Evaluation of D-amino Acid in Combination with Flucloxacillin on the Formation and Dispersal of Staphylococcus aureus Biofilm

Khalid Faisal Alahmadi, Abdulrahman Mohammed Alalyani, Salman Ali Abdali, Emad Alharbi, Abdulaziz Jafar almussallam, Khalid Ibrahim Alahmadi and Ammar AL-Farga

1King Fahad Hospital, Ministry of Health, Al Madinah Al Munawwarah, Saudi Arabia
2Almeqat Hospital, Ministry of Health, Al Madinah Al Munawwarah, Saudi Arabia
3Biochemistry Department, College of Sciences, University of Jeddah, Saudi Arabia

*Corresponding author: Ammar AL-Farga, Biochemistry Department, College of Sciences, University of Jeddah, Saudi Arabia

Received: October 10, 2019
Published: October 16, 2019

Abstract

Staphylococcus aureus is one of the most common bacterial strains and has been linked to various infections ranging from light respiratory and skin infections to fatal conditions such as endocarditis osteomyelitis and periodontitis. However, S. aureus has been resistant to antibacterial drugs and body immune system response mechanisms owing to its efficient adaptation mechanisms such as biofilm formations. D-Amino acids are believed to play a significant role in the structural formation of the biofilm's peptidoglycan. The amino acids form the peptide chains and crosslinks with N-acetylmuramic acid. Altering the structural composition of the compounds would compromise the mechanical integrity of the biofilm and, thus, could be used as a potential target for preventing biofilm formation. The aim of the given study is to evaluate the effect of D-amino acid in combination with flucloxacillin on the formation and dispersal of Staphylococcus aureus biofilm. This is achieved by determining the minimum inhibitory and lethal concentrations of D-Amino acids (D-glutamate, D-aspartate and D-methionine) in combination with flucloxacillin against Staphylococcus aureus. The effectiveness of D-Amino acids and flucloxacillin is investigated separately and the results compared. D-aspartate and D-methionine show antibacterial activity whereas D-Glutamate promotes bacterium multiplication. The minimum lethal concentration of flucloxacillin is 1.25mg/mL, whereas the minimum inhibitory concentration was 0.625mg/mL. D-methionine improved the flucloxacillin's antibacterial efficacy and demonstrated a potential clinical significance by ANOVA test.

Keywords: D-amino acid; Staphylococcus aureus; Flucloxacillin

Introduction

Staphylococcus aureus (Staph aureus, S. aureus or Golden staph) is a Gram-positive bacterium that belongs to the phylum Firmicutes (Marking & Shaw). The bacterium is non-moult, spherical shaped and grows in clusters that resemble a bunch of grapes. When grown on blood agar plate, S. aureus forms round golden-yellow colonies with haemolysis. Staphylococcus aureus is facultative-an aerobic, catalase and coagulase positive and, therefore, can be isolated from other Staphylococci by a coagulase test. Staph aureus is a human commensal parasite that has been identified to colonise the anterior nares of about 30-50% of people. The bacterium commonly grows in the respiratory tract, particularly the nasal cavity and the skin. However, Golden staph has been identified with several respiratory and skin infections (Hood, 2015) [1]. The bacterium is said to be an opportunistic microgram that tends to cause infections due to several factors such as a weakened immune system.

The type of infections that arise from S. aureus range from simple skin lesions, boils and sties to more serious infections such as endocarditis [2], osteomyelitis (Tang, et al., 2017), periodontitis and peri-implantitis [3] and fatal septic shock meningitis and pneumonia (Jansen (Van Vuuren, 2015). Like all other pathogens, it has evolved mechanisms to establish itself in the host and cause infection by means of virulence factors. These include surface proteins that help bacterium attach to the host cell and prevent phagocytosis. Invasions such as kinases and hyaluronidase are employed to help
the spread of the bacterium. In addition, biochemical processes and toxins enhance survival [4]. Additionally, *S. aureus* secretes agr-controlled virulence factors such as immune avoidance and toxic antibodies that lyse inflammatory effectors of the immune system such as polymorphonuclear leukocytes (PMNs).

