Evaluation of the Effect of Sub-inhibitory Concentrations of Ceftriaxone on Combating Multi-drug Resistant Staphylococcus aureus

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ABSTRACT

Staphylococcus aureus is the deadliest of all the common Staphylococcal bacteria and is regarded as one of the most common bacteria that cause illnesses. S. aureus's ability to build biofilms and other virulence variables determines whether it can cause superficial or invasive infections. It can also rapidly alter its virulence and metabolic responses in various tissues. Anti-virulent medications that stop the development of biofilms and virulence factors are therefore becoming increasingly necessary. As a result, we focused on an antibiotic that had already been approved and investigated whether changing the dosage would have an impact on S. aureus virulence. Most of the studied isolates exhibited a noteworthy decrease in S. aureus biofilm and virulence factors production (protease, hemolysin, and coagulase) upon treatment with 1/4 and 1/8 MICs of ceftriaxone, according to the data. It might therefore be regarded as a survival strategy to enhance patient outcomes and reduce germ resistance.

Keywords: Staphylococcus aureus, Biofilm, Virulence factors, Sub-MIC concentrations, Ceftriaxone
1. INTRODUCTION

Staphylococcus aureus is one of the most prevalent infectious microorganisms that cause illness and death worldwide. In addition to being a human pathogen, S. aureus is a commensal bacterium. About 30% of people on the Earth have S. aureus infection. In addition, it is a major contributor to infections connected to devices, pleuropulmonary, skin and soft tissue, osteoarticular, and infective endocarditis.

S. aureus strains generate a multilayered biofilm entrenched in the slime layer and expressing a diverse protein. Antibiotics of various kinds can’t treat S. aureus infections, especially those linked to their biofilms. The ica genes create a polysaccharide intercellular adhesion (PIA), which makes it possible for “biofilm” formation by the bacteria that enables them to grow on both biotic and abiotic surfaces. In addition to protecting the bacteria against various environmental challenges, biofilm stops antibiotics from penetrating and interacting with the bacterial cells. Thus, one of the most effective ways to stop the development of antibiotic resistance from occurring in S. aureus is to target the biofilm formation. Biofilm formation is the mechanism by which S. aureus clings to and persists on the native host tissues, such as bone and heart valves, thus causing infective endocarditis and osteomyelitis, respectively. These are in addition to the implanted medical devices, prosthetic joints, artificial heart valves, catheters, and orthopedic implants, which cause severe and enduring infections in hospitalized patients within a healthcare setting.

Furthermore, the pathogenic strains of S. aureus invade and cause infection by expressing various distinct virulence factors that remarkably participate in the host-pathogen interactions, such as proteases, coagulases, hemolytic toxins (a, b, c, and d), and toxic shock syndrome toxin 1 (TSST1). By releasing toxins and exoenzymes, these virulence factors not only aid in the pathogen's entry into host tissues, immune system evasion, and adherence to host cells, but they also inflict tissue damage. The degree of bacterial infection is determined by the expression of these virulence factors, which are regulated by multiple regulatory loci, including the Staphylococcal accessory regulator (sarA) gene and the accessory gene regulator (agr) gene.

In our research, we looked for a medication that could be used to counteract the growth in microbial resistance. To that end, we reexamined the use of ceftriaxone by assessing how it affected bacterial biofilm formation and virulence factors.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

Nutrient agar, nutrient broth, mannitol salt agar, Mueller-Hinton agar, Luria-Bertani agar, skim milk agar, Luria-Bertani broth, Tryptic soy broth, ceftriaxone, casein, Trichloroacetic acid, Folin, hydrogen peroxide, glacial acetic acid, methanol, ethyl alcohol, sulfuric acid, phenol, and crystal violet.

2.2. Bacterial isolation and identification

The clinical samples of sputum, urine, wound, and blood were collected from patients who were admitted to Tanta University Hospitals, Tanta, Egypt, and cultivated immediately on nutrient agar (NA) after being grown in nutrient broth (NB) (Oxoid, UK). After incubation for 24 h, the recovered bacterial colonies were subjected to traditional identification procedures, such as Gram-staining, growth on mannitol salt agar (MSA) plates, and standard biochemical identification techniques like catalase and coagulase tests.

