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CHARACTERIZATION OF A CLINICAL ISOLATE OF STAPHYLOCOCCUS AUREUS, AND THE ACTION OF LINEZOLID ON GROWTH PROPERTIES AND TOXIN PRODUCTION

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ABSTRACT

Staphylococcus aureus emergence as a significant pathogen has been enhanced by its increased resistance to many antibiotics due to its ability to express several virulence factors, and extracellular toxins, as seen in Methicillin-resistant *S. aureus* (MRSA) strains. This study, investigated an *S. aureus* clinical isolate obtained from Glasgow Royal infirmary MRSA reference laboratory, using phenotypic and molecular methods in assessing its susceptibility to linezolid, ability to produce biofilms, known toxins, and the impact of treatment with sublethal linezolid concentration on toxin(s) expression. Result revealed Minimum Inhibitory Concentration (MIC) of linezolid on *S. aureus* planktonic cells as 4mg/L, Minimum Bactericidal Concentration (MBC) as 32mg/L, and MBC/MIC ratio of 8. Antibiotic time kill assay revealed linezolid effect on the planktonic cells of the *S. aureus* isolate as bacteriostatic; as viable count reduction was approximately 2log10. Biomass measurement of the *S. aureus* isolate by comparison with RP62a; a known biofilm-producing strain, indicated that it formed strong biofilm. In biofilm, linezolid concentration at 10×MIC had no significant effect ($p>0.01$) on its viability. Whereas at 40×MIC, linezolid effect was significantly greater ($p<0.01$), but unable to eliminate its viability. The *S. aureus* clinical isolate was shown to produce Staphylococcal Enterotoxin A and toxic shock toxins, and when challenged with sublethal linezolid concentration (0.25×MIC), its expression of both toxins proteins was downregulated by 2 folds. This study suggests linezolid ability to limit expression of vital *S. aureus* virulence factors and reinforces linezolid for consideration, in the treatment of severe *S. aureus* infections.

KEYWORDS: *Staphylococcus aureus*, MRSA, Linezolid, Planktonic, Biofilms, Toxins.

INTRODUCTION

The *Staphylococcus* genus, is composed of Gram positive cocci, non-spore forming, non-motile facultative anaerobic bacteria, possessing complex nutritional growth requirements and a low G+C DNA content. It is categorized into two main groups on the basis of coagulase enzyme production; of which coagulase producing *S. aureus*, is most pathogenic and implicated in nosocomial and community acquired infections.[1] As a commensal, *S. aureus* colonizes about 20-30% of humans permanently, while 30% of individuals are transiently colonized thus, increasing the likelihood of possible infections. *S. aureus* can be an opportunistic pathogen, causing a range of infections including; mild skin and soft tissue infections, infective endocarditis, bacteraemia, necrotizing pneumonia, and toxin-mediated diseases, which significantly increases morbidity and mortality.[1,2] In 1961, an *S. aureus* drug resistant strain was first reported in the United Kingdom.[3] MRSA, emerged based on the acquisition of *mec*A gene, and has evolved to become the commonest antibiotic resistant strain in most countries including the UK, where it constitutes about 45% of isolated *S. aureus* strains.[2,4]

S. aureus infection diversity, is linked to its ability to express virulence determinants including adherence factors, cell surface factors (Staphylococcal protein A, clumping factor proteins, collagen-binding protein, staphyloxanthin, capsular polysaccharides), secreted factors, pyrogenic superantigens (TSST -1, Staphylococcal enterotoxins A, B, C, D, E, G, H, I), exfoliative toxins (ETA, ETB), cytolytic toxins (cytolysins, leucocidin family), exoenzymes (nucleases, proteases, lipases), and miscellaneous proteins (staphylococcal complement inhibitor, chemotaxis inhibitory protein, extracellular fibrinogen binding protein, extracellular adherence protein).[1,5] Furthermore, some *S. aureus* strains form biofilms on damaged tissues and implanted biomaterials, which

enhances treatment difficulty by reducing antibiotic penetration and limiting host immune responses.[6]