**Staphylococcus aureus Antibiotic Resistance Mechanism**

*Staphylococcus aureus* is the leading cause of many bacterial infections in the world. According to Pantosti et al. (2007), the bacterium quickly evolves into multidrug-resistant clones upon invading the body. The transfer of this antibiotic resistance trait within the *S. aureus* colony can be linked to multiple gene transfer mechanisms. Many of the strains found in hospitals are often resistant to many antibiotics. In fact, vancomycin intermediate resistant strains have also been reported [5] Methicillin resistance *Staphylococcus aureus* (MRSA) is the most common and widespread resistant strain. MRSA is resistant to antibiotics and disinfectants, thereby aiding its survival in the hospital environment [6].

Several mechanisms have been deployed by the bacterium to acquire resistance such as by acquisition of extrachromosomal plasmids or additional genetic information in the chromosome and by mutations in chromosomal genes [6]. In the biofilm environment, *S. aureus* can transfer genes through conjugation and mobilization (Savage et al. 2013). The bacteria also have an increased rate of bacteriophage release and, hence, the transfer can take place through transduction (Resch et al., 2005). Some scientific experiments have observed that mobile genetic elements that are transferred primarily between the bacteria consisting of the antibiotic resistance genes might be responsible for the formation of biofilm as well [7,8]. Notably, *S. aureus* developed a strain that hinders the body’s immune response mechanism of iron starvation by obtaining the supply from transferrin and heme using alpha-hemolysin toxins (Dahners, 2015). The toxin forms pores on red blood cells to access hemoproteins. The bacterium also develops circumvention mechanisms to obtain other ions such as copper and manganese from the blood cells.

The current medical response to the growing clinical complexity of bacterium infections includes the application of carbolic acid, typically, chlorhexadine and mupirocin [9]. An attempt to supplement the conventional treatment by triggering the host’s immune system and the microbial virulence factors is currently under study. Dryden et al. [10] report a prospect vaccination solution to the outmaneuvering antibiotics resistant bacterium’s strain as a permanent solution to *S. aureus*’ antibiotic resistance adaptation.

**Staphylococcus aureus Biofilms Mechanism**

A biofilm is defined as an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002). Biofilms are a bacterial community embedded in a matrix produced from polysaccharides, protein and DNA and attached to a tissue surface. The matrix of bacterial biofilms is responsible for structural stability and prime protection of the group. *S. aureus* consortia have shown significant resistance to antibiotics, biocides and all other chemical toxicants (Høiby et al., 2010). In addition, the matrix can resist the human body’s immune system and is not susceptible to phagocytosis contrary to planktonic growth.

The development of *S. aureus* biofilm typically proceeds through five stages: attachment, multiplication, exodus, maturation and dispersal (Figure 1). During the attachment phase, planktonic bacterium attaches to the surface of its host tissue. Throughout the process, the cells utilize microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) for cell-to-cell attachment and bind to the host matrix components. The multiplication stage begins with asexual reproduction. The cells undergo multiple cell divisions and accumulate, thereby forming the cluster. In the process, the bacteria release extracellular proteins (FnBPs, ClfB and SDRC) and polysaccharide intracellular adhesin (PIA) that promote cellular binding at the initial stages of the attachment and maintain the stability of the immature biofilm. Cytoplasmic nucleoid-associated proteins bind to eDNA and form a component of the extracellular matrix (ECM).

**Figure 1:** Stages of biofilm formation (Woehl, 2017).
By the end of the multiplication stage, a specific and coordinated bacterium cell's release is observed. This controlled cell movement marks the exodus phase. The process typically begins six hours after the bacterium's attachment and coincides with microcolony formation. The detachment is mediated by nuclease-initiated eDNA degradation. This stage is then followed by the development of a micro-colony structure. Other than increased surface area, the structure also provides the site for nutrient exchange and waste removal. High-density biofilm develops to adapt to the environmental stressor. These processes mark the maturation stage of the biofilm.

