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Characterization of a Strain of Staphylococcus Aureus Isolated from the Catheter of a Patient from the Glasgow Infirmary

Victoria Temitope Agbede
M.Sc. Postgraduate Student, School of Health and Life Sciences,
Glasgow Caledonian University, Scotland

Abstract:
In this study, a strain of Staphylococcus aureus isolated from the catheter of a patient of the Glasgow Infirmary was investigated. Isolate was exposed to gentamicin antibiotic at both the minimum inhibitory concentration and at the sub-minimum inhibitory concentration. The impact of the antibiotic exposure on the toxin and biofilm production of the test strain was assessed. Test strain expressed MIC at 0.12mg/L of gentamicin. Kill curve showed a bactericidal effect of the antibiotic. The test strain was found to tolerate the antibiotic in its biofilm state than the free-living cells in a concentration-dependent manner. The test strain was discovered to be capable of producing enterotoxin C and this was expressed in its genes. This study confirmed that complications can arise from catheter use in patients especially when used for prolonged periods.

Keywords: *Minimum inhibitory concentration, gentamicin, aminoglycoside, antibiotic susceptibility testing, biofilms*

1. Introduction

Staphylococcus aureus (*S. aureus*) is a leading cause of human bacterial infections which could be life threatening. *S. aureus* occurs as normal flora on the skin, skin glands, mucous membranes, intestine, vagina and upper respiratory tract of warm-blooded animals. They are opportunistic organisms which are capable of causing infection when conditions become favorable. *S. aureus* has been implicated in infections ranging from food poisoning, superficial skin abscesses to life threatening diseases such as pneumonia, meningitis, osteomyelitis, septicaemia, bacteraemia, sepsis and toxic shock syndrome (Lowy F. D., 1998).

Virulence and pathogenicity are directly linked to the bacteria genome make-up. Pathogen genomes encode many virulence factors which are responsible for the expression of pathogenicity. The virulence factors can be classified into these categories: adhesins, evasins, impedins, invasins and toxins. Pathogenicity may be lost as a result of mutation in the pathogenic genes or gained by acquisition of resistance genes via horizontal gene transfer.*S. aureus*expresses a variety of virulence factors and resistance mechanisms which are pivotal in the establishment and maintenance of infections as well as the evasion of host defense mechanism(s) (Ford, 2014)

Infection caused by *S. aureus* can be treated by antibiotics. However, the organism's resistance to several antibiotics has made treatment difficult. Strains of *S. aureus* that have become resistant to several specific antibiotics are known as Methicillin-Resistant Staphylococcus aureus (MRSA). The ability of *S. aureus* to adapt rapidly to unfavorable conditions imposed on them by the environment or human host has contributed to its survival and evolution. *S. aureus* has evolved over the years to tolerate antibiotics. Antibiotic use has led to the natural selection of resistance genes (Malachowa & DeLeo, 2010). An antibiotic is any agent that kills or inhibits the growth of bacteria. Antibiotics that kill bacteria are referred to as bactericidal while those that only inhibit the growth of the bacteria are referred to as bacteriostatic. Antibiotics are usually grouped based on their chemical structure, mode of action or spectrum of activity. Most antibiotics work by aiming at specific bacterial physiological functions and cause disruption. While some target the bacterial cell wall, some aim at protein synthesis of the bacteria. Bacteria can grow resistance to antibiotics with continued use or misuse of the antibiotic (Finberg, et al., 2004).

Antibiotic resistance can occur spontaneously via mutation or by acquisition of resistant genes either vertically or horizontally. Vertical acquisition of resistant genes is hereditary, that is, acquired from parents, whereas, horizontal acquisition of resistant genes is acquired from other bacteria on mobile genetic elements such as plasmids (Read & Woods, 2014). Mobile genetic elements (MGEs) play a vital role in the adaptation process (Malachowa & DeLeo, 2010). In addition to various enzymes and toxins secreted by various Staphylococcus strains, they are also capable of biofilm formation resulting in a slime layer. The ability to produce slime has conferred pathogen city to this organism. Slime is a viscous, extracellular glyconjugate that allows bacteria attach to smooth surfaces such as implanted medical devices and catheters. Slime has the tendency to inhibit neutrophil chemotaxis, phagocytosis and antimicrobial agents (Schommer, Christner, Hentschke, Ruckdeschel, Aepfelbacher, & Rohde, 2011). Microbial biofilms represent an important determinant of chronic infections. Biofilms have a variety of features that contribute to their resistance to antibiotics. These features include lower growth rate, an exo-polysaccharide matrix, an optimal 3-dimentional structure, a change in gene expression