2.3. Minimum inhibitory concentrations of the recovered isolates

The minimum inhibitory concentration (MIC) values of ceftriaxone against S. aureus isolates were ascertained using the agar dilution technique. Briefly, the molten Mueller-Hinton agar (MHA) was poured into Petri dishes after ceftriaxone (CTR) was added at escalating concentrations (multiple of two, i.e., 0.25, 0.5, 1, 2, 4, 1024 µg/ml) using serial two-fold dilutions. Using an automated inocette, an aliquot of each isolate's prepared suspension was delivered to a specific well in the sterile multi-inoculator seed plate following the record key setup. To prevent the carryover issue, each well in the seed plate was only partially filled. The sterile inoculating rods were used to gently lower the head into the seed plate's well, lift it, and then lower it again onto the agar medium to inoculate the prepared plates. Every rod deposited roughly 1 µl, resulting in a final inoculum of 104 CFU/spot per isolate on the agar surface. Every plate was incubated for one night at 37°C.

2.4. Screening of biofilm production by S. aureus isolates

Screening of biofilm formation was performed using 96-well flat bottom plates briefly. The colonies were then homogenized using a vortex mixer and diluted to achieve a turbidity level equivalent to 0.5 McFarland standard in two ml of Tryptic soy broth (TSB) (Oxoid, USA) from overnight cultures. After inoculating each well with 100 µl of the bacterial suspension, the plates were incubated at 37°C for 24 h. The formed biofilm was fixed using 100% methanol for 20 min. After that, gently cleaning the plates using phosphate-buffered saline (PBS), 100 µl of 0.1% crystal violet (CV) was added to each well and left for 30 min. Following washing the wells with distilled water (DW), the excess CV was removed, and then dissolving CV using a 33% (v/v) glacial acetic acid microtiter reader.
(Sunrise TM, TECAN, Switzerland) was used to evaluate the biofilm formation by measuring the OD at 595 nm.  

2.5. Screening for virulence factor expression by S. aureus isolates

Using Luria-Bertani agar (LB) plates made with 5% skim milk and 1.5% agar (Oxoid, USA) on them, the tested isolates were grown. After incubating the plates for 48 h at 37°C, we examined the plates to check if any clear zones had formed around the implanted bacteria. To test the hemolytic activity of S. aureus isolates, 4% human blood was added to LB agar. The lysis zones surrounding the inoculated bacteria were examined on the plates after 48 h at 28 °C to ascertain their development.

2.6. Growth curve of S. aureus isolates before and after ceftriaxone treatment

The isolates (n = 13) that produced all virulence factors under investigation were cultivated in LB broth with and without 1/2, 1/4, and 1/8 MICs of ceftriaxone (CTR) at 37°C. The OD value for treated and untreated isolates was maintained at 0.3 and samples from each culture were taken every 30 min (at zero time, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, and 420 min.), and the absorbance was measured at 600 nm.

2.7. Testing the effect of ceftriaxone at sub-MIC on S. aureus biofilm formation

2.7.1. Crystal violet microtiter plate assay

The impact of CTR on S. aureus isolate biofilm formation was investigated, as prescribed by. Briefly, using a 96-well microtiter plate, the isolates were cultured in TSB for 24 h at 37°C with and without 1/8 MIC and 1/4 MIC of CTR. The formed biofilm was fixed using 100% methanol for 20 min, dried, and stained with 200 µl of 0.1% CV for 15 min. The plate was then dried after rinsing with water. The stained biofilms were dissolved in 200 µl of 33% (v/v) glacial acetic acid, and then the OD at 595 nm was evaluated using a microplate reader (Sunrise TM, TECAN, Switzerland). Negative control was used to assess the quality of the process and performed with the same previously mentioned steps with only one exception as no antibiotic or bacteria was added to it. The experiment was performed in triplicate.