In the maturation stage, Golden staph undergoes relatively high cell division forming the extracellular matrix of the host factor and extracellular DNA (eDNA). Recent studies have revealed that the array also contains biomolecules such as polysaccharides, proteins and polysaccharide intercellular antigens (PIA) (Wallace, 2017) [8]. PIA is primarily composed of β-1, 6-linked N-acetylglucosamine residues but also contains anionic fraction and some traces of non-N-acetylated D-glucosaminy1. The compound is synthesized by the in vitro UDP-N-acetylg glucosamine activities and gives the biofilm its toxic properties.

The structure has diversified protein components with coordinated gene expression. This diversity is vital for its survival mechanism against antibiotics. This biofilm then keeps on growing, sometimes forming mushroom- or tower-like structures that are maximally resistant to antibiotics or other antimicrobial agents. After a specific period, auto-inducing peptide (AIP or octapeptide) accumulates within the matrix and activates the histidine kinase. The histidine kinase then phosphorylates the response regulator and establishes P3 promoter transcription. The process releases RNA molecules which regulate the release of virulence factors causing the dispersal of the biofilm cells.

The bacterial cells can move to other areas and initiate the development of new biofilms. The biofilm's bacteria clusters can be recognized under a light microscope. The pathogen's precise identification can only be conducted through DNA hybridization and specialized staining techniques (Høiby et al.). Benson, however, presents several incidences of PIA-independent biofilm developments including are gene locus coded activities, fibronectin-binding proteins, biofilm-associated protein (Bap) and Bap-related proteins. The processes of cell division and extracellular matrix deposition continue as the film accumulates into glycocalyx or slime layers. Golden staph cells within the matrix sometimes reactivate the planktonic state, causing the dispersal of the film (Bookenberger et al.) (Figure 1).

**Biofilm Antibiotic Resistance Mechanisms**

Different antibiotics and antimicrobial agents have been tested against S. aureus biofilms. However, these studies have consistently shown that the antibiotic's susceptibility has significantly reduced in the biofilm mode of growth as compared to when they are grown in planktonic mode [7,8]. The increased antibiotic resistance is linked to their targeted mutations mechanism, decreased cell permeability to the antimicrobial agents and chemical modifications of the enzymes.

Variable mutation across these biofilm layers and hyperactivity at the bacterium are the two aspects that increase the matrix's resistance to antimicrobial agents. The bacteria have a higher rate of mutation and transfer their genetic material horizontally, thus becoming highly resistant to multiple antibiotics. The bacteria cell can also alter the target so that its affinity for binding to the antimicrobial is significantly reduced.

**Effect of Flucloxacillin against Staphylococcus aureus**

Flucloxacillin is a narrow-spectrum beta-lactam antibiotic that belongs to the class of antibiotics called penicillin. Historically, it has been shown to be effective against penicillinase-producing strains of the bacteria such as S. aureus. It is an isoxazole penicillin antimicrobial agent that is well absorbed after oral or intramuscular administration. As compared to other types of isoxazole penicillin’s, flucloxacillin has been found to be effective against Gram-positive cocci which are now inherently penicillin-resistant (Sutherland et al., 1970). The mechanism of action of flucloxacillin is the same as for other beta-lactams; it acts by inhibiting the cross linking of the linear peptidoglycan polymer, thus inhibiting synthesis of the bacterial cell wall. Because the cell wall in Gram positive bacteria such as S. aureus has a thick peptidoglycan, the integrity of the cell wall is compromised, and cell death occurs.

**Problem Statement**

S. aureus was first discovered in human abscesses and presented as a causative agent in wound infection in 1880 at Berlin Surgical Congress by a Scottish surgeon, Alexander Ogston [3]. From then on, the bacterium has continued to colonize the human population and emerged as one of the significant skin and soft tissue infectious pathogens. S. aureus became a significant clinical challenge and community health concern because it was linked to several cases of recurring, persistent and chronic infections including rhinosinusitis, osteomyelitis and periodontitis.

The bacterium rapidly evolved and developed different resistance mechanisms against the human immune system and antibacterial drugs. S. aureus has an immunomodulatory virulence mechanism and adhesion characteristics that alter the immune response activities of its host. Consequently, a series of clinical preventive and treatment techniques have been established. Such methods include carbolic acids (chlorhexadine and mupirocin), glycol peptides (teicoplanin vancomycin) and oxazolidinones (OXAs).

