Effect of Ciprofloxacin on the Growth and Biofilm Formation Ability of *Staphylococcus aureus*

Abstract

Staphylococcus aureus is part of the normal bacterial flora of the skin, intestine and upper respiratory tract of both humans and animals and has the potential of causing staphylococcal infections if there is a breach in the hosts' defense mechanism. These infections could range from mild superficial skin infections to more severe and even fatally invasive diseases such as sepsis and toxic shock syndrome. The infectivity of *S. aureus* is attributed to its ability to withstand extreme conditions and its possession of various virulence factors. The aim of this project was to study the effect of ciprofloxacin on the growth and biofilm forming ability of CM10 strain of *Staphylococcus aureus* using time kill study, resazurin and live/dead staining of biofilms and Real-time polymerase chain reaction. The identity of the given CM10 strain was confirmed when the result of the API-Staph was in total accordance with the results obtained from the colony morphology and phenotypic characterization tests (Coagulase/protein A, Gram, and Catalase tests). CM10 strain of *S. aureus* was not susceptible to 0.25mg/L of ciprofloxacin used for the time kill experiment but was susceptible to a minimum inhibitory concentration of 0.5mg/L. The difference between the ciprofloxacin treated biofilms of CM10 strain and the untreated biofilms was significant ($P<0.05$) showing that ciprofloxacin has an adverse effect on the cells in the biofilm. The results of this study provide an insight on the growth as well as the biofilm forming ability of CM10 strain of *Staphylococcus aureus.* Ciprofloxacin has been shown to be an effective antibacterial against this strain of *S. aureus* by its inhibitory effect on the growth as well as biofilm forming ability of this strain of *S. aureus*. This information would assist in developing novel anti-biofilm therapies to help in the management of biofilm mediated infections thereby reducing the morbidity and mortality rate of staphylococcal infections.

Key Words: *Staphylococcus aureus*, Ciprofloxacin, antibiotics, minimum inhibitory concentration, Biofilm Formation.

INTRODUCTION

Staphylococcus aureus is a bacterium which was first identified in the 1880s by Sir Alexander Ogston in Aberdeen, Scotland to be the causative agent in wound suppuration [1 & 2]. *Staphylococcus aureus* is a Gram positive facultative anaerobic coccus belonging to the family *Micrococcacea* which reproduces asexually via binary fission. After reproduction, daughter cells do not entirely detach from each other and subsequent replication results in formation of clusters [3].

S. aureus is part of the normal bacterial flora of the skin, intestine and upper respiratory tract of both humans and animals [4 & 3]. It has the potential of becoming pathogenic if there is a breach in the host defence mechanism such as breakage of skin or epithelial layer and reduction of host immunity [5 & 6]. *Staphylococcus aureus* is known to colonise the upper respiratory mucosa of about 15% to 35% of healthy individuals without causing any infection, however under conducive environment, about 38% of these carriers could develop a staphylococcal infection [3]. These infections could range from mild superficial skin infections to more severe and even fatally invasive diseases such as sepsis and toxic shock syndrome [7]. The infectivity of *S. aureus* is attributed to its ability to withstand extreme conditions including temperatures of 7- 48^{o}C, pH levels of 4.2 - 9.3, sodium chloride concentrations of up to 15% and a combination of various other mechanisms it possesses to overcome its host defences including tissue invasion, toxin production and antibiotic resistance [7].

However, various approaches which leads to drug tolerance and persistence have been developed by the *Staphylococcus aureus* bacteria thereby enhancing their survival rate during adverse conditions [8]. Based on previous observations, it has been proven that the use of antibiotics for bacterial resistance and virulence is as a result of pre-existing selection of mutants in a bacterial population [9]. Although, according to a current research, the emergence of de novo mutations known as ‘adaptive resistance’ has been revealed after the exposure of bacteria in non-lethal stress conditions [10]. Moreover, this event goes in relation to the activation of the SOS system which is foremost to increased rates of recombination and mutation thereby influencing the dissemination and evolution of bacterial resistance [11].

Staphylococcus aureus bacteria is able to successfully invade its host due to its ability to produce certain exoproteins which include: Coagulase, Hyaluronidase, Hemolysins and Beta-lactamase. Other exoproteins produced include

Dnase, lipase, and staphylokinase which dissolve fibrin [12]. It can also form a compact layer of cells bound together by an adhesin called Polysaccharide Intercellular Antigen (PIA). This layer of cells typically called a biofilm varies in thickness (mostly multi-layered for *S. aureus*) and is embedded in a slimy layer made up of teichoic acid and host proteins [13]. The cells in the biofilm usually exhibit diverse patterns of growth, gene expression and consequently, protein production. This is as a result of the diverse nature, generation and stages of growth of the cells in the biofilm [14]. The cells in the biofilm have the ability to detach from the film and spread to previously uninvaded regions leading to progressive invasion. The biofilm layers control the inflow and outflow of substances and in the case of the multi-layered staphylococcal biofilm; this control is instrumental to its pathogenicity, its ability to withstand unfavourable changes in its environment as well as to tolerate high doses of antibiotics [14 & 12]. This attribute has made it one of the major causative agents of both hospital and community acquired infections thus a pathogen of increasing medical concern [7].

Natural resistance of staphylococcal strains to antibiotics is usually attributed to their possession of a thin capsid (visible only under an electron microscope) [6] which serves as a protective covering for the cell coupled with their location within the pus-filled carbuncles which limit antibiotic access [15]. However, most of its antibiotic resistance abilities are acquired via horizontal gene transfer.

Resistance to antibiotics exhibited by *S. aureus* was first recorded shortly after the introduction of penicillin in the 1940s and was closely followed by the emergence of Methicillin-Resistant strains of *S. aureus* (MRSA) in 1961 [12]. Resistance to beta-lactam antibiotics gave rise to the development and use of other classes of antibiotics with wider spectrum of activities including quinolones and fluoroquinolones which were first synthesized in the 80s and have *in vitro* activity against a wide range of Gram positive and Gram-negative bacteria. Ciprofloxacin, a synthetic antibiotic usually in form of a hydrochloride salt is a quinolone [16]. It is a well-tolerated antibiotic which has a bioavailability of 70% and a half-life of 4 hours in mammals [17 & 18]. It elicits its antimicrobial activity by inhibition of DNA synthesis by acting on topoisomerase II; encoded by the *gyr* A and *gyr* B genes and responsible for regulation of DNA supercoiling and topoisomerase IV which is responsible for the recategorization of daughter genome strand after replication. Hence, its broad-spectrum activity has been highly reported [18].

The aim of this project was to study the effect of ciprofloxacin on the growth and biofilm forming ability of a strain of *Staphylococcus aureus* (CM10) isolated from a clinical sample and preserved at -80^{o}C on beads in Dr Sue Langs'

library (Microbiology laboratory, Faculty of Health and life sciences, Glasgow Caledonian University) using time kill study, resazurin and live/dead staining of biofilms. For the purpose of this study, it would be referred to as CM10 strain.

Materials and Methods

Bacterial Isolation and Identification

To determine the effect of ciprofloxacin on CM10 strain of *S. aureus*, a comprehensive approach involving a combination of quantitative and qualitative analysis was employed. *S. aureus* 6571 (Oxford strain) which has a known minimum inhibitory concentration of ciprofloxacin and *S. epidermidis* RP62a which has ability to form biofilm, were used as control strains for this research.