The expression during infection, of these virulence factors is coordinated, lending evidence to the presence of global regulators such as staphylococcal accessory regulator (including its homologues), accessory gene regulator, etc., which aid in *S. aureus* adaptability, survival and infection.[5]

The need for a potent treatment option informed linezolid development; the first developed oxazolidine class of antibiotic approved for use clinically, due to its wide range of effectiveness against Gram positive bacteria, some Gram negatives, *Norcadia* spp, *Actinomyces* spp, MRSA, vancomycin resistant enterococci, several species of mycobacteria, and penicillin resistant pneumococci.[7] Linezolid is a bacteriostatic antibiotic that prevents the 70S initiation complex formation, by binding to the 50S subunit of bacterial ribosome via 23S rRNA interaction thus, inhibiting protein synthesis. It is available in oral and intravenous formulation and could be utilized for empiric monotherapy of community associated MRSA and beta haemolytic streptococci infections.[7,8]

Linezolid is effective in the treatment of skin and soft tissue infections, nosocomial pneumonia, ventilator associated pneumonia, MRSA meningitis, infective endocarditis, and biofilm associated MRSA. It is more effective compared to glycopeptides, has high bioavailability, easy switch option to oral therapy, good pharmacokinetic and safety profile, inhibits staphylococcal toxins production and bacterial growth.[9,10] However, reports of linezolid resistant *S. aureus* clonal dissemination, demands active surveillance, to ascertain the emergent risk of resistance strains while establishing guidelines for its appropriate use.[11]

In order to differentiate *S. aureus* clinical isolates, antimicrobial susceptibility testing is the most common phenotypic technique used. However, the limitation of strains having differences in genetic profiles, while possessing same antibiogram patterns, informs the use of more advantageous molecular methods such as PCR-based methods. Also, *S. aureus* strain typing has proven vital in detecting outbreak-related strains, and in the control of new waves of infections involving resistant strains.[12]

The aim of this present study is to characterize a clinical isolate of *S. aureus*, and provide insights into the impact of linezolid on the isolate in its planktonic and biofilm forms, while determining its ability to express known staphylococcal toxins in the presence or absence of linezolid, including the impact of treatment with sublethal linezolid concentration on toxins expression.

MATERIALS AND METHODS

Antimicrobial Agent and Bacterial Strains Used

The antibiotic used in the present study was linezolid. The study was conducted using an *S. aureus* clinical isolate (labelled Saur06) as the test strain, obtained from Glasgow Royal infirmary MRSA reference laboratory, Scotland, United Kingdom. Other strains utilized include; quality control antibiotic susceptibility strain *S. aureus* subsp. *aureus* Rosenbach (ATCC 29213) and biofilm positive strain (RP62a) from Glasgow Caledonian University reference library of Dr. Susan Lang. Pure culture(s) of all strains were obtained by streaking strains preserved in cryobeads on blood agar; prepared using Columbia blood agar base (Oxoid Limited, Basingstoke, United Kingdom) supplemented with 5% defibrinated horse blood (E & O Laboratories, UK). Brain Heart Infusion agar (Oxoid Limited, Basingstoke, UK), was utilized for viable count determination, and overnight cultures of strains were prepared by inoculating a single colony of required strain into either Mueller Hinton Broth (Oxoid Limited, Basingstoke, UK), Tryptone Soya Broth (Oxoid Limited, Basingstoke, UK), and Brain Heart Infusion Broth (Oxoid Limited, Basingstoke, UK) depending on requirements. Broths were incubated for 18h whilst shaking (120rpm) at 37^0C. Phosphate Buffered Saline Dulbecco 'A' tablets (Oxoid Limited, Basingstoke, UK) was prepared and utilized for serial dilutions and biofilm washing protocols. Strains were subcultured on blood agar based on requirements.