Current developments in the medical field include the introduction of solithromycin (SOL), fluoroquinolones, ozenoxacin, nemonoxacin and zabofloxacin. However, the efficacy of such technologies is limited by several factors and most importantly antibiotic agents are only effective in the early phase of infection because they cannot penetrate the biofilm matrix. Clinical protocols such as aminoglycoside nephrotoxicity and the surgical removal of the bacterium biofilm have been found to be complex and costly. Advanced antibacterial therapy is, therefore, an unmet requirement.
Resolution

Consequently, we introduce a novel antibacterial technology where Flucloxacillin and D-amino acids (D-glutamate, D-aspartate and D-methionine) are combined to develop a new *S. aureus* biofilm dispersal polytherapy. The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of flucloxacillin were determined and the inhibitory effect of each of the D-amino acids on biofilm was separately assessed. Subsequently, flucloxacillin was combined with the amino acid and its effectiveness was examined at the optimum amino acid concentration, comparing it to that of pure flucloxacillin. Statistical analysis was also undertaken to establish the clinical significance of the novel idea.

Objectives

The aim of the study is to investigate the antibacterial effectiveness of flucloxacillin when combined with D-amino acids (D-glutamate, D-aspartate and D-methionine) on *S. aureus* biofilm. The data are compared with the effectiveness of flucloxacillin alone. We hypothesise that D-Amino acids will improve the antibacterial efficacy of flucloxacillin when combined. The specific objectives include:

a) To examine minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of flucloxacillin.

b) To analyse three D-amino acids’ efficacy on the *S. aureus* biofilm separately.

c) To explore the efficacy of flucloxacillin when combined with each of the three D-amino acids at optimum concentration.

d) To compare the antibacterial effectiveness of the combinatorial agents and flucloxacillin alone.

e) To undertake a statistical test (ANOVA) to validate the clinical significance of the newly developed antibacterial agent.

Materials and Methods

Health and Safety

All experiments were conducted using aseptic techniques and in accordance with the University of Wolverhampton’s code of practice regulations for project work in the School of Pharmacy.

<table>
<thead>
<tr>
<th>Tube</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimicrobial/TSB dilution factor</td>
<td>1/2</td>
<td>1/4</td>
<td>1/8</td>
<td>1/16</td>
<td>1/32</td>
<td>1/64</td>
<td>1/128</td>
<td>1/256</td>
<td>Only TSB</td>
<td>Only TSB</td>
</tr>
<tr>
<td>Inoculum (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>Positive control</td>
<td>Negative control</td>
<td></td>
</tr>
<tr>
<td>Observation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In the present study, serial antimicrobial agents with varying concentrations were prepared by a sequential double dilution of the antimicrobial agents provided using TSB prepared in the previous laboratory session. 5ml AgNO₃ of the initial level was added to 5ml TSB to obtain ½ the dilution. Half the volume of the resulting solution (5ml) was transferred to 5ml TSB to achieve a dilution of ½. This procedure was repeated until a dilution of 1/256 was achieved. The dilution series is as shown in Table 1. Tubes 9 and 10 were left undiluted for control tests. 100µl dilute inoculum was added to the first antimicrobial samples in the dilution series (Table 1). The samples were incubated for 18 hours at 37°C in a static incubator and growth was recorded.

Biofilm Formation

The biofilm formation was conducted in 96-well plates according to the directions of Toole (2011). 100 μL aliquots of the adjusted bacterial suspension were inoculated into individual wells of the 96-well flat-bottomed polystyrene plate under aseptic conditions overnight at 37°C for 72 hours in the static incubator. The excess culture was discarded, and the plate gently washed using 100μl of 1X phosphate buffered saline (PBS; pH 7.4) using a multichannel pipette.