and production of potentially resistant genes(Cirioni, et al., 2006). Gentamicin isan aminoglycoside antibiotic that kills bacteria by binding to the 30S subunit of the bacteria's ribosome thereby inhibiting protein synthesis(Jensen & Lyon, 2009). This study aims to investigate the impact of gentamicin exposure on the test isolate in the planktonic and the biofilm states, toexamine the effect of sub-minimum inhibitory concentration of gentamicin on the virulence factors harbored by this clinically important strain, and ultimately to enable physicians make informed choices on the appropriate antibiotics when treating *S. aureus* associated infections.

2. Materials and Methods

2.1. Bacterial Isolation and Identification

Bacterial isolate were retrieved from the catheter of a patient of the Glasgow Royal Infirmary. The organism was stored on beads at a temperature of -80°C until needed. Media used for culturing organism were prepared according to manufacturer's instructions. The blood agar plates were incubated at 37°C and examined for growth between 18-24 h. The same procedure was carried out for Oxford strain *S. aureus* NCTC 6571 and *S. epidermidis* (RP62A). These two organisms served as control. Isolate of Staphylococci were identified based on colonial morphology, Gram stain, and the catalase test. Staphylococci isolates were subjected to coagulase test to determine whether they are *S. aureus* (positive for coagulase test). Analytical Profile Index test were carried out on the isolate to confirm its identity as *S. aureus*. Isolate was named *S. aureus* CM11.

2.2. Growth Curve of S. Aureus CM11

Growth curve of *S. aureus* CM11 was carried out. A 10ml nutrient broth was inoculated with the CM11 strain and incubated with shaking at 37°C overnight. 100 ml of Mueller Hinton broth (Oxoid limited, Basingstoke Hampshire, England) in a flat bottom flask was placed in a water bath at a temperature of 37^0C overnight. The flask was then inoculated with 2mls of the overnight culture. The optical density (O.D. 600nm) was measured with the aid of a spectrophometer at time 0 and recorded and serial dilutions were prepared to 10^{-6} and plated out. This procedure was repeated at 30 minutes interval up to 3.5 hours. At the 4th hour, serial dilutions were increased to the 10^{-7} and plated out. This was repeated every 30 minutes up to the 6th hour. Bacterial count cfu/ml (colony forming unit per ml) was recorded at every hour.

2.3. Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *S. aureus* CM11. Oxford strain NCTC 6571 served as control. Gentamicin (Sigma Aldrich, Germany) was the antibiotic used to check for the organism susceptibility. Stock solutions were prepared using the Andrews (2001) method. The MICs of the planktonic cells of*S. aureus* CM11were determined using the broth microdilution method as described in the Andrews (2001) paper.

2.4. Kill Curve of S. AureusCM11

A kill curve including a growth control of the organism was set up to determine the effect of gentamicin on the growth of the organism. An overnight culture of CM11 and Oxford strain were prepared. 2 X 100ml of broth autoclaved in conical flasks were placed in a water bath overnight at a temperature of 37°C. The O.D. of overnight cultures were checked and corrected to O.D of 0.3 estimated to contain about 10^8cfu/ml of bacteria and placed on ice to prevent further growth. 0.5ml each of the *S. aureus*CM11 culture was pipetted into both flasks. The O.D of both flasks were measured and recorded. Inoculum of both flasks were plated out at 30 minutes interval up to the sixth hour.