Phenotypic Tests for the Identification of the Isolate

Gram Staining

A single colony of the *S. aureus* test strain was smeared on a glass slide, heat-fixed, and air-dried. Crystal violet was applied on smear, and allowed for one minute at room temperature, before rinsing with water. Iodine was then flooded on smear, and allowed for one minute, before rinsing, followed by application of alcohol (95%), and rinsing after 30 seconds. Safranin was applied and rinsed after one minute. Cells were imaged by microscopy (40× objective and 100× objective under oil immersion). RP62a served as control.

Staph latex agglutination test

The Staph latex test kit (ProLab diagnostics, UK) was utilized. A drop of blue staph latex test reagent was placed on a slide card, followed by smearing of a colony of the *S. aureus* test strain and gentle rocking for some few seconds while observing for possible clump formation. RP62a served as control.

Catalase test

A single colony of the *S. aureus* test strain was smeared into a glass universal, containing 6% (w/v) hydrogen peroxide and observed for bubbles formation. A positive result unique to Staphylococci was observed for after some seconds.

Analytical Profiling Index (API) Staph test

A single colony of the *S. aureus* clinical isolate was mixed with a solution of barium sulfate (0.5 McFarland) to prepare a suspension. Each microtubule of APISTAPH test strip v5.0 (BioMerieux) was filled with the suspension, with the exception of ADH and URE cupules which was supplemented with mineral oil. The strip was incubated for 18h at 37^0C, and reactions developed based on the provided manufacturer's instruction. [13] A database

(apiWeb™ v.4.1), was used to interpret the 7 digits' numerical profile to obtain the clinical isolate percentage relatedness to *S. aureus*.

Determination of the Bacteria Growth Pattern

Growth studies of the *S. aureus* test strain planktonic cells, was determined by Optical Density (OD600) measurement and viable count (CFU/ml) determination, within a 6h time point at 30min interval. 2ml of the test strain overnight culture was inoculated into a flask containing 100ml of sterile Mueller Hinton Broth (1:50 dilution), in shaking (120rpm) incubation at 37^0C. Culture turbidity was measured at time 0, and at intervals of 30min, and viable count determined by plating selected dilutions (10-fold serial dilution) at each time point and subsequent incubation.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against the Planktonic *S. aureus* Clinical Isolate

The *S. aureus* clinical isolate was tested against a range of linezolid concentrations (mg/L) to determine the MIC, with broth dilution susceptibility method utilized as detailed in the guideline of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[14] Eleven concentrations including the linezolid MIC range (1-4mg/L) was prepared, and 75µL of each was aliquoted unto corresponding wells of a microtiter plate, followed by the inoculation of 75µL ($5x10^4$ CFU/well) of the test strain, and ATCC 29213 cells (antibiotic susceptibility control). The setup included sterility and growth controls, followed by overnight incubation at 37^0C. MIC was determined as the least linezolid concentration which demonstrated complete inhibition of growth after overnight incubation at 37^0C. All clear wells were subcultured on BHIA plates and incubated at 37^0C for 24h to determine the MBC (mg/L); the least concentration of linezolid which achieved ≥99.9% killing of the *S. aureus* clinical isolate cells. MBC/MIC ratio was thereafter determined.

Antibacterial Time Kill Assay

To assess the antimicrobial activity of linezolid on planktonic culture of the test strain, the time kill experiment was carried out *in vitro*. The set up included a control flask (untreated), which contained 100ml MHB and 0.5ml of $1x10^8$ CFU/ml of the *S. aureus* clinical isolate cells ($5x10^5$ CFU/ml), and a test flask (treated), which contained the same initial content as the untreated, in addition to treatment with 1ml of 1 X MIC of linezolid. The both flasks were placed in water bath shaking (120rpm) incubation at 37^0C, and the bacteria growth pattern was determined by OD600 measurements and viable count determination (CFU/ml). Each measurement was performed at an interval of 30min for 6h and linezolid impact, was observed for either a bactericidal (≥3log10 decrease) or bacteriostatic (<3log10 decrease) effect in the clinical isolate viable count.