The regulations guiding toxic sample disposal were observed. The wells were stained using 0.1% Crystal Violet for 30 minutes at room temperature. Excess crystal samples were washed out four times using distilled water and dried using a paper towel. The plate was then set upside down to dry overnight and a snapshot captured the appearance of the dry wells coated by the biofilm. The 96-well plates were incubated for 15 minutes in preparation for biofilm quantification. 125µl solubilize biofilm (95% ethanol) was added to the wells and the samples were incubated for another 15 minutes. The 96-well plate containing the bacteria biofilm. The experiment was then incubated for 24 hours at 37°C and the biofilm bacterium inhibition effect investigated. The biofilm dispersal protocol was conducted in triplicate for all three of the D-amino acids tested and the averaged result compared to the previous lab exercise.

Biofilm Dispersal Using D-Amino Acid Solutions

Initially, 2.13g, 2.35g and 5.66g of D-aspartic acid, D-glutamic acid and D-Methionine acid were dissolved in sterilised distilled water to obtain 100mL with a concentration of 160mm. The samples were left to stir on a magnetic stirrer for 30 minutes until the amino acid was fully dissolved. After that, amino acids were filter sterilized. The amino acid stock samples were stored in aliquots at a temperature below freezing point (-30°C).

The solutions were diluted to provide two-fold the recommended concentration by the protocol. Double strength MHB was then prepared, autoclaved and added to the D-amino acid samples to obtain the serial of two-fold dilution of the D-amino acid/ MHB combination. 80mM, 40mM, 20mM, 10mM and 5mM were added to the 96-well plate containing the bacteria biofilm. The experiment was then incubated for 72 hours at 37°C and the biofilm bacterium inhibition effect investigated. The biofilm dispersal protocol was conducted in triplicate for all the D-amino acids tested and the average effect compared to the previous lab exercise.

Biofilm Dispersal Using D-Amino Acids/ Flucloxacillin Combinatorial

Each of the D-Amino acids was prepared to the optimum concentration marked as the most efficacious dilution in the previous exercise. Four times the MLC (5mg/ml) Flucloxacillin was added to the D-Amino acid solution. Three other samples were prepared and used for comparison; typically, 5mg/ml Flucloxacillin, pure MHB and D-Amino acid at its optimum inhibition concentration. 100µL of 1 × 107 cells/mL inoculum was added to the 96-well plate containing the bacteria biofilm. The experiment was then incubated for 24 hours at 37°C and the biofilm bacterium inhibition effect investigated. The biofilm dispersal protocol was conducted in triplicate for all four sample acids tested and the averaged result compared to the D-amino acid and Flucloxacillin separate tests. Antibacterial efficacy was investigated based on the spectrophotometer concentrations readings.

Statistical Analysis

In order to determine the clinical significance, an analysis of variance (ANOVA) test was conducted on the data that demonstrated potential antibacterial efficacy.

Results

Broth Dilution Assay

The values of flucloxacillin’s MLC and MIC against S. aureus were 1.25 mg/ml and 0.625 mg/mL respectively (Figure 2.1). The minimum concentration was observed in the test tube with the respective concentration level. Bacteria growth was observed to be increasing in concentration from test tubes 4 to 9. Test tubes 1, 2, 3 and 10 were clear (Table 2.1). The minimum inhibitory level was taken as the lowest concentration with no bacteria growth observed [T4 = 0.625]. Test tube 10 was used as a negative control test. Similarly, an MLC test is also presented below (Table 2.2).

Figure 2.1: Broth dilution test for flucloxacillin MIC. The lowest concentration with no bacteria growth was recorded in test tube 4.
The study investigated the impact of adding amino acids to the antimicrobial *Staphylococcus aureus* biofilm growth. Three D-amino acids (D-Aspartic acid, D-Methionine acid and D-Glutamic acid) were first tested separately. The assessment of the antibacterial efficacy of the amino acids was made by taking series of optical absorbance of each biofilm after they had been exposed to varying concentrations of the samples (Figure 2.2). The effectiveness of the tested samples was analyzed from their ability to reduce the bacteria concentration in the biofilm.