2.7.2. Phenol-sulfuric acid assay

This assay was conducted to investigate how CTR affected the exo-polysaccharide (EPS) production in the S. aureus biofilm matrix. The bacterial isolates were grown for 24 h at 37°C in LB broth with and without 1/8 and 1/4 MIC CTR. After incubation, the samples were centrifuged at 8000 g for 10 min., and the pellets were re-suspended in PBS before being centrifuged once again. After centrifugation, an equivalent volume of ethyl alcohol was added to the supernatant. Finally, 1 ml of EPS solution, 5 ml of concentrated sulfuric acid, and 1 ml of cold 5% phenol were carefully mixed. OD was measured at 490 nm, and the percentage of EPS decrease was calculated using the following equation: \[
\text{Percentage of EPS decrease} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100
\]

2.7.3. Auto aggregation assay

The visual test tube settling method was used to evaluate the sedimentation rate, which is in turn related to bacterial auto-aggregation; therefore, we used it to evaluate the impact of CTR on S. aureus auto-aggregation. Briefly, 5 test tubes of overnight cultures with and without 1/4 and 1/8 MIC of CTR in LB broth were adjusted at 600 nm to the same optical density and then vigorously vortexed. The OD was measured at (0 h, 2 h, 4 h, 8 h, and 24 h) by taking one time from each test tube superfluous supernatant.

2.8. Testing of sub-MIC of ceftriaxone on virulence factors

2.8.1. Protease production

The bacterial isolates were screened on skimmed milk for protease production after being cultivated in LB broth with and without CTR for 24 h at 37°C. After incubation, the broth was centrifuged at 5000 rpm and then filtered using a Millipore filter (0.45 µm). The skim milk agar plates' wells were filled with bacterial supernatants (100 µl). After incubation for 24 h at 37°C, the diameter of the formed lysis zones developed around the wells was measured using a calibrated ruler. Moreover, protease assay using casein as a substrate was conducted. A bacterial suspension in LB broth was prepared with and without CTR and incubated for 24 h at 37°C. After incubation, 1 ml of the supernatant obtained after centrifugation and filtration was added to 1 ml of 0.05 M phosphate buffer and 0.1 M NaOH containing 2% casein and then incubated at 37°C for 10 min. The reaction was stopped by adding 2 ml of 0.4 M trichloro-acetic acid (TCA) and incubated at 37°C for 30 min. The obtained solution was centrifuged at 5000 rpm for 15 min. Finally, 3 ml of filtrate was mixed with 5 ml of Na2CO3 and 2 ml of Folin, and then the absorbance was measured at 660 nm. Negative control was performed as the same as the previously mentioned steps expect only the first step as LB broth was used without CTR and without bacteria. The experiment was performed in triplicate.
2.8.2. Hemolysin production

Using both qualitative and quantitative approaches, hemolysin production was found to be impacted by CTR. In the qualitative approach, 180 μl of LB broth, either with or without CTR, was mixed with bacterial overnight culture (20 μl) or incubated at 37 °C. After incubation for 18 h, the samples were streaked on human blood agar plates for 24 h at 37 °C. The resulting clear zone surrounding the inoculated bacterium was observed. In the quantitative approach, S. aureus strains were inoculated in LB broth with and without CTR. After incubation, centrifuging, and filtration of the resulting supernatant, 600 μl of a 2% red blood cell (RBC) suspension was mixed with 600 μl of the supernatant and incubated for 2 h at 37 °C. After incubation, the obtained suspension was centrifuged for 8 min. at 4 °C at 10,000 g. Hemoglobin release was recorded by measuring the absorbance at 540 nm.

2.8.3. Coagulase production

The bacterial supernatants were double-fold diluted serially in 96-well, round-bottom microtiter plates using brain heart infusion (BHI) (Oxoid, USA) broth. All wells received 100 μl of a citrated plasma solution (20% v/v) before being combined with serial dilutions of CTR. The plates were incubated at 37 °C and observed after 4 h of incubation. The maximum dilution at which plasma coagulation was observed, or the titer, was measured (the titer, which is the reciprocal of the greatest dilution, shows plasma coagulation). Negative control treatments were used by combining citrated rabbit plasma with BHI broth. The experiment was repeated three times.