2.5. The Effect of Gentamicin on Biofilm of S. AureusCM11

Growth of biofilm was set up by preparing an overnight culture of the test strain and *S. epidermidis*, RP62a which served as control as they are good biofilm formers. The procedure was carried out using the resazurin assay kit (Fisher Scientific, England). Resazurin test was used to check for the presence of viable cells of CM11 biofilm after treatment with gentamycin in the 96-well microtiter plate and a 6-well plate. The effects of 1X, 4X and 10X MIC were tested on the biofilm formed by the organism. For the 96-well plate, the supernatant from the treated and incubated plate was removed from all the wells. Wells were washed three times with Phosphate Buffer Saline (Oxoid limited, Basingstoke Hampshire, England). 100µl of 0.001% resazurin was then pipetted into all the wells. The plate was sealed, wrapped with foil and incubated for 2 hours at a temperature of 37°C. The fluorescence (excitation 544 and emission 590) of the 96-well plate was measured on plate reader. The content of the 6-well plate was treated with antibiotic and incubated, after which supernatant was discarded. Wells were washed thrice with PBS and once with distilled water. Biofilm cell viability was determined with live/dead staining following the manufacturer's instructions. Coverslips were observed with the aid of EVOS inverted microscope to view the live and dead cells.

2.6. Biochemical Tests for Detection of Enterotoxins

Protein agglutination and ELISA tests were employed to determine the enterotoxins carried by the isolate. Protein agglutination test was carried out using StaphTEX™ Blue kit. ELISA was performed using Staphylococcus aureus Enterotoxin ELISA Kit.

2.7. Molecular Analysis

Extraction of genomic DNA was performed on overnight broth cultures of the test strain and the positive control strain (oxford strain NCTC 6571) using the QiagenDNeasy Mini Prep kit. For quality control purpose, the DNA was quantified by the agarose gel electrophoresis. 4µl of hyper ladder was loaded into the first well. 2µl of loading dye and 8µl of the extracted DNA was loaded into well. Gel was visualized using the ultraviolet transilluminator machine. The quality was checked by pipetting 2µl of the DNA extract unto the epoch machine. Presence of genes coding for toxins A, B, C, D, E and Toxic shock were determined by conventional PCR amplification using the extracted DNA samples as templates. 10.5µl of PCR water, 12.5µl of master mix, 0.5µl each of both the forward reaction primer and backward reaction primer coding for toxin C (5'-GGC GAT AAG TTT GAC CAA TCT AAA TAT-3') and 1µl of template DNA were pipetted into the PCR tubes and then amplified using the PCR machine. 1µl of template DNA and 1µl of water were used respectively as positive and negative controls for the experiment. 1% agarose gel was set up and the toxin genes were observed and quantified.

The effect of sub-minimum inhibitory concentration on the RNA of isolate was determined by treating the isolate with 0.25X MIC of the antibiotic (Gentamicin). There were 2 batches of inoculum/overnight cultures. One batch was treated with the sub-MIC of the antibiotic and the second batch was untreated.

Isolation of the treated and untreated RNA was performed using the Invitrogen Trizol Max Bacterial RNA Isolation Kit. DNAse treatment was carried out with the use of a kit (Turbo DNase kit; Ambion, cat no AM 2239). RNA (combined with 2µl loading dye) was run on a 2% agarose gel at 85V for 45 min and viewed for quality and quantity using the EPOCH machine. RNA clean-up was carried out with the use of RNeasyMinElute Cleanup kit (Qiagen; cat no 74204). cDNA preparation was carried out using M-MLV Reverse Transcriptase 200U/µl (Invitrogen; 28025 - 13). The RNA yield was assessed by UV absorbance. Real time PCR was performed using the Opticon 2 instrument. Each reaction contained 12.5µl SYBRgreener mix, 0.5µl of forward primer (final concentration 200nM), 0.5µl of reverse primer (final concentration 200nM), 200-400ng of cDNA, and sterile DNase-free water to a final volume of 25µl.

2.8. Statistical Analysis

P-value of below 0.05 was considered statistically significant. Analyses were made using the Minitab Statistical Software for Windows.