**Table 2.1:** Broth dilution MIC test.

<table>
<thead>
<tr>
<th>Tube</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5000</td>
<td>1.2500</td>
<td>0.6250</td>
<td>0.3125</td>
<td>0.1563</td>
<td>0.0781</td>
<td>0.0391</td>
<td>0.0391</td>
<td>0.0391</td>
<td>Only inoculum</td>
</tr>
<tr>
<td></td>
<td>0.0195</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Only TSB</td>
</tr>
<tr>
<td>Inoculum (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>Positive control</td>
<td>Negative control</td>
</tr>
<tr>
<td>Bacterial growth</td>
<td>Clear</td>
<td>Clear</td>
<td>Clear</td>
<td>4</td>
<td>15</td>
<td>25</td>
<td>29</td>
<td>37</td>
<td>40</td>
<td>Clear</td>
</tr>
</tbody>
</table>

**Table 2.2:** Broth dilution MLC test.

<table>
<thead>
<tr>
<th>Tube</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5000</td>
<td>1.2500</td>
<td>0.6250</td>
<td>0.3125</td>
<td>0.1563</td>
<td>0.0781</td>
<td>0.0391</td>
<td>0.0391</td>
<td>0.0391</td>
<td>Only inoculum</td>
</tr>
<tr>
<td></td>
<td>0.0391</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Only TSB</td>
</tr>
<tr>
<td>Inoculum (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>Positive control</td>
<td>Negative control</td>
</tr>
<tr>
<td>Bacterial Growth</td>
<td>Clear</td>
<td>Clear</td>
<td>Clear</td>
<td>4</td>
<td>13</td>
<td>23</td>
<td>29</td>
<td>37</td>
<td>73</td>
<td>Clear</td>
</tr>
</tbody>
</table>

**Effect of D-Amino Acids**

Figure 2.2: D-Amino acid test; a) D-aspartic; b) D-Glutamate; and c) methionine.

Figure 2.3: Antibacterial test for D-Methionine acid; average absorbance against concentration.
D-Methionine and D-Aspartic acid indicated uniform antibacterial effectiveness on the biofilm. The concentration of the bacteria was observed to drop with the concentration of the antibacterial agents (Figures 2.3 & 2.4) (Tables 2.3 &2.4). The trend continued until an optimum level was reached above which the bacteria concentration was observed to rise with the increase in antibacterial agent concentration. Both D-Amino acid samples reach their optimum efficacious concentration at 20mM.

Figure 2.4: Antibacterial effect of D-Aspartic acid test.

Table 2.3: D-Methionine acid activity ANOVA.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>0.263632667</td>
<td>7</td>
<td>0.03766</td>
<td>1.545</td>
<td>0.161</td>
<td>2.104</td>
</tr>
<tr>
<td>Columns</td>
<td>1.659013367</td>
<td>14</td>
<td>0.11850</td>
<td>4.86</td>
<td>9.9E-07</td>
<td>1.793</td>
</tr>
<tr>
<td>Error</td>
<td>2.389309833</td>
<td>98</td>
<td>0.024381</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.311955867</td>
<td>119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: D-Aspartic acid antibacterial activity test.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>0.3286009</td>
<td>7</td>
<td>0.04694299</td>
<td>3.008001</td>
<td>0.006632</td>
<td>2.104448</td>
</tr>
<tr>
<td>Columns</td>
<td>1.995803217</td>
<td>14</td>
<td>0.14255737</td>
<td>9.134757</td>
<td>1.78E-12</td>
<td>1.793981</td>
</tr>
<tr>
<td>Error</td>
<td>1.52939185</td>
<td>98</td>
<td>0.01560604</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3.853795967</td>
<td>119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

However, D-Methionine acid showed similar effectiveness at 40mM, contrary to D-Aspartic whose activity started to decline with a rise in acid concentration above its optimum value. Again, a significant drop in antibacterial activity at 10mM was observed in both samples against the trend. This discrepancy was attributed to experimental errors including sample contamination, measurement error and non-uniform incubation conditions. Analysis of variance, however, showed that only D-Methionine was of clinical importance (P-value = 0.161>0.05). D-Aspartic has less P-value and would be rejected for clinical application.

