methods in Shahrekord infectious samples, where 73.5% of their isolates had a high ability (strong adhesive) to form biofilm, 5.33% had moderate attachment, and 15.4% were able to form biofilms. They had a weak connection in biofilm production with the frequency of different genes such as *icaC* and *icaB*. The results of this study showed that the frequency of genes with biofilm formation was also significant in the direction of overlapping each other and the relationship between them (14, 16). In line with our study, they reported that adhesive factors in *S. aureus* bacteria with a high frequency caused biofilm formation.

Motamedi et al. (2016) investigated adhesion factors in MRSA clinical isolates to determine antibiotic-resistance patterns. They found a significant relationship between the expression of genes of adhesion factors in *S.aureus* and antibiotic resistance of the same isolates (3, 22, 23).

Our study also demonstrates that exactly adhesion factors cause the formation of biofilm and Quorum Sensing activity, ultimately causing the transfer of antibiotic-resistance genes in other strains and isolates.

Atefeh Karimi et al. (2018) investigated the phenotype and genotype of biofilm formation among *S. aureus* strains isolated from patients with urinary tract infections in Isfahan hospitals (24, 25). They proposed 72% of the strains as biofilm producers, and the frequencies of ica*A, icaD*, *clfA*, *fnbA*, and *cna* genes were 43, 86, 75, 61, and 84%, respectively. They discussed biofilm formation and the expression of virulence genes. Similar to our results their results showed a significant relationship between biofilm formation and the expression of virulence genes.

Finally, this study showed a significant relationship between the *clfA* gene and biofilm formation, and on the other hand, biofilm formation had a significant relationship with antibiotic resistance ($P < 0.05$).

Limitations of this study included a low number of isolates, no evaluation of other surface adhesion expression, and the lack of patients' demographic data.

Conclusion

Urinary and genital infections (cystitis, urethritis, vaginitis, urethritis, etc.) in the world are usually caused by Enterobacteriaceae and coliforms, especially *E. coli*, which arise due to the lack of public hygiene and a small number of genital and urinary infectious samples (20, 22, 24). Gram-positive bacteria are present, but in elderly people or immunosuppressive people (immune system deficiency) and also as secondary infections or infections transferred from a patient. Due to the frequent use of antibiotics, especially Beta-lactams, following respiratory infections and pharyngitis, the resistance of *S. aureus* is increasing rapidly, and biofilms formation and virulence factors, such as clfA and clfB, are a concern of the WHO for eradication, especially among patients suffering from sepsis or toxemia.

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Conflict of Interest

The Authors declare no conflict of interest

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