2.9. Statistical analysis

One-way analysis of variance (ANOVA), the T-test, and a significance cut-off of 0.05 for p-value were used to examine the data. Every assay was conducted three times, and the results were expressed using the mean standard deviation (±SD).

3. RESULTS

3.1. Bacterial isolates

Regarding the sources of the clinical samples, most bacterial isolates were recovered from sputum (n = 41), urine (n = 19), wound (n = 7), and blood (n = 3) as shown in Fig. 1.

3.2. Determination of minimum inhibitory concentrations of the Ceftriaxone

The MICs of CTR were determined against the bacterial isolates (n = 70). Ceftriaxone MICs ranged from 8 μg/ml to 256 μg/ml. Most of the isolates (n = 36) have MIC = 8 μg/ml, followed by MIC = 16 μg/ml (n = 15), then MIC = 256 μg/ml (n = 8), then MIC = 64 μg/ml (n = 4), and finally MIC = 128 μg/ml (n = 1).

3.3. Bacterial growth rate in the presence of sub-MIC of Ceftriaxone

The effect of CTR at 1/2, 1/4, and 1/8 MICs on the bacterial growth of the selected isolates (n = 13) was tested at different intervals. The obtained data showed that 1/2 markedly affected bacterial growth, 1/4 MIC showed lower activity, and the effect of 1/8 MIC was negligible as shown in Fig. 2.

3.4.1. Crystal violet microtitration assay

The biofilm formation of the selected bacterial isolates (n = 13) was significantly affected (p < 0.05), as shown in Fig. (3), and demonstrated in Table (1). In the presence of CTR, most isolates showed a reduction in biofilm formation at both 1/4 and 1/8 MIC treatments. The recorded reduction of CTR ranged from -34 to 63 %, and 1.6 to 66 %, with 1/8 and 1/4 MICs treatments, respectively. The previous percentage reduction calculated using the following equation = (OD untreated – OD treated) / OD untreated * 100
Figure 2. Growth curves of the selected 13 *S. aureus* isolates.
Table 1. Percent (%) of biofilm reduction in the presence of sub-MIC of Ceftriaxone. CTR: Ceftriaxone. The asterisks represents the statistical significance (p<0.05).

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<thead>
<tr>
<th>Isolate Code</th>
<th>% Reduction in biofilm formation with sub-MIC of CTR</th>
<th>Isolate Code</th>
<th>% Reduction in biofilm formation with sub-MIC of CTR</th>
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<td>63***</td>
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3.4.2. Phenol-sulfuric acid method for assessment of exo-polysaccharide formation

Further investigation on the effect of CTR on biofilm formation was done by studying its effect on matrix formation (EPS). There was a significant (p < 0.05) reduction in EPS formation in most of the tested bacterial isolates that may be up to 85% after 1/4 MIC treatment and up to 80% with 1/8 MIC treatment (Fig.4).

3.4.3. Auto aggregation measurement

The impact of 1/8 and 1/4 MIC of CTR on bacterial auto-aggregation was determined by measuring the sedimentation rate. From OD measurement, we observed that sub-MIC of CTR decreased the rate of bacterial auto-aggregation in most of the tested isolates, and this was highly correlated with the reduction in bacterial biofilm formation. (Fig. 5).

3.5. The impact of ceftriaxone at sub-MIC on S. aureus virulence

The results of sub-MIC of CTR on the production of protease, hemolysis, and coagulase were evaluated.

3.5.1. Protease production

The effect of sub-MIC of CTR on S. aureus protease production was evaluated. The treated S. aureus isolates were significantly (p< 0.05) affected by CTR treatment as previously shown in Fig. 6. Exposure to CTR at 1/4 and 1/8 MICs caused a reduction in protease production by 28-100 % and -33-44 %, respectively.
Figure. 5. Auto aggregation measurement through measuring the % reduction of initial OD value for 13 isolates

Figure. 6. A representative result about the effect of CTR at sub-MIC on S. aureus protease production (A) The most common result that was detected with treatment. (B) Percent reduction of protease production with different concentrations of CTR. The error bars indicate standard deviations. The asterisks represent statistical significance (P< 0.05).
3.5.2. Hemolysin production

The hemolytic activity of S. aureus isolates was detected with and without treatment by 1/4 and 1/8 MICs of CTR. The degree of hemolysis was determined using the spectrophotometric method. The exposure to CTR at 1/4 and 1/8 MICs caused a reduction in hemolysin production by 18-100 % and -32–46 % (Fig. 7).