3. Results

3.1. Confirmation of the Isolate

Identification tests carried out on the isolate proved to have the characteristics of *S.aureus.* The test strain was named *S. aureus* CM11.

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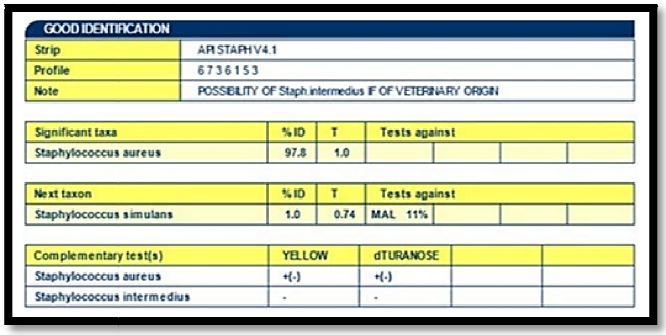

GOOD IDENTIFICATION	
Strip	API STAPH V4.1
Profile	6736153
Note	POSSIBILITY OF Staph.intermedius IF OF VETERINARY ORIGIN

Significant taxa	%ID	T	Tests against			
Staphylococcus aureus	97.8	1.0				

Next taxon	%ID	T	Tests against			
Staphylococcus simulans	1.0	0.74	MAL 11%			

Complementary test(s)	YELLOW	dTURANOSE		
Staphylococcus aureus	+(-)	+(-)		
Staphylococcus intermedius	-	-		

Figure 1: API Identification Test Result Displayed 97.8% Similarity with S.Aureus and 1% Similarity with S. Simulans

3.2. Growth Curve of S. AureusCM11

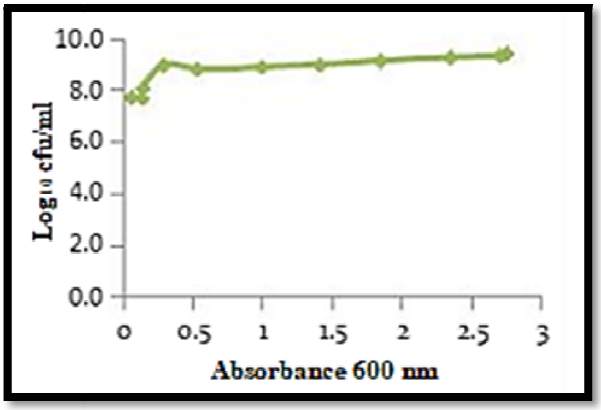

Figure 2: Curve of Viable Count and Optical Density of S. Aureus CM11

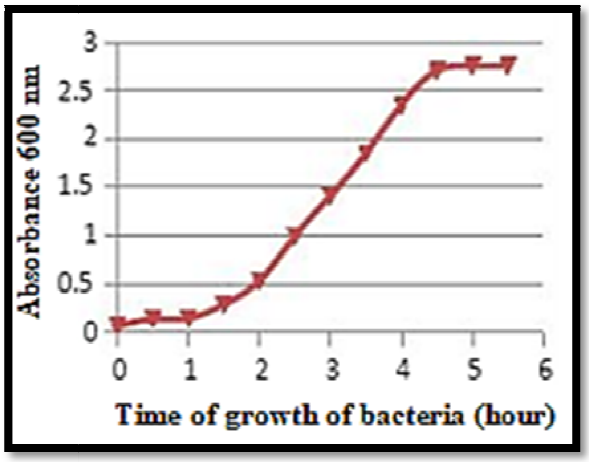

Figure 3: Graph of Absorbance against Time of Growth of S. Aureus CM11

It was observed that the lag phase lasted till about the first hour before the rapid exponential phase began and lasting till about the 4.5th hour making a total of about 3.5 hours for cells to grow exponentially. The test strain entered into the stationary phase at about the 4.5th hour.