Various culture media were prepared according to manufacturers' instructions, sterilised by autoclaving at 121^{o}C for 15 minutes (with the exception of Phosphate buffered saline which was sterilised at 110^{o}C for 10 minutes) and used for the culture of CM10, *S. aureus* 6571 (Oxford strain) and *S. epidermidis* RP62a at various times including; Brain Heart Infusion Agar, Brain Heart Infusion Broth, Tryptone Soya Broth, Mueller-Hinton Broth, Columbia Blood Agar Base and Phosphate buffered saline, all of which were manufactured by Oxoid (UK).

CM10 strain, *S. aureus* 6571 and *S. epidermidis* RP62a were obtained from Dr Sue Langs' library (Microbiology laboratory, Faculty of Health and life sciences, Glasgow Caledonian University), stock culture preserved at -80^{o}C on beads. These were allowed to thaw and reconfirmed by being cultured onto blood agar plates in duplicates using the streaking method as described by Cheesbrough (2006) [19] to obtain pure and discrete colonies. The inoculated blood agar plates were incubated at 37^{o}C for 18 hours. Colonies were then characterised morphologically; size, shape, margin, texture, appearance, pigmentation and their ability to form zones of haemolysis on blood agar. Furthermore, a Gram staining procedure was carried out using the method described by Cheesbrough (2006) [19].

Other biochemical tests were also carried out to further characterize and reconfirm the test organisms as stipulated by Baron (1996) [20]. A catalase test was performed to ascertain the organisms' ability to produce catalase which breaks down hydrogen peroxide to form oxygen and water. This was achieved by emulsifying a colony of the organism in a loop full of 3% hydrogen peroxide solution while watching for the escape of oxygen in form of air bubbles. CM10 strain was also tested for its ability to produce coagulase and protein A which converts fibrinogen to fibrin using a Staph latex kit for identification of *S. aureus* manufactured by Pro-lab diagnostics (UK). This was interpreted by the presence or absence of agglutination after a colony of the test organism was emulsified into a drop of the latex reagent. In addition to the above test, CM10 was identified using a standardized system for the

identification of Staphylococcus using a series of miniaturised biochemical test called Analytical Profile Index Staph (API-Staph) manufactured by Bio merieux (France).

The API-Staph test is based on the principle that when dehydrated substrates for biochemical tests are inoculated with bacterial suspension prepared in an API Staph medium and incubated at 37^{o}C for 18 hours they give rise to a characteristic reaction in each micro tube [21]. These reactions were interpreted according to the reading table provided by the manufacturer and the identification code was obtained. The identification code was typed into a specially adapted identification database to obtain the identity of CM10.

Bacterial Growth Curve

A bacterial growth curve experiment was carried out on the CM10 strain of *S. aureus* using aseptic technique for a duration of 6 hours to determine its growth pattern, the duration of its exponential phase and also to estimate the viable count at distinct optical densities and times. This was done using the methods described by Kloos, and Schleifer (1975) [22]. An overnight culture of CM10 was prepared by inoculating 10 ml of Mueller-Hinton broth in a universal bottle with a single colony of CM10 strain obtained from a blood agar plate and incubating the inoculated broth at 37^{o}C for 18 hours. Aliquots (2ml) of overnight culture of CM10 was added to 100 ml of sterile Mueller-Hinton broth pre-heated to 37^{o}C in a 250 ml conical flask. This mixture was homogenised by gentle shaking then, an aliquot of 1 ml was dispensed into a cuvette and its optical density at zero time was read using a bench top spectrophotometer (Jenway 6300, UK) at 600 nm. The reading was appropriately recorded. In the same way, 100 µl was taken from the conical flask and diluted in 9.9ml of Phosphate buffered saline (PBS) to give a 10^{-2} concentration. This was further diluted to a 10^{-6} concentration in PBS. Aliquots of 100µl of the 10^{-4} to 10^{-6} bacteria concentration range were plated on brain heart infusion agar plates in duplicates and incubated for 18 hours at 37^{o}C. A repeat of the above procedure was carried out every 30 minutes until the 4th hour when an extra dilution (10^{-7}) was made and plated out continuously until the 6-hour period elapsed. After incubation, colonies for every time point where counted and recorded.

On completion of the experiment, a purity plate of the bacterial culture from the conical flask was made on blood agar to check for contaminants.

Antibiotic Susceptibility Test

CM10 strain of *Staphylococcus aureus* was exposed to ciprofloxacin (Sigma, UK) using the broth micro dilution method described by Garcia to obtain the precise concentration at which visible growth of CM10 would be inhibited after 18 hours incubation at 37^{o}C [23]. This concentration termed the Minimum inhibitory concentration (MIC) which is defined as the lowest concentration of a drug preventing visible growth of bacterium, is usually determined by the appearance of the first clear well in a range of doubly diluted concentrations of antibiotic in a micro titre plate. The procedure includes preparation of stock solutions of ciprofloxacin, then a suitable antibiotic dilution range was chosen, and the test was appropriately carried out aseptically.

Preparation of ciprofloxacin stock solution

Ciprofloxacin has a known potency of 980 µg/mg as stated by the manufacturer (Sigma, UK). This was used to calculate the quantity of antibiotic required to make stock solution A using the formula:

$W=1000/P \times V \times C$

Where W is the weight of the antibiotic required

P is the potency of the antibiotic which is 980µg/mg.

V is the required volume of the stock solution being 10ml.

C is the required concentration of the stock solution, which was 10,000mg/l

Following the calculation using the above formula, 102.04mg was weighed out and dissolved in 10ml of sterilized distilled water to form stock A. From stock A, a 1 in 10 dilution using 500µl of stock A and 4.5ml of sterile distilled water was made to obtain stock B with a concentration of 1000mg/l. Likewise, a 1 in 100 dilution of stock A with distilled water was made to obtain stock C with a concentration of 100mg/l and another 1 in 100 dilution of stock B was made to obtain stock D with a concentration of 10mg/l. These stock solutions were used to constitute the dilution range used for the test.

Dilution range

According to the British Society for Antimicrobial Chemotherapy standards, the minimum inhibitory concentration (MIC) of ciprofloxacin for *Staphylococcus species* is within 0.06 – 128 mg/l dilution range [24]. A control strain (*S. aureus* 6571) with an established MIC of 0.12mg/l was used to check that the antibiotic dilution was prepared correctly [24]. Consequently, a dilution range was chosen that incorporates the MIC of *S. aureus* 6571 (Oxford strain) with a minimum of 2 wells before its established MIC and a total of 11 concentrations ranging from 0.03 to 32mg/l. For this range, calculations of constituents were made using the formula:

$$C_1V_1=C_2V_2$$

Where C_1 is the antibiotic concentration of the stock solution

V_1 is the required volume of stock solution

C_1 is the required antibiotic concentration

V_2 is the final/required volume.

The antibiotic concentrations for the dilution range were prepared and 75µl of each dilution beginning with the lowest concentration (0.03mg/l) was dispensed into each well of the micro plate in individual columns beginning from column 2 to column 12. In a similar way, 75µl of Mueller-Hinton broth was dispensed into each of the wells in column 1. The micro plate was firmly sealed with a sterile adhesive tape and stored frozen at -20^{o}C for 18 hours. Overnight cultures each of CM10 and *S. aureus* 6571 (Oxford strain) in 10ml of sterile Mueller-Hinton broth were prepared and incubated at 37^{o}C for 18 hours to be used for inoculation of the micro plate.