![Fig. 7. Percent reduction in hemolysin production with different concentrations of CTR. The error bars indicate standard deviations. The asterisks represent statistical significance (P< 0.05).](image)

3.5.3. Coagulase production

Through the determination of the coagulation titer, the effect of treatment with sub-MIC of CTR on coagulase production was evaluated. Results showed that CTR at 1/4 and 1/8 MICs significantly (p< 0.05) affected coagulase production as seen in Fig. 8. Most bacterial isolates showed a reduction in the coagulation titer that in some cases reached 100% reduction.

![Fig. 8. A representative result about the effect of CTR at sub-MIC on S. aureus coagulase production. (A) Decrease of coagulation titer with sub-MIC treatment. (B) Percent of the reduction in titer of coagulase after CTR treatment. The error bars indicate standard deviations. The asterisks represent statistical significance (P< 0.05).](image)

4. DISCUSSION

The Gram-positive Staphylococcus aureus germs cause a broad range of clinical illnesses. This bacterium is frequently the main source of infections in both hospital and community settings. On healthy skin, S. aureus often does not cause infections, but if it gets into the circulation or internal tissues, it can cause several potentially dangerous illnesses. This trait makes S. aureus among the most frequent reasons for people to get infections. A biofilm is a collection of linked cells from microorganisms that are covered in a matrix made of proteins, various organic compounds, and extracellular polymeric substances (EPSs). Polysaccharide intercellular adhesion (PIA), which is generated by the intercellular adhesion (ICA) locus, is a significant constituent of S. aureus biofilms. Biofilm production is also connected to the expression of the ICA gene. The bacteria that form biofilms are up to 1000 times less sensitive to antibiotics, which makes them considerably harder to cure.

Apart from the conventional resistance mechanisms of S. aureus, it is characterized by its capacity to persist in the biofilm state on biotic and abiotic surfaces. This trait makes S. aureus among the most frequent reasons for people to get infections. A biofilm is a collection of linked cells from microorganisms that are covered in a matrix made of proteins, various organic compounds, and extracellular polymeric substances (EPSs). Polysaccharide intercellular adhesion (PIA), which is generated by the intercellular adhesion (ICA) locus, is a significant constituent of S. aureus biofilms. Biofilm production is also connected to the expression of the ICA gene. The bacteria that form biofilms are up to 1000 times less sensitive to antibiotics, which makes them considerably harder to cure.

In this study, we tried to indicate the impact of the sub-MIC of CTR on S. aureus biofilm production. From our study, it was detected that CTR at 1/8 and 1/4 MIC caused a reduction in biofilm formation, which ranged from -34 to 63% and from 1.6 to 66%, respectively. Moreover, the impact of CTR at various concentrations on the S. aureus biofilm matrix was investigated by measuring EPS formation using the phenol-sulfuric acid assay and through OD measurements. The reduction in color intensity in most of the tested isolates was detected, which indicated that there was a decrease in EPS formation by sub-MIC treatment.

Moreover, it was revealed that there was a significant
reduction in bacterial auto-aggregation in most of the tested isolates, which was highly correlated to their reduction in biofilm formation except for the S8 isolate. Additionally, the pathogenicity of an infection caused by S. aureus is determined by its virulence factors, which include toxins, secreted enzymes (i.e., lipase and protease), and adhesins (i.e., fibronectin-binding protein and protein A).