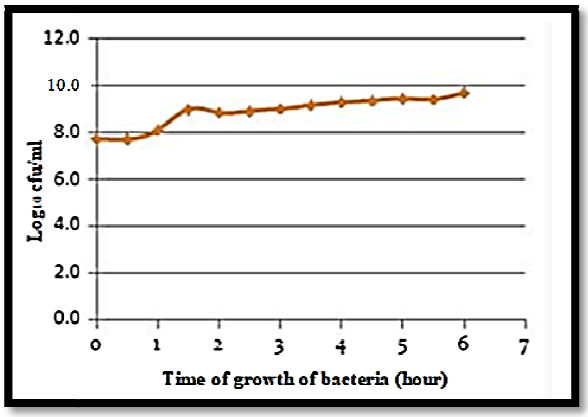

Figure 4: Graph of Viable Count against Time of Growth against the Time of Growth of S. AureusCM11

The bacterium lag phase lasted for about an hour. The exponential phase began from the first hour up until the 6th hour, making a total of 5 hours for the exponential phase

3.3. Minimum Inhibitory Concentration of S. aureus CM11

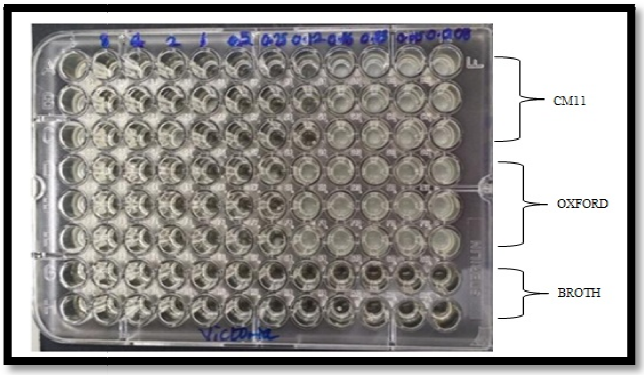

Figure 5: MIC Plate Showing the Lowest Concentration to Inhibit Bacteria Growth

The wells in rows 1-3 (from the top) contain the test strain, CM11 and the different concentrations of the antibiotic, gentamicin. The wells in rows 4-6 contain the control strain, Oxford and the different concentrations of the antibiotic. The wells in rows 7-8 contain sterile broth and antibiotic. The test strain, CM11, exhibits a clear zone from 0.12mg/L concentration of the antibiotic indicating its MIC at this concentration. The control strain, Oxford, exhibited a MIC of 0.25. The sterile broth in rows 7 and 8 serving as control exhibited a clear zone. Photo was taken at the clinical microbiology laboratory, Glasgow Caledonian University, Scotland.

3.4. Effect of antibiotic on S. aureus CM11

Time-kill curve plotted showed that gentamicin induced a bactericidal effect on the test strain

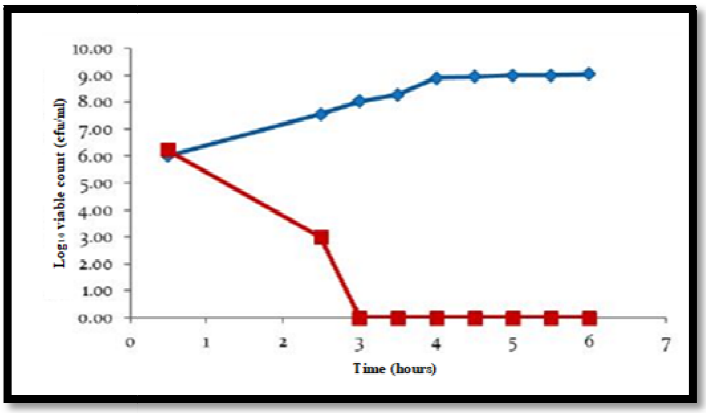

Figure 6: The Growth and Kill Curve of S. Aureus CM11

The exponential phase of the growth curve (represented in blue) began at about the 0.5th hour and progresses steadily into its stationary phase. The kill curve (represented in red) shows a decline in the number of bacterial cells from the 0.5th hour till the 3rd hour. At the 3rd hour, there were no viable cells left

Growth curve of S. aureus CM11

Kill curve of *S. aureus* CM11

3.4. The effect of Gentamicin on Biofilm of CM11 Isolate

CM11 biofilm treated with gentamicin showed lesser growth when compared to that without the antibiotic. This is shown in the figure below