Inoculation

From the graph of viable count (log10) against absorbance, the absorbance at which the viable count would be 10^8cfu/ml was calculated to be 0.3(±0.02). Consequently, dilutions of the overnight cultures of CM10 and Oxford strains were made as follows:

CM10 strain: 910µl of sterile Mueller-Hinton broth: 90µl of overnight culture of CM10 giving an absorbance of 0.308.

Oxford strain: 900µl of sterile Mueller-Hinton broth: 100µl of overnight culture of *S. aureus* 6571 giving an absorbance of 0.304.

A 1 in 100 dilution of the 10^8cfu/ml of each of the *Staphylococcal* strains was made with Mueller-Hinton broth as the diluent to give a count of 10^6cfu/ml. Then an aliquot of 75µl of the 10^6cfu/ml dilution was added in 3 rows for each of the *Staphylococcal* strains to 75µl of the antibiotic already added to each well to give a final concentration of 10^5cfu/ml of the organism per well. An aliquot of 75µl of sterile broth was added to each well of the last 2 rows to serve as a control to check that the broth used was sterile. The inoculated micro plate was incubated for 18 hours at 37^{o}C after which the value for the MIC was recorded.

Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) of ciprofloxacin on CM10 strain of *S. aureus* was carried out using the methods of Andrews J.M, (2001) to ascertain the concentration at which the antibiotic would kill the organism and also to determine if the organism exhibited tolerance to the antibiotic [24]. This was achieved by plating out the cells in different concentrations of ciprofloxacin from the clear wells in the 96-well plate used for the minimum inhibitory concentration on brain heart infusion agar plates in duplicates. The inoculated plates were incubated for 18 hours at 37^{o}C after which they were checked for growth of colonies. Turbidity on the plate indicates growth of the microorganism, the MBC will be determined by the lowest concentration at which ciprofloxacin inhibited growth of CM10 strain of *S. aureus*.

Kill Curve

A bacterial time kill curve is a basic microbiological way of assessing the antimicrobial activity of antibacterial compound. This experiment was carried out to determine the potency of ciprofloxacin on the CM10 strain of *S. aureus* by observing the effect the antibiotic has on the growth of the organism. An overnight culture of CM10 was prepared as stated above and diluted to give an optical density of 0.3 at 600nm. An aliquot of 500µl of the 0.3

optical density was dispensed into each of two 250ml conical flasks containing 100ml of Mueller-Hinton broth with 1 conical flask serving as test and the other as a control with which to compare the test.

Both flasks containing Mueller-Hinton broth were preheated at 37^{o}C in a water bath. Stock solution C of ciprofloxacin with a concentration of 100mg/l was prepared as stated in Preparation of ciprofloxacin stock solution above. and an aliquot of 250µl was added to the test flask to obtain a concentration of 0.25mg/L in the flask. At times 0 and 30 minutes, the optical densities of cultures in both flasks were read at 600nm and a serial dilution of 10^{-2} and 10^{-3} of the cultures in both flasks were made using a similar technique as that used for the growth curve. An aliquot of 100µl of these bacterial concentrations were plated out in duplicates on brain heart infusion agar plates and incubated at 37^{o}C for 18 hours. Following the observation from the previous growth curve experiment, the expected time for the commencement of the exponential growth phase was 3 hours thus, the optical densities of the cultures in both flasks was read at 600nm every 30 minutes and beginning from the 3rd hour, every 30 minutes until the 7th hour, a serial dilution was made from 10^{-2} to 10^{-7} for the control flask and 10^{-2} to 10^{-4} for the test flask. An aliquot of 100µl of 10^{-2} to 10^{-4} dilutions from test and 10^{-5} to 10^{-7} dilutions from the control flask were plated out on brain heart infusion agar plates and incubated for 18 hours at 37^{o}C after which the viable count was recorded.

At the end of the experiment, a purity plate to check for contaminants was made for each flask and a minimum inhibitory concentration experiment on CM10 strain of *S. aureus* and *S. aureus* 6571 respectively using a 96well plate was set up as a control to check that the antibiotic stock solution was prepared accurately.

Biofilm formation

CM10 strain of *S. aureus* was tested for its ability to form biofilm in a 96-well plate and the effect of ciprofloxacin on the biofilm formed was also assayed for using resazurin staining and live/dead staining procedures according to the protocols of Chavant *et al*, 2007 [25]. *S. epidermidis* RP62a was used as a positive control and Mueller-Hinton broth was also used as a negative control. Resazurin staining procedure was used because it measures the metabolic activity of the live cells by their reduction of resazurin to pink resorufin which is fluorescent, and its fluorescence measured using a plate reader at an emission of 590nm and excitation of 540nm is directly proportional to the number of living cells in the biofilm. It is also a preferred method as it is easy to use, stable and non-toxic to the bacterial cells [26].

For this reason, an overnight culture of CM10 and RP62a in Mueller-Hinton broth was diluted to give an optical density of 0.3 at 600nm and a 1 in 100 dilution of the $0.3OD_{600}$ of both cultures was made using Mueller-Hinton broth as the diluent.

Table 1: Showing the Inoculation of a 96well Plate to test for Biofilm formation.

<table>
<tr><th></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><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="12">75µl of diluted RP62a culture in each well from 1-12</td></tr>
<tr><td>B</td><td colspan="12" rowspan="3">75µl of diluted CM10 culture in each well of rows B, C, D and E</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td colspan="12">75µl of sterile Mueller-Hinton broth in each well from 1-12</td></tr>
<tr><td>F</td><td colspan="12" rowspan="2">Wells in row G and H were left empty.</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

A 96well plate was inoculated as shown in Table 1 below and incubated at $37^{o}C$ for 18 hours to check for biofilm formation which would be characterized by a thick layer of bacterial growth on the walls of the wells.

Effect of ciprofloxacin on the biofilm:

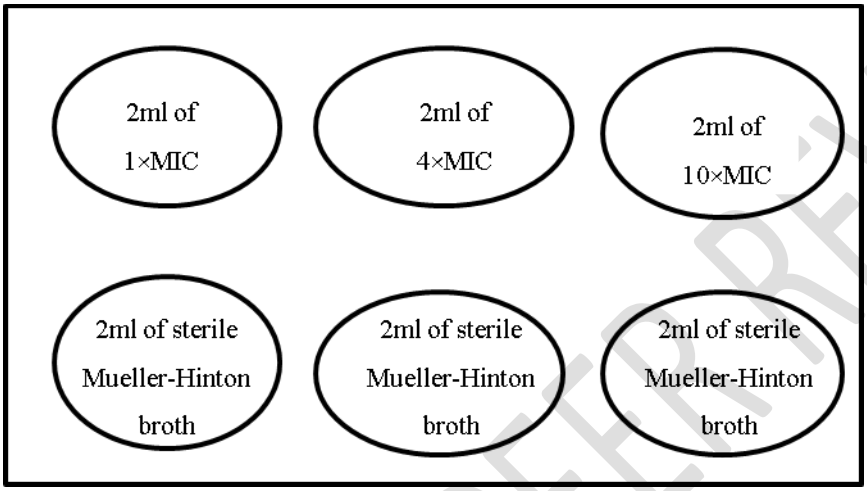
After incubation, the plate was washed 3 times with sterile phosphate buffered saline (PBS). A stock solution of ciprofloxacin with a concentration of 100mg/l (Stock C) was diluted to give 1×MIC (0.5mg/L), 4×MIC (2mg/L) and 10×MIC (5mg/L) concentrations and an aliquot of 100µl of the different concentrations was dispensed into wells of separate rows as shown in Table 2 below:

Table 2: Table Showing Inoculation of a 96well Plate with Ciprofloxacin.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100µl of sterile Mueller-Hinton broth in each well containing RP62a biofilm from A1-A12											
B	100µl of sterile Mueller-Hinton broth in each well containing CM10 biofilm from B1-B12											
C	100µl of 1×MIC concentration of ciprofloxacin in each well containing CM10 biofilm from C1-C12											
D	100µl of 4×MIC concentration of ciprofloxacin in each well containing CM10 biofilm from D1-D12											
E	100µl of 10×MIC concentration of ciprofloxacin in each well containing CM10 biofilm from E1-E12											
F	100µl of sterile Mueller-Hinton broth in each well from F1-F12											
G	Wells in row G and H were left empty.											
H												

In a similar way, A 6 well plate containing a single sterile coverslip in each well was inoculated with 2ml of the diluted CM10 culture in each well and incubated at 37^{o}C for 18 hours. After incubation, the plate was washed 3 times with sterile phosphate buffered saline (PBS). 2ml of 1×MIC, 4×MIC and 10×MIC was dispensed into different wells of the 6well plate as shown in Figure 1**Error! Reference source not found.** below and incubated at 37^{o}C for 18 hours.

Figure 1: Figure showing inoculation of a 6well plate containing coverslips with ciprofloxacin.



Resazurin staining and live/dead staining procedures:

After incubation, the wells in the 96well micro plate were washed 3 times with sterile phosphate buffered saline after which the cells were stained by dispensing 100µl of 0.001% resazurin (Sigma, UK) into each well in rows A – F. The micro plate was incubated for 2 hours at 37^{o}C and the optical density of the dye was measured using a Fluostar Optima micro plate reader at an emission of 590nm and excitation of 540nm.

The 6well plate containing coverslips was also washed 3 times with sterile phosphate buffered saline and rinsed in distilled water after which it was stained in the dark for 15minutes with 1ml of the live/dead stain (made up of 1µl each of 3.34mM SYTO 9 nucleic acid stain and 20mM propidium iodide (Invitrogen, UK) in 98µl of distilled water), washed in distilled water and air dried. The dried coverslips were removed, mounted on clean grease free cavity slides and viewed under the Evos inverted microscope (Life technologies, UK) using × 100 objective.

Statistical analysis

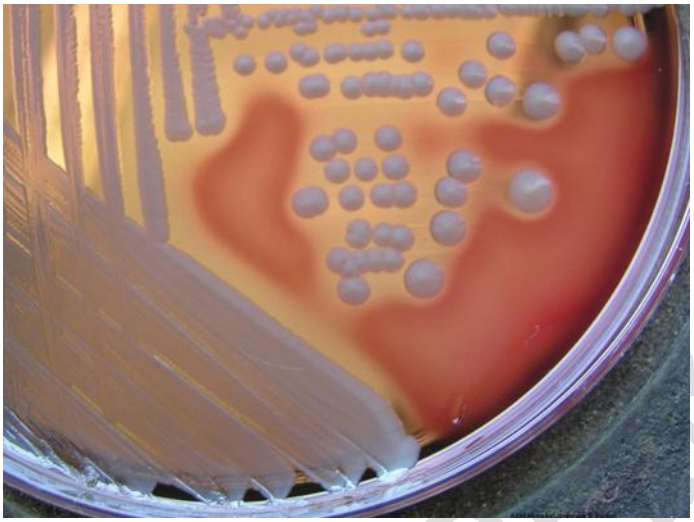
Statistical analysis for this research was performed using GraphPad prism 5. One-way analysis of variance (One-way ANOVA) was used for the kill curve experiment. More so, Mann-Whitney U test was performed on the optical density of the resazurin stained biofilms of *Staphylococcus epidermidis* RP62a and *Staphylococcus aureus* CM10 to show the differences between the two strains. Additionally, Dunnett's multiple comparison test was performed to compare each antibiotic treated population of CM10 strain to the untreated population.

Results

Bacterial identification/reconfirmation

Culture of CM10 on blood agar yielded 1mm in diameter small, circular colonies with smooth entire margins, which were raised, greyish and non-pigmented but exhibited zones of beta haemolysis on blood agar (Figure 2).

Figure 2: Morphology of CM10 on Horse Blood Agar Plate.



A Gram stain of the colonies viewed under the light microscope showed the colonies to be Gram positive cocci in clusters. Catalase test and coagulase/protein A test carried out on the organism both yielded positive results.

Figure 3: CM10 strains API-Staph Reaction. Growth curve

An API-Staph identification as a result of biochemical test results as shown in Figure 3 confirmed the organism to be a 97.8% *Staphylococcus aureus* match with only 1% similarity to *Staphylococcus simulans*.

Following the bacterial growth curve experiment, results obtained are as follows:

Table 3: Table showing the optical density and viable count of CM10

Time in hours	Optical density at 600nm	Viable count in cfu/ml	Log10 of viable count
0	0.057	5.15×10^{7}	7.71
0.5	0.086	3.90×10^{7}	7.59
1	0.151	5.50×10^{7}	7.74
1.5	0.285	1.03×10^{8}	8.01
2	0.472	1.82×10^{8}	8.26
2.5	0.72	4.15×10^{8}	8.62

3	0.924	5.45×10^{8}	8.74
3.5	1.4	1.19×10^{9}	9.08
4	1.68	1.19×10^{9}	9.08
4.5	1.93	1.77×10^{9}	9.25
5	2.08	1.94×10^{9}	9.29
5.5	2.24	2.04×10^{9}	9.31
6	2.31	2.12×10^{9}	9.33