Therefore, the ability of an antibiotic to inhibit the release of virulence factors by a bacterium may be just as crucial to the effectiveness of antibiotic therapy for infections caused by S. aureus as its bacteriostatic or bactericidal properties.36, 37

In this study, the impact of CTR on S. aureus virulence factor production was studied. The obtained results indicated that there was a significant reduction in protease production that ranged from 28–100% and -33–44% after exposure to 1/4 MIC and 1/8 MIC, respectively. Ceftriaxone also reduced hemolysin production that ranged from 18 to 100% and from -32 to 46% with 1/4 and 1/8 MIC treatments, respectively, which was detected using the spectrophotometric method from which the amount of released hemoglobin was calculated. This was directly proportional to hemolysin production. Furthermore, coagulase production was significantly reduced after treatment with 1/4 and 1/8 MIC of CTR, which ranged from 0-100% and -50-100%, respectively.

El-Mowafy et al., findings corroborated with our results, showing that modest β-lactam dosages drastically altered the Quorum sensing QS signals and reduced the virulence factors38. Moreover, Caixeta et al., study showed that sub-MICs of β-lactam suppressed the virulence gene expression and biofilm formation.

Additionally, Kumar demonstrated how several β-lactams had anti-virulence and anti-QS properties. A previous study conducted by Viedma et al., showed that the relative levels of agr locus expression were impacted by oxacillin at sub-MIC, which affected the virulence and biofilm formation. Previous studies reported that, when compared to the untreated control, the isolates treated with a β-lactam antibiotic at sub-MIC exhibited lower levels of hemolysis.42, 43

Stoitsova et al., study revealed that the various β-lactam antibiotics at various sub-MIC doses affected the formation of biofilms in different ways; either by inducing or inhibiting the growth of the distinct bacterial strains.44

According to Frank et al., the cell wall-active antibiotics either did not affect S. aureus biofilm formation or had an inhibitory effect at sub-MIC doses.45 In accordance, Majidpour et al., study except for a single strain that showed an increase in biofilm formation, S. aureus exposed to sub-MICS of oxacillin significantly reduced the production of biofilms.46

Moreover, García-Torrico et al., revealed that sub-MIC caused irreversible physiological alterations, including delayed growth, aggregation, modification, and changes in outer membrane proteins (OMPs) and lipopolysaccharide (LPS) profiles, resulting in a complete diminution of Yersinia ruckeri's virulence.47 A previous study revealed that Pseudomonas aeruginosa exposed to β-lactam antibiotics at sub-MIC increased extracellular toxins production which in turn led to worsening in the Pseudomonas aeruginosa infection model.48

Also, it was reported previously by Hodillé et al., that treatment with sub-MIC of β-lactams has the potential to increase Staphylococcal pathogenicity and worsen the infection.49 The synthesis of α-toxin, PVL toxin, enterotoxins, toxic shock syndrome toxin (TSST), and LukED is induced by the β-lactam antibiotics at non-lethal concentrations, which led to therapeutic modifications and worse outcomes.49

5. CONCLUSION

The rise in microorganism resistance makes it more difficult for the patient to heal fully, ultimately worsening their prognosis. It is highly desirable to find new drugs rather than conventional ones to reduce the severity of the infection and compact bacterial resistance; as the ability of bacteria to form biofilms and secrete various virulence factors is a crucial factor in establishing the infection and reducing bacterial sensitivity. It was discovered that the current antibiotic, when taken at its 1/4 or 1/8 MICs, significantly reduced the development of both biofilm and virulence factors in the majority of the studied bacterial isolates without the need for studying their safety as it had been used for several decades, but further studies may be required to confirm the inhibitory effect of sub-MIC and exclude any undesired results that may occur, and until that, it must be taken at its recommended dose.

AUTHOR’S CONTRIBUTIONS

Conceptualization: Tarek El-Banna, Fatma Sonbol, and Maisra El-Bouseary; Data curation: Amira Omar and Maisra El-Bouseary; Formal analysis: Amira Omar, Tarek El-Banna, Fatma Sonbol, and Maisra El-Bouseary; Methodology: Amira Omar, Tarek El-Banna, Fatma Sonbol, and Maisra El-Bouseary; Visualization: Tarek El-Banna, Fatma Sonbol, and Maisra El-Bouseary; Writing – original draft: Amira Omar and Maisra El-Bouseary; Writing – review & editing: Amira Omar, Fatma Sonbol, and Maisra El-Bouseary. All authors have read and agreed to the published version of the manuscript.

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