Biofilm Establishment

Biofilm establishment was performed using a modified methodology reported by Cha *et al*. [5] 100µL of adjusted overnight cultures of the clinical isolate and biofilm positive strain (RP62a) adjusted to ($1 x 10^6$ CFU/ml) were aliquoted into seperate rows of a 96 well microtiter plates.

MHB was included as sterility control, and plate was incubated at 37^0C in a rocking platform for 24h.

Biofilm Biomass Quantification

The *S. aureus* clinical isolate and RP62a biomass were measured by crystal violet (CV) assay using a modified methodology reported by Smith *et al*. [4] Following incubation, biofilm cells in microtiter plate were gently rinsed using PBS (Oxoid Limited, Basingstoke, UK) to remove residual planktonic cells. Plate was heat-fixed at 60^0C for 20 min and 100µL CV stain 0.5% (w/v) was applied on adherent cells. After 5min, excess stain was removed by washing gently with sterile water, 100µL of 70% ethanol was applied to wells, and absorbance was measured at 595nm wave length (Fluostar Optima, BMG - Labtech).

Resazurin (Biofilm Metabolic) Assay

Metabolic activity of the *S. aureus* clinical isolate biofilms was measured using resazurin assay. 100µL of resazurin dye 0.001% (w/v) was applied to washed wells of a biofilm plate, and plate was foil-wrapped and incubated in dark conditions at 37^0C for 2h. Fluorescence was measured to quantify viable cells in biofilm at Ex540/590Em (Fluostar Optima, BMG - Labtech).

Antibiotic Susceptibility and Resistance Assay of the *S. aureus* clinical isolate biofilms

Linezolid treatment of biofilms and quantification of killing effect by fluorescence

Different linezolid concentrations impact on the susceptibility of clinical isolate biofilm cells was investigated. Selected wells of a sterile 96 well microtiter plate was inoculated with adjusted test strain inoculum ($5 x 10^5$ CFU/well), and controls which included; RP62a, untreated test strain inoculum, and sterile MHB. The setup was incubated at 37^0C in a rocking platform for 24h. Following incubation, wells were washed with PBS, and 100µL of linezolid concentrations (1xMIC, 10xMIC,

40xMIC) was added to corresponding wells. The microtitre plate content was re-incubated at 37^0C for 24h, to achieve interaction with linezolid. Wells were thereafter washed with PBS, followed by staining and fluorescence measurement.

Linezolid treatment of biofilms and quantification of killing effect by live - dead analysis

1% (w/v) Poly L lysine pre-treated coverslips were transferred into each well of a 6-well plate, and 2ml of adjusted *S. aureus* test strain inoculum (1 x 10^6 CFU/ml) was inoculated into each well and incubated at 37^0C for 24h. Following incubation, wells were washed with PBS, and cover slips were treated with 2ml of 10xMIC and 40xMIC linezolid concentrations (in duplicate). Coverslips in wells serving as control, were untreated. The set up was re-incubated at 37^0C for 24h, after which wells were re-washed and 1ml of live-dead stain composed of 1.5µL SYT09 + 1.5µL propidium iodide + 997µL of molecular grade water (Live/Dead™ BacLight, Molecular Probes kit, USA), was applied on 40xMIC treated coverslips and untreated coverslips. Stained coverslips were incubated in the dark for 15min, air dried, mounted on slides and imaging under EVOS fluorescence microscope (AMG, Washington, USA), with transmitted light, GFP and TEXAS RED at ×40 magnification.