Figure 2.5: The graph of observance against different D-glutamate concentrations.
On the other hand, the D-Glutamic acid test showed that sample concentration increased the rate of bacteria growth (Figure 2.5). A uniform rise in S. aureus concentration in the biofilm samples was observed with an increase in the D-Amino acid except for 40mM where a significant drop was evident. The observation in the three D-Amino acid samples used is summarized as shown in Figure 2.6.

The antibacterial activity of the three D-amino acid samples was also investigated when combined with flucloxacillin (Figure 2.7). Only D-Methionine showed an improved rate of antibacterial activities when used in combination with flucloxacillin in comparison to the pure flucloxacillin and amino acid samples used separately. The sample recorded OD595 of 0.057 compared with MLC of 0.009 and Methionine of 0.153. Additionally, the ANOVA test indicated that the sample was of clinical significance as compared to its pure amino acid test Figure 2.8 & Table 2.5.

![Figure 2.6: The comparison of the three-amino acid sample performance.](image)

![Figure 2.7: Flucloxacillin/D-Amino acid antibacterial test; a) D-Aspartic; b) D-Glutamate; and c) D-Methionine](image)

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Source of variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td></td>
<td>0.045395219</td>
<td>7</td>
<td>0.006485</td>
<td>1.385956</td>
<td>0.262509</td>
<td>2.487578</td>
</tr>
<tr>
<td>Columns</td>
<td></td>
<td>0.001934094</td>
<td>3</td>
<td>0.000645</td>
<td>0.137782</td>
<td>0.936317</td>
<td>3.072467</td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td>0.098261156</td>
<td>21</td>
<td>0.004679</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>0.145590469</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5: D-Methionine ANOVA test.

On the other hand, the other two antibacterial agents prepared showed an increasing effect on bacteria growth when combined with flucloxacillin instead. Higher concentrations of the bacteria in the biofilm were observed when the inoculum was subjected to the combinatorial culture as compared to the two cases where the samples were used separately. The mixture recorded an OD595 of 0.89, compared to pure D-Amino acid of 0.49 and MLC of 0.18 in D-Aspartic acid (Figure 2.9). Similarly, D-Glutamate recorded OD595 of 0.89 when combined with flucloxacillin, which was relatively higher compared to MCL (0.19 OD595) and the pure sample (0.49 OD595; Figure 2.10).
In summary, only two D-Amino groups (D-Methionine and D-Aspartic acid) out of the tested samples showed the inhibition effect when used separately. However, only D-Methionine indicated significant consistency in the ANOVA test. The other sample showed an increased bacterial growth rate. When used in combination with flucloxacillin, only D-Methionine was effective. ANOVA indicated that the test would be consistent and, therefore, is of clinical significance. The MLC and MIC values of flucloxacillin against S. aureus were 1.25 mg/ml and 0.625 mg/mL respectively.

Discussion

In the given study, an analysis was undertaken to investigate the efficacy of pure flucloxacillin and in combination with D-amino acids (D-glutamate, D-aspartate and D-methionine) against S. aureus. Although the mechanism of D-amino acids against biofilm remains a mystery, studies have indicated that these compounds have a significant effect on the matrix complex [12,13]. Von Dach [12], for instance, determined that the bacterial signalling agents responsible for inducing biofilm dispersal are functionally analogous to D-amino acids. Huppert (2015) isolated sample D-Amino acids from Bacillus subtilis biofilms and concluded that the substance was a biofilm synthesis inhibitor. According to Marking & Shaw [13], D-Amino acid molecules disrupt extracellular bonding and disassemble the structure. As such, they offer a potential clinical medication.

From the experiment, only D-Methionine and D-Aspartic acid demonstrated a significant antibacterial effect on the biofilm when the amino acids were used separately. D-Aspartic acid, however, showed non-significant P-value in the ANOVA test, indicating that the agent may not be of clinical value. The D-Glutamic acid test, on the other hand, indicated an increase in bacteria growth with the concentration of the antimicrobial level. Flucloxacillin recorded a 1.25 mg/ml MLC and 0.625 mg/mL MIC against the bacterium [14].