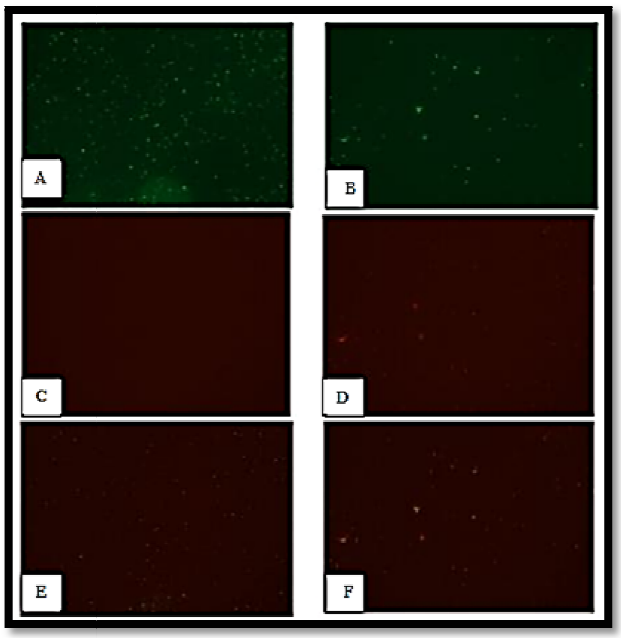

Figure 7: Picture Showing the Effect of Antibiotic Induced Biofilm at 10x Mic of the Antibiotic. A, C, E Are the Untreated Biofilm. B, D, F Are Images of the Biofilms Treated with 1x, 4xand 10x Mic of Gentamicin Respectively, Photo Was Taken atthe Clinical Microbiology Laboratory, Glasgow Caledonian University, Scotland

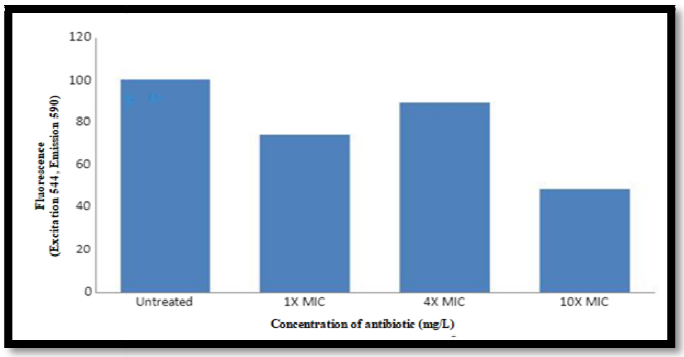

Figure 8: A Bar Chart Showing the Effect of Gentamicin on Biofilm Formed by the Test Strain, CM11, at the Different Concentrations of the Antibiotic (1X, 4X, and 10X MIC Respectively), the 10X MIC Was the Most Effective of the Three Concentrations of the Antibiotic

3.5. Biochemical Tests for Detection of Enterotoxins

Protein agglutination detected the presence of toxin C in CM11. ELISA test carried out on CM11 detected the presence of toxin C in CM11 due to color change.

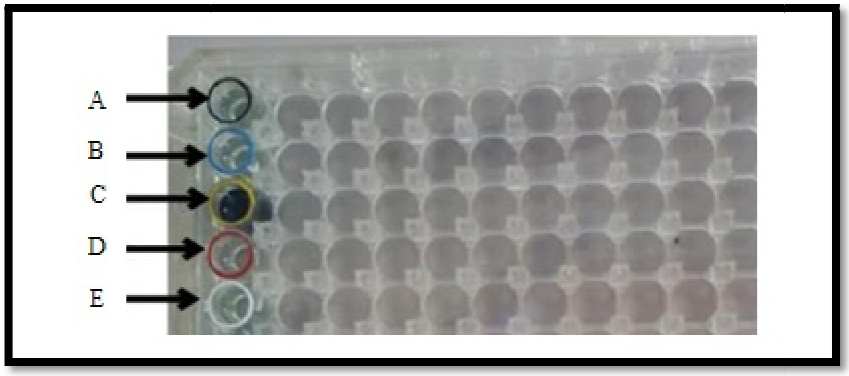

Figure 9: An Enzyme-Linked Immunosorbent Assay (ELISA) Plate for the Detection of Enterotoxins A-E. The Deep Blue Color in Well C Represents a Positive Result for Staphylococcal Enterotoxin C (SEC). Photo Was Taken at the Clinical Microbiology Laboratory, Glasgow Caledonian University, Scotland