Figure 4: Time against Viable Count and Absorbance of CM10 strains' growth curve

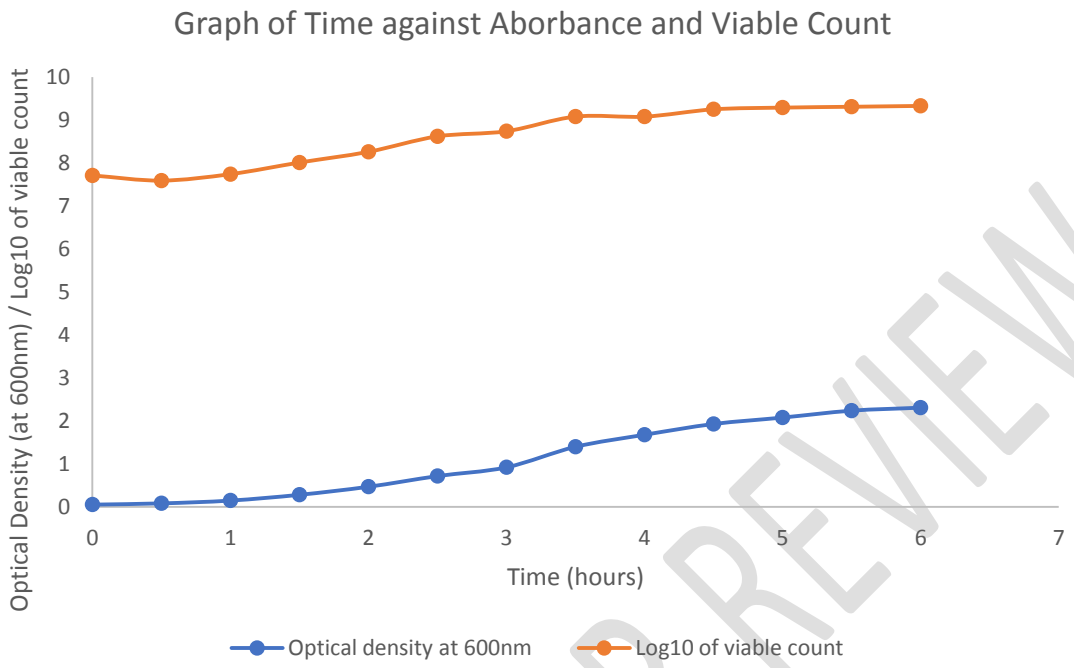
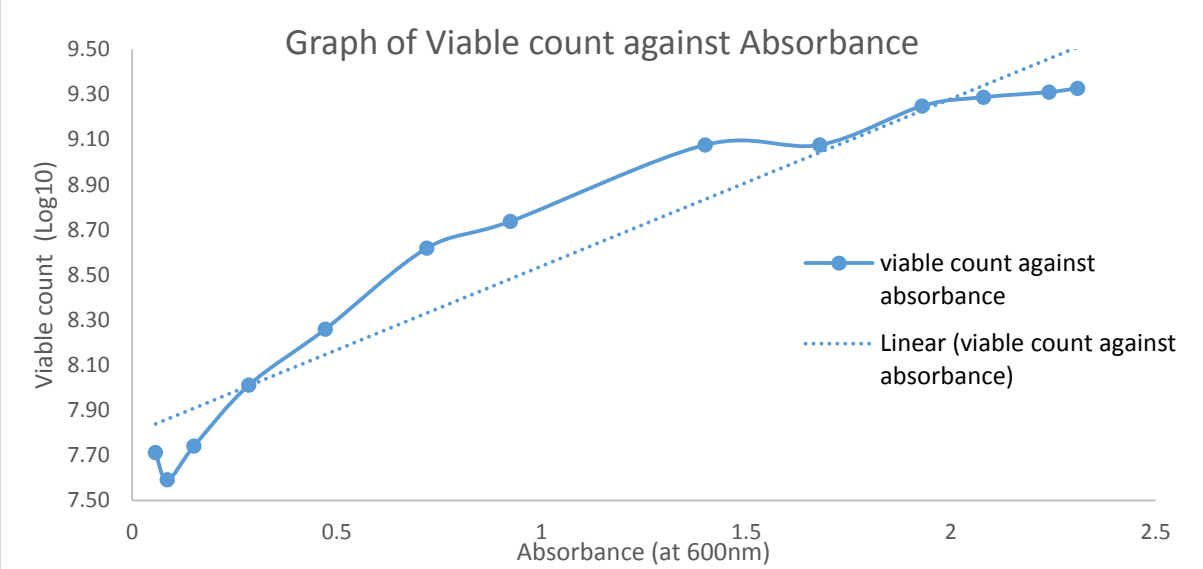


Figure 5: Viable count against Absorbance of CM10 strains' growth curve



The purity plate inoculated at the end of the bacterial growth curve experiment yielded a pure culture of CM10, there by validating the results of the growth curve.

Antibiotic susceptibility test results

The minimum inhibitory concentration of ciprofloxacin for CM10 strain of *S. aureus* was found to be 0.5mg/l and 0.25mg/l for *S. aureus* 6571 while the Minimum bactericidal concentration of ciprofloxacin for the CM10 strain of *S. aureus* was found to be 2mg/l after plating out the clear wells from the 96well MIC micro titre plate.

Figure 6: MBC plate of CM10

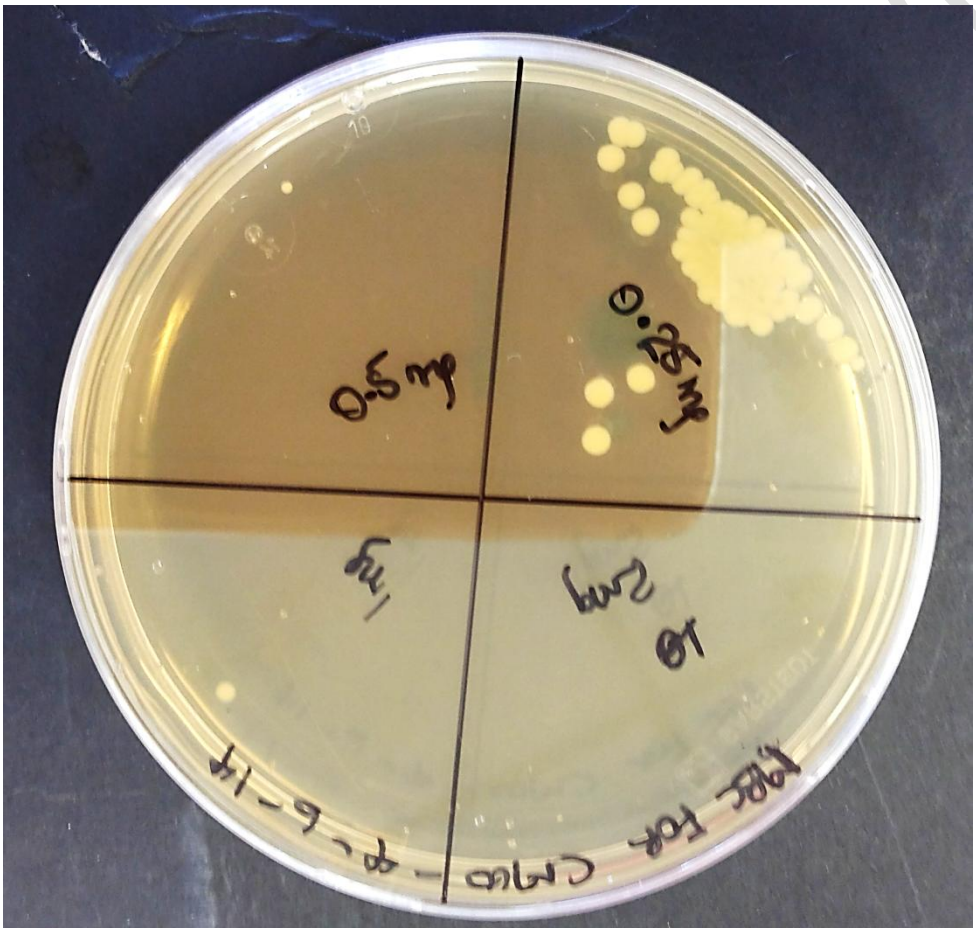


Figure 6 shows heavy growth of CM10 strain at 0.25mg/L concentration of ciprofloxacin, a much more reduced growth at 0.5mg/L and 1mg/L concentrations (2 colonies and 1 colony respectively) and no growth at 2mg/L concentration of ciprofloxacin.

Kill curve.

Results of the kill curve experiment carried out to assess the effect of ciprofloxacin on the growth of the organism are as follows:

Figure 7: Viable count of CM10 treated with 0.25mg/l of Ciprofloxacin and Untreated CM10 cultures plotted against time.