Genomic DNA Extraction

Bacteria from 1ml of overnight culture (grown in TSB), was sedimented by centrifugation (8000×*g*) for 5min using an Eppendorf AG Minispin microcentrifuge (Hamburg, Germany). The pellet was resuspended in 185µL of bacterial lysis buffer (20Mm Tris-HCl, 2mM EDTA, 1.2% Triton ×100, 25U/ml lysostaphin, 20mg/ml lysozyme) and incubated at 37^0C for 1h. Genomic DNA extraction was achieved using DNA extraction kit (DNeasy blood and Tissue kit (50), Qiagen GmbH, Germany), and all protocol performed according to the manufacturer's instruction provided. [15] Quantitative and qualitative evaluation of extracted *S. aureus* test strain genomic DNA was determined using EPOCH Take3 Micro-Volume plate reader (BioTek Instruments, Inc., USA and Gen.5.1.10 Software) at 260/280nm wavelengths. Gel electrophoresis (Flowgen Bioscience), was performed on DNA extract through a 1% agarose gel, with run conditions at 120V for 60min, followed by gels visualization (UVP Bio Doc-It 220 imaging system).

Polymerase Chain Reaction (PCR) Amplification and Agarose Gel Electrophoresis for Toxin Genes Detection

The *S. aureus* clinical isolate DNA template was analysed for the presence of staphylococcal toxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *spa*, *tst*). PCR mix was prepared composing of a master mix (12.5 µL), forward and reverse primers (0.5 µL), and water (10.5 µL) after which, 24µL of the prepared master mix was aliquote into PCR tubes, which had been inoculated with 1µL of DNA template. Amplification was performed (40 cycles) using PCR T3 thermocycler (Biometra, Germany) and the different primer pairs (Table A.1), had different PCR conditions. Following amplification, the products of PCR were loaded into wells of agarose gel (2%), with run conditions of 120V for 60min and gels were visualized (UVP Bio Doc-It 220 imaging system), for toxin genes detected.

Reverse Passive Latex Agglutination (RPLA) Assay

The production of Staphylococcal enterotoxins (Sea, Seb, Sec, Sed, See) and TSST proteins by the *S. aureus* clinical isolate was investigated by double dilution method using the methodology detailed in the manufacturer's instruction.[16,17] TSB and BHI supernatants were recovered after centrifuging 10ml of the test strain overnight cultures in TSB and BHI at 900× *g* for 20min at 4^0C (Allegra X-12R). SET-RPLA TD 900 and TST-RPLA TD 940 kits (Oxoid LTD), containing diluents, sensitized latex, and latex controls were used to setup reactions in a v-well microtiter plate. Result was observed for agglutination after 24h incubation at room temperature on a vibration-free platform.

Genomic RNA Extraction

5ml of the test strain overnight culture adjusted to 1 x 10^8 CFU/ml was inoculated into 2 flasks containing 45ml sterile MHB, to achieve a final concentration of 1 x 10^7 CFU/ml. The flasks were placed in a water bath at 37^0C shaking (120rpm) incubation to mid exponential growth, at which point one flask was treated with 0.25 x MIC (1mg/L), while the second flask served as untreated control. The flasks were then allowed for continued incubation for 2h after which cells pellet were recovered from 1ml of culture from each flask by centrifuging at 3000×*g* for 2min using Eppendorf AG Minispin Micro-centrifuge (Hamburg, Germany). 200µl of RNA protect (Qiagen) was added to pellet, followed by the extraction of RNA with Nucleospin kit, following the provided manufacturers procedures. Quantitative and qualitative integrity of RNA extract was determined using EPOCH Take3 Micro-Volume plate reader (BioTek Instruments, Inc., USA and Gen.5.1.10 Software) at 260/280nm and through agarose gel electrophoresis as described in a previous study.[18]

Reverse Passive Latex Agglutination (RPLA) assay to determine titre level of toxins (proteins) expression

The test strain titre expression levels of staphylococcal enterotoxin A, and TSST proteins when challenged with linezolid at sub MIC (0.25 x MIC), was determined using SET-RPLA TD 900 and TST-RPLA TD 940 detection kits (Oxoid LTD), according to the provided manufacturer's instructions.[16,17] Treated and untreated culture supernatants utilized, were obtained by centrifuging 5ml of each culture at 900×*g* for 20min at 4^0C (Allegra X-12R). The diluents, sensitized latex, latex controls, treated and untreated supernatant samples were reacted as provided in the instruction in separate rows in a v-well plate. Each well was observed for agglutination

after 24h incubation at room temperature, on a vibration-free platform.