3.6. S. aureus CM11 expressed genes encoding enterotoxin C and Protein A

PCR carried out on CM11 proved the presence of a DNA containing genes that express toxin C and protein. RNA extracted from both treated (with sub-MIC of gentamicin) and untreated CM11 were analyzed. Both were of good quality.

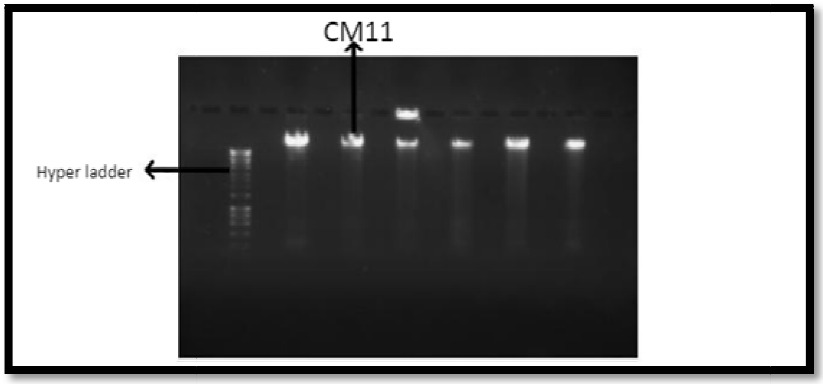

Figure 10:Gel Electrophoresis Results of a Conventional PCR Reaction of DNA of S. Aureus CM11 Photo Was Taken at the Clinical Microbiology Laboratory, Glasgow Caledonian University, Scotland

4. Discussion

S. aureus CM11 attained growth early suggesting they grow and multiply rapidly. The ability of the test strain to give out bubbles when catalase test was carried out on it indicates the presence of the enzyme catalase, which breaks down hydrogen peroxide, hence resulting in the production of oxygen in the form of bubbles. This is present in most cytochrome-containing aerobic and facultative anaerobic bacteria with*S. aureus* belonging to the family of facultative anaerobes. *S. aureus* CM11 is coagulase-positive suggesting that it possesses the enzyme coagulase that clots blood plasma, which is a good indicator of a pathogenic *S. aureus*. In the human host, the action of the coagulase enzyme produces clotting of the plasma by converting fibrinogen to fibrin in the immediate vicinity of the bacterium as a means of

protection. This fibrin formed has the capacity to surround bacterial cells or the infected tissues thus protecting the bacterial cells from host resistance mechanisms such as phagocytosis. *S. aureus*CM11 was screened for the presence of various genes which are capable of causing diseases in humans. The pan-genome of *S. aureus* strain is a combination of the core genome of *S. aureus* and unique genes which are exclusive to the strain. The core genome is highly conserved and is present in most of the strains. The uniquegenes account for the variability among strains of the organism. About 2,400 sequence types for *S. aureus* have been discovered till date. The PCR result showed that *S. aureus* CM11 strain lacked the genes for toxin A, B, D, E and toxic shock syndrome toxin. *S. aureus*CM11 however expressed the gene for Staphylococcal enterotoxin C (SEC). Results from the ELISA confirmed the secretion of enterotoxin C by *S. aureus*CM11 (Figure 9). Enterotoxin C is known for causing abdominal pain, vomiting and diarrhea in humans (Grumann, Nübel, & Bröker, 2013).Furthermore, studies have also shown that SEC production by pathogenic *S. aureus*was associated with persistent infections, bovine mastitis, and toxic shock syndrome (Booth, Pence, Mahasreshti, Callegan, & Gilmore, 2001)(Edwards, et al., 1997)(Fitzgerald, et al., 2001)(Rasheed, et al., 1985). Majority of MRSA strains secrete large amount of this toxin. Mutant toxin strains, mSEC, were found to induce toxin-specific antibodies that can lead to its clearance in the body of the host (Hu, et al., 2005).