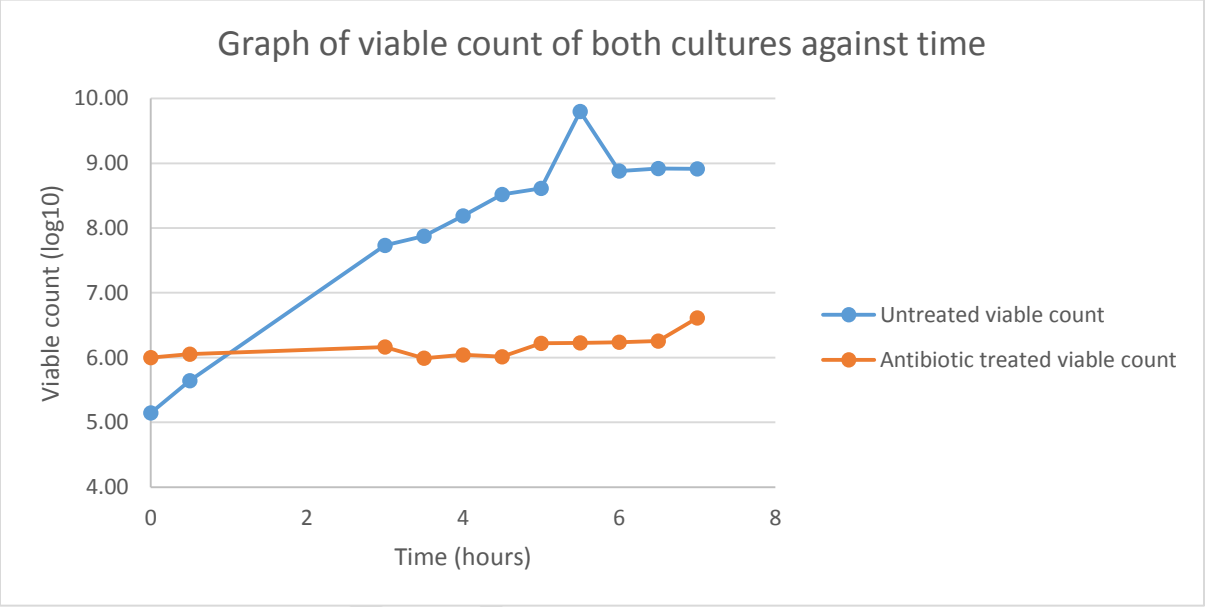
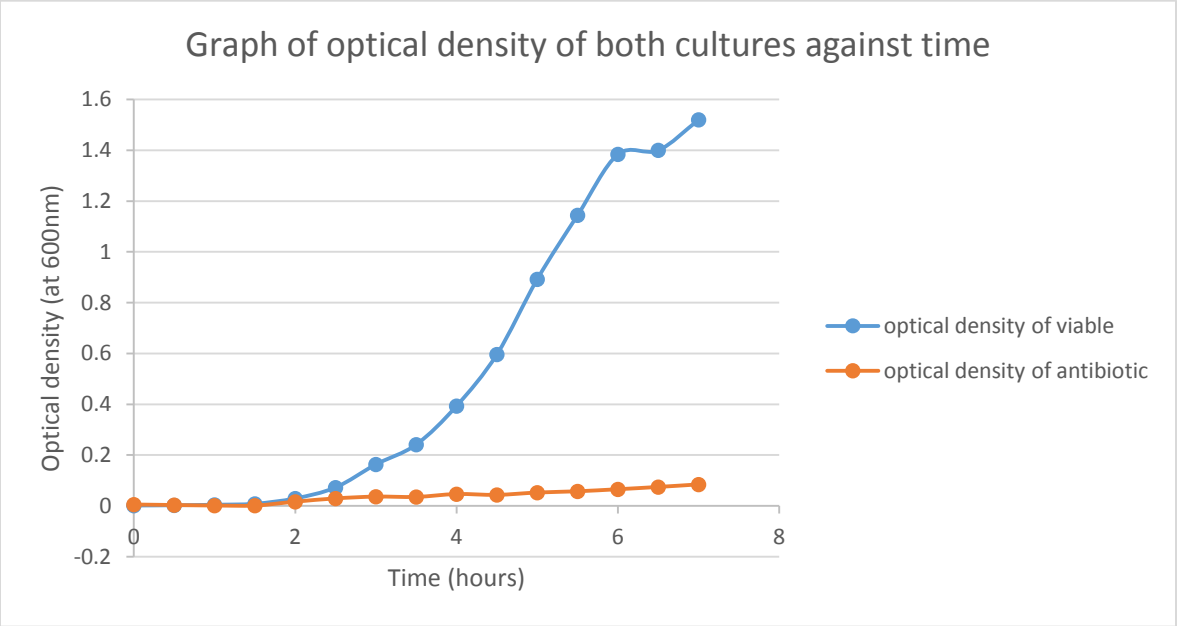


Figure 8: Optical density of CM10 treated with 0.25mg/l of Ciprofloxacin and Untreated CM10 cultures plotted against time.



One way analysis of variance performed on the data obtained from the kill curve experiment yielded no significant results with a p value of 0.22 was greater than 0.05 ($P>0.05$). The purity plate inoculated at the end of the time kill curve experiment yielded a pure culture for the untreated flask of CM10; however, the flask treated with ciprofloxacin yielded a mixed growth of 2 similar colonies but with 1 having zones of beta haemolysis and the other none.

Biofilm formation

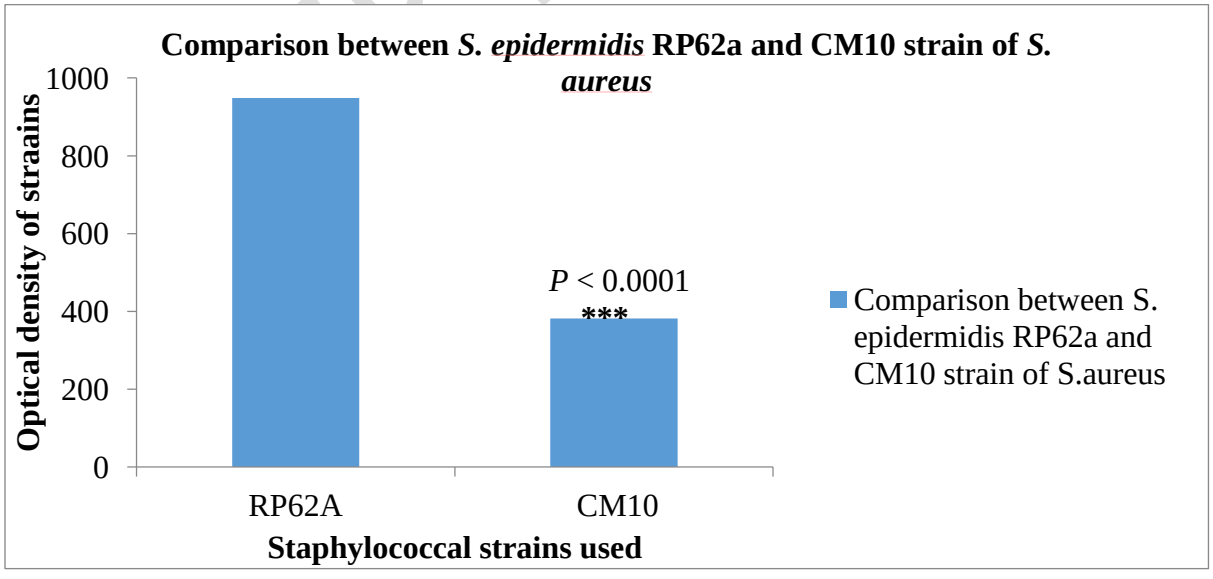
After inoculating and incubating a 96well plate at 37^{o}C for 18 hours, a biofilm characterized by a thin layer of bacterial growth at the base of the inoculated wells was observed thereby confirming that the CM10 strain of *S. aureus* was positive for biofilm formation.