Only D-Methionine demonstrated significant growth inhibitory activity against S. aureus biofilm when used in combination with flucloxacillin. In the remaining two samples, a higher concentration was recorded for the mixture as compared to when constituents were used separately. From ANOVA, the D-Methionine test demonstrated a significant consistency, indicating that the compound offers a promising potential clinical antibiotic therapy [15].

The results in this study are comparable to several other studies in the field (Dahners; Wallace, 2017). Wallace [7] for example, identified D-Amino acids as the components of biofilm peptidoglycan. According to the study, the amino acids form peptide chains and crosslinks with N-acetylmuramic acid and develop a three dimension-like layer that gives the bacterium its biofilm mechanical integrity. Additionally, the study identified that the structure was relatively thick in Gram-positive bacteria and forms 80% of the biofilm’s dry weight.

In reference to Woehl [7], penicillin binds to DD-transpeptidases, thereby altering oligopeptide crosslinks of peptidoglycan. The deformation not only compromises the mechanical integrity and allows accessibility of immune response agents but also inhibits gene transfer across the biofilm. S. aureus, therefore, is incapable of developing antibiotic resistant genes. It was also shown that N-acetylmuramide glycanhydrolyase which is a protein in nature hydrolyses glycosidic bonds in the peptidoglycan matrix. This therefore indicates that amino acids play a significant role in building and breaking the biofilm matrix [16-18].

The therapeutic target of the structural protein component within the matrix can be a potential clinical tool against biofilm dispersal. More importantly, D-amino acid therapy can prevent biofilm development and make the bacterium susceptible to other antimicrobial and body immune response agents. The concept, therefore, explains the improved performance observed with D-Methionine acid on flucloxacillin antibacterial activity. Additionally, flucloxacillin is reported to bind with penicillin-resistant N-acetylmuramide glycanhydrolyase [19-21].

Dahners (2015) also presents D-Amino acids as an officious Pseudomonas aeruginosa biofilm dispersant. The study investigated the effect of adding sample D-Amino acids to three antibacterial agents; typically penicillin, chlorhexidine and nisin and showed that D-Trp and D-Met have significant antibacterial influence against Pseudomonas aeruginosa biofilm. Dahners (2015) agrees with Wallace (2017) that the D-Amino acids are structural components of peptidoglycan and play a significant role in regulating the formation and breakdown of biofilm components. Notably, Dahners (2015) postulated that amino compounds alter the exopolysaccharide components of peptidoglycan, thereby reducing their mechanical integrity and decomposing the biofilm matrix.

Damian (2017) further adds that amino compounds demonstrated a significant inhibitory effect against biofilm formation. The process of biofilm formation involves selective protein uptake. The structural function of the protein blocks in the biofilm matrix could be associated with the trend observed with D-Glutamate. The sample facilitated bacteria multiplication, implying that the D-Amino acid was a prerequisite material for biofilm development. Only very few studies, however, have examined amino acid consumption during biofilm development. We thus propose the role of amino acid in biofilm formation as a topic for future study [21-24].

Conclusion

The current study has established a novel antibacterial therapeutic therapy. Three groups of D-Amino acids (D-glutamate, D-aspartate and D-methionine) were investigated with regards to their role in S. aureus biofilm dispersal activity. The samples were then tested in combination with flucloxacillin. The minimum lethal concentration of flucloxacillin was 1.25 mg/mL, while the minimum inhibitory concentration was 0.625 mg/mL. D-aspartate and D-methionine showed a significant antibacterial effect when used separately. However, there was an inconsistency in D-aspartate, thereby nullifying for clinical treatment [0.016<0.05]. D-Glutamate was observed to facilitate the growth rate. On the other hand, only D-methionine was observed to improve the antibacterial activities of flucloxacillin. The ANOVA indicated that the D-Methionine
test had a significant consistency and that the technique offers a promising potential clinical antibacterial therapy.

Appendices

References

15. Bookenerger L, Hoet A (2017) Presence, Distribution, And Risk Factors Associated with Staphylococcus Aureus Among Veterinary Health Care Workers at The Ohio State University Veterinary Medical Center.