Another very importantvirulent factor expressed by*S. aureus* CM11 is theStaphylococcal protein A (SpA).Protein A in *S. aureus*(SpA) is situated in the bacterial cell wall envelope and released during growth of the bacteria. It plays a key role in the organism's evasion of the host immune system by binding to the IgG/IgM thus inhibiting opsonization and phagocytosis. Studies have shown that SpA mutants that were unable to bind to the IgM were phagocytosed eliciting the B-cell response in the process unlike the wild type strains that were capable of making void the humoral immune responses (Falugi, Kim, Missiakas, & Schneewind, 2013)

MIC of gentamicin on *S. aureus*CM11 is shown to be 0.12mg/L (Figure 5). At this concentration, it is expected to inhibit the growth of planktonic cells of this organism (Andrews, 2001), however the outcomes obtained invitro may not be obtainable under in vivo conditions.Gentamicin was effective against *S. aureus*CM11 biofilms, albeit at a much higher concentration of the antibiotic (10X MIC) than was required for the free-living state of the bacterium. This finding was consistent with a study by Coraca-Huber *et al.,* (2012) which showed that Gentamicin was the most effective antibiotic tested on *S. aureus*both in the planktonic and the biofilm states. Organisms have been shown to develop adaptive resistance to gentamicin (Mohamed, Nielsen, Cars, & Friberg, 2012).*S. aureus* CM11 may have been able to tolerate the antibiotic at the 4X MIC. This resistance may emerge at the start of therapy and can be promoted by exposure to higher concentration of the antibiotic, after which the bacteria become susceptible to the antibiotic(Mohamed, Nielsen, Cars, & Friberg, 2012). This is probably what was demonstrated in the result obtained in figure 8, where there was increased growth at the 4X MIC compared to the 1X MIC and drastically decreased growth at the 10X MIC. Results support study carried out by Coraca-Huber *et al.,* (2012) where *S.aureus* biofilms treated with gentamicin assumed some form of resistance. The 10X MIC was the most effective of the three concentrations. This indicates that higher concentration of antibiotic will be required in treating bacteria in the sessile state. This is highly disadvantageous as it poses greater health risk resulting from antibiotic resistance and the spread of resistant bugs.

Studies have shown that sub-MIC has a significant effect on the toxin production by bacteria. Antibiotics at sub-MIC levels have been shown to inhibit the virulence factors of *S. aureus.* Studieshave shown that there was a significant decrease in the toxin and enzyme production by the bacterium (Gemmell & Ford, 2002)(Bernardo, et al., 2004)(Herbert, Barry, & Novick, 2001). A study by Mohsin M *et al.,* (2010) also revealed that sub-MIC of gentamicin tested on Shiga Toxin Escherichia coli (STEC) resulted in decrease of the Shiga toxin produced by Escherichia coli. The effect of sub-MIC of gentamicin was investigated on the virulence factors of the test strain, S. aureus CM11. The RNA treated with 0.25MIC of gentamicin was of good quality but unfortunately the graph produced by the real time PCR could not be read at the final phase. This is likely as a result of contamination. This study thereforecannot state if the sub-MIC of gentamicin inhibits the virulence factors possessed by*S. aureus* CM11.In summary, the virulence factors expressed by *S. aureus* CM11 are important factors for the establishment of infection and the organism's survival in the body of the host. The ability of the organism to form biofilms further increases its chances of survival and evasion of host immune system.

5. Conclusion and Recommendation

The test strain, *S. aureus* CM11, isolated from the catheter explored in this study is considered to be pathogenic. Biofilm formation by the bacteriumenabledits tolerance to the antibiotic. This can potentially make infection caused by this strain difficult to treat. Research can be conducted to develop novel anti-biofilm agent.

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