Table 3: Results of Resazurin staining of CM10

Concentration of ciprofloxacin	Average viability of CM10	Percentage viability of CM10
Untreated	382	100
1×MIC (0.5mg/l)	206	54
4×MIC (2mg/l)	104	27
10×MIC (5mg/l)	58	15

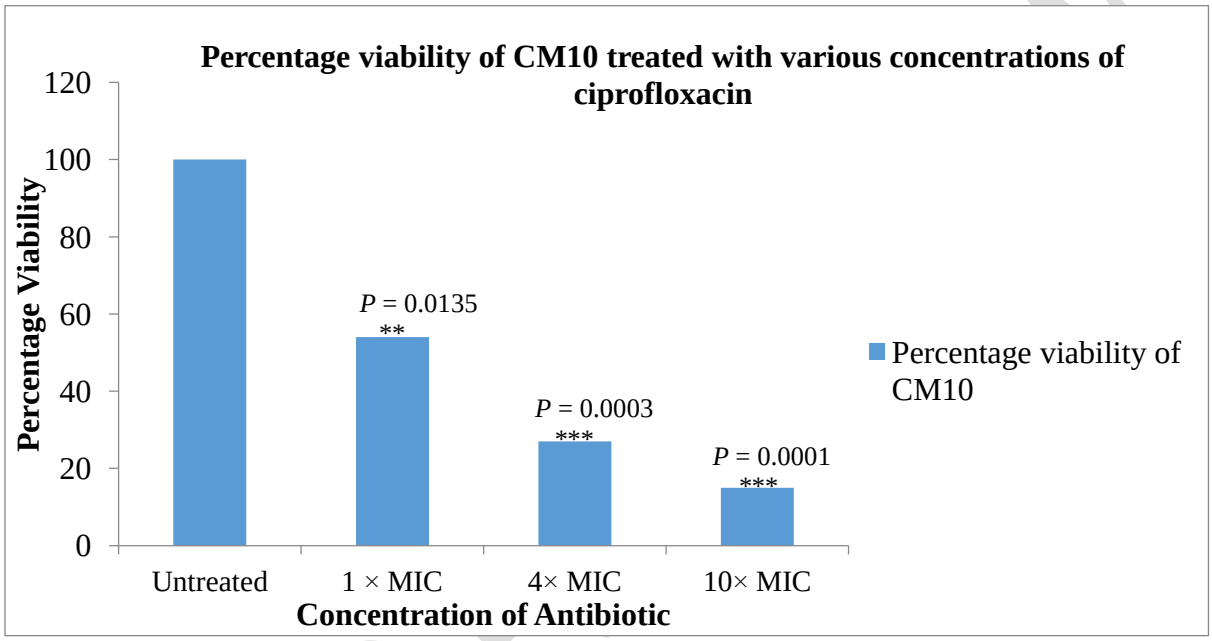
Subsequent treatment with ciprofloxacin and staining with resazurin and live/dead staining procedure yielded the following results on table 3.

Figure 9: Comparison of the effect of Resazurin staining on *S. epidermidis* RP62a and CM!0 strain of *S. aureus*



Mann Whitney U test performed on the optical density of the resazurin stained biofilms of *Staphylococcus epidermidis* RP62a and *Staphylococcus aureus* CM10 showed statistically significant differences (P <0.0001) between the two strains. This data is represented in Figure 9.

Figure 10: A representation of Resazurin staining results showing Percentage viability of CM10 treated with various concentrations of ciprofloxacin compared to the untreated CM10 strain.



Dunnett's multiple comparison test revealed the difference between the untreated strain of CM10 and each treatment was to be statistically significant (P=0.05). This data is represented in

10.

Furthermore, biofilms of CM10 strain treated with varying concentrations of ciprofloxacin and stained with resazurin were assigned percentage values using their means in comparison with the untreated biofilm of CM10 strain of *S. aureus* which was regarded as 100%. Results of the comparison (Figure 11) shows the significant difference between the treated and untreated CM10 strains.

Figure 11: Showing an overlay of live and dead population of untreated population of CM10

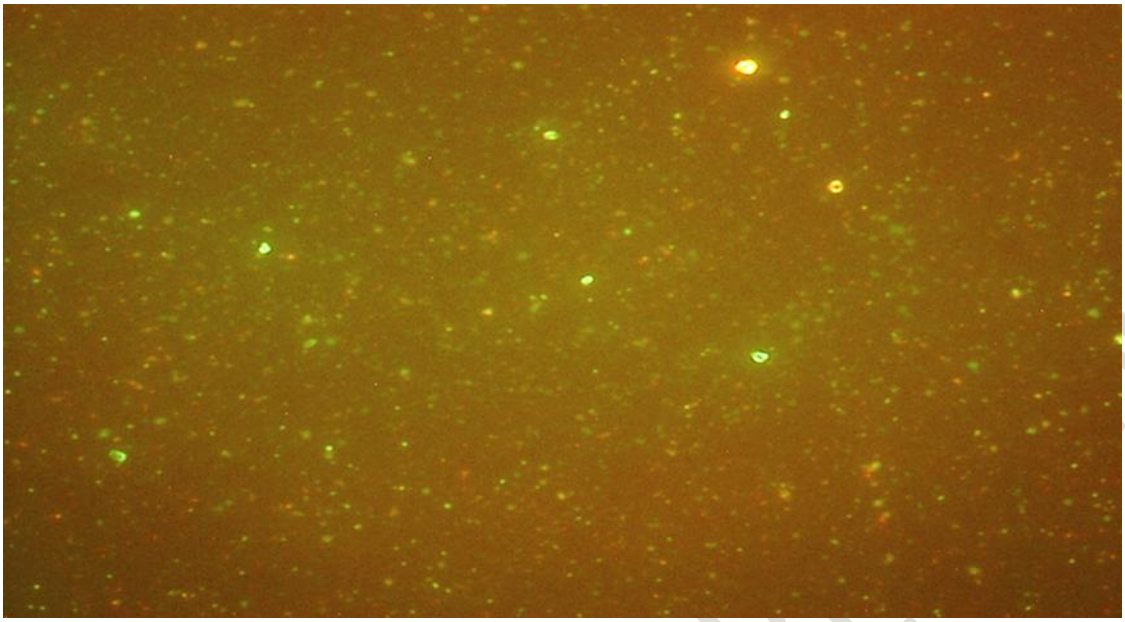
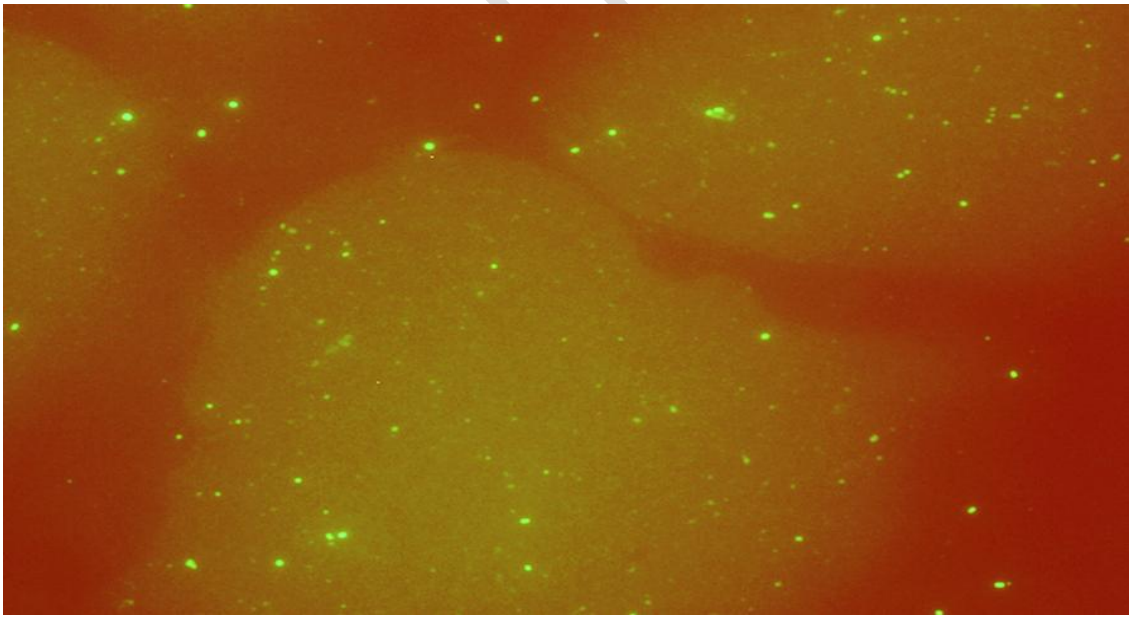


Figure 12: Showing an overlay of live and dead population of CM10 Treated with 4X Minimum Inhibitory Concentration of ciprofloxacin



In Figure 11 and Figure 12 above, the green fluorescence shows the population of the live cells while the red fluorescence shows the population of the dead cells after staining with the live/dead stain.

Discussion

Since the identification of *Staphylococcus aureus* as the causative agent in wound suppuration, it has become prevalent with its involvement in more infections including food poisoning, sepsis and toxic shock syndrome [2 & 7]. A combination of resilience on the part of the organism and possession of virulence factors including exotoxins has made it a major causative agent of both community and hospital acquired infections [12 & 7]. The emergence of antibiotic resistance encoded on mobile transferable genetic elements has further increased the morbidity and mortality rate associated with staphylococcal infections [2, 27 & 12]. With the issue of resistance, one would seek the possibility of using a higher concentration of antibiotics and even an alternative antibiotic to tackle the infection, but these antibiotics are active against planktonic cells and usually fail to completely eradicate biofilms, leading to persistent infections [14 & 26].

To confirm the identity of the given CM10 strain, the results obtained from the colony morphology, phenotypic characterization tests (coagulase/protein A, Gram, and catalase tests) as well as the results of the API-Staph were in total accordance with typical *S. aureus* characteristics recorded in previous studies [28 &29]. However, genetic identification by sequencing of the 16S rRNA genes is a preferred but more expensive method [30 & 31] as relying on characteristics such as haemolysis on blood agar and rapid slide agglutination test are less sensitive and non-specific [30, 32 & 33].

Results from the optical density of *S. aureus* obtained from the bacterial growth curve experiment showed that CM10 strain of *S. aureus* has a doubling time of approximately 37 minutes (±2). The spectrophotometry measurement was based on the assumptions that the bacterial cells were evenly distributed in the Mueller-Hinton broth thus the optical density would be proportional to the viable count at any given time. Shaking of the flask during incubation in the water bath helped distribute the cells in the suspension and also eradicated the possibility of the bacterial cells settling to the bottom of the flask during the experiment.

Antimicrobial susceptibility testing is important for monitoring resistance in commensal bacteria and various clinically important pathogens [34] such as this. Results for the susceptibility of CM10 strain to ciprofloxacin were interpreted according to the British society for antimicrobial chemotherapy guidelines (BSAC) [35]. CM10 strain of *S. aureus* was found to be susceptible to 0.5 mg/l concentration of ciprofloxacin which is within the range which has been established [24] and below the 1.0 mg/l susceptibility limit for ciprofloxacin [36]. This result agrees with a

study that demonstrated a 98% sensitivity and a 2% intermediate susceptibility of 147 *Staphylococcus aureus* strains to ciprofloxacin as well as two other study. [28, 29 & 37] However, a study carried out on Methicillin-Resistant strains of *S. aureus* yielded resistant results to ciprofloxacin susceptibility with concentrations ranging from 128 mg/l to 256 mg/l. [38].

This suggests the possibility of CM10 being a Methicillin Sensitive strain of *S. aureus*, but such conclusions could not be drawn as the presence of the *mecA* gene was not assayed for. It is worth to note that results for the susceptibility of CM10 strain differed from the 0.25mg/l ciprofloxacin susceptibility of *S. aureus* 6571 control strain as expected. Nevertheless, ciprofloxacin was still able to inhibit the growth of CM10 strain thus can be used to treat infections caused by it. Ciprofloxacin at a concentration of 4×MIC achieved a 99.9% kill of CM10 strain.

The time kill experiment carried out to find out the effect ciprofloxacin has on the growth of CM10 strain of *S. aureus* was carried out using half the minimum inhibitory concentration because as at the point of this experiment, the minimum inhibitory concentration of ciprofloxacin on CM10 was yet to be established. However, using an initial inoculum of 5×10^5cfu/ml; at the conclusion of the experiment at 7 hours, the viable count was 4.10×10^6 after treatment with 0.25mg/l concentration of ciprofloxacin. Nevertheless, there was a slight inhibition of the growth of cells as shown in Figure 7 and Figure 8 which is in accordance with the bacteriostatic mechanism of action of ciprofloxacin [18]. One way analysis of variance performed on the data obtained from the kill curve experiment yielded no significant results with a P value of 0.22 was greater than 0.05 ($P>0.05$). Invariably, there is a possibility of experiencing a decrease in the viable count if a higher concentration of ciprofloxacin is used for this time kill experiment. The seemingly mixed growth obtained from the purity plate of the organisms in the ciprofloxacin treated flask could have been as a result of contaminants during the experiment as well as the loss of function as a result of mutation due to the activation of SOS repair mechanism to repair the damage caused by the antibiotic [39]. The ability of CM10 strain of *S. aureus* to form a biofilm was assayed for using a micro titre plate. Although it is not an ideal method because of the possibility of cells depositing at the bottom of the wells [27] this limitation was overcome by shaking of the micro titre plate during incubation. Results of the resazurin stained biofilm of CM10 strain were compared with that of *S. epidermidis* RP62a. Results suggest that RP62a is a better biofilm former that CM10 however, conclusions cannot be drawn as the biofilms were not stained with crystal violet to determine the biofilm mass in order to classify them into fully established, moderately attached or weakly adherent biofilms [27].

Microscopic comparison between the overlay of live and dead cells of untreated CM10 strain and that of 4×MIC treated CM10 strain showed that more cells were dead on the untreated coverslip than on the treated coverslip. Similarly, there seemed to be a somewhat equality in the number of live and dead cells on the untreated coverslip. This suggests that the cells on the untreated coverslip were most probably in the stationary phase of growth as they had been incubated for 48 hours. On the other hand, the appearance of the cells on the coverslip treated with 4×MIC of ciprofloxacin suggests inhibited growth of the organism and possible growth of resistant or mutated strains of CM10 by selective pressure.

Conclusion

The results of this study provide an insight on the growth as well as the biofilm forming ability of CM10 strain of *Staphylococcus aureus.* Ciprofloxacin has been shown to be an effective antibacterial against this strain of *S. aureus* by its inhibitory effect on the growth as well as biofilm forming ability of this strain of *S. aureus*. Thus, this antibacterial can be used for the treatment of patients suffering from infections caused by the CM10 strain of *Staphylococcus aureus*.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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