Data and statistical analysis

The unpaired two tailed t-test was applied to evaluate differences between means in the assessment of the impact of different linezolid treatment concentrations on test strain biofilms viability, and to determine absorbance difference in the *S. aureus* clinical isolate and RP62a biofilms. Result were considered significant at $p<0.01$. Graphs and statistical analysis generated were processed using GraphPad Prism v7.03 (GraphPad Prism Software Inc., California, USA) and Microsoft Excel Application 2016 v16.0 (Microsoft Corporation, USA).

RESULT

All experimental strains are staphylococcal isolates by phenotypic relatedness

Gram staining analysis, revealed all experimental strains (*S. aureus* clinical isolate, ATCC 29213, and RP62a) as Gram positive bacteria as well as being catalase positive. The test strain was also shown to produce a positive reaction in the antibody-antigen based Staph latex agglutination test while RP62a (control) was negative (Table 1). By API STAPH test, the test strain showed a 97.7% similarity with *S. aureus* (Fig.1).

The *S. aureus* clinical isolate growth pattern is typical of the normal bacterial growth curve

The growth pattern of the *S. aureus* test strain using viable count (CFU/ml) and OD600 measurements were in conformity with the normal bacterial growth curve; possessing characteristic bacterial growth phases (Fig. 2a and 2b). Additionally, calibration graph of viable count (CFU/ml) against OD revealed a linear relationship between Saur06 viable count and OD, at OD600: 0.3 as approximately 1 x 10^8 CFU/ml (Fig. 2c).

Susceptibility of the *S. aureus* clinical isolate planktonic cells to linezolid was achieved *in vitro*

Linezolid MIC for planktonic *S. aureus* clinical isolate cells and ATCC 29213 (control) was 4 mg/L (within the EUCAST acceptable range of 1-4 mg/L), while linezolid MBC for the test strain and control strain (ATCC 29213) was 32 mg/L. The ratio of MBC/MIC = 8 (Table 2).

Linezolid treatment impact on planktonic cells was bacteriostatic

Linezolid effect on treated *S. aureus* test strain planktonic cells (at 1×MIC) was bacteriostatic and treatment was unable to achieve ≥3log10 CFU/ml reduction in viable count, within the post-treatment experimental period (Fig. 3a). Viable count reduction of the test strain treated cells reached was approximately 2log10, compared with the untreated culture, which characteristically followed the normal bacterial growth curve pattern. Furthermore, OD6OO absorbance measurements at each experimental time points, revealed a consistent reduction effect, in the treated setup compared to the untreated (Fig. 3b).

The *S. aureus* clinical isolate biofilm formation ability is relatively high

Based on biomass measurement by CV staining assay, *S. aureus* test strain revealed good biofilm forming ability (Fig. 4a). Although cells of the test strain possessed a relatively lesser ability to bound CV compared to RP62a (control), the difference was not significant ($p > 0.01$). Evidence of metabolic activity was also confirmed in both strains by fluorescence measurement, and this was shown to be lesser in the *S. aureus* test strain compared to RP62a, but the difference wasn't significant ($p > 0.01$) (Fig. 4b).

Total killing of *S. aureus* test strain cells in biofilm not achieved with selected linezolid treatment concentrations greater than MIC

Linezolid treatment impact at different concentrations (1xMIC, 10xMIC, and 40xMIC) on the *S. aureus* clinical isolate biofilms, revealed highest viability in biofilms at treatment concentration of 1xMIC (97.7%), followed by 10xMIC (78.6%) and 40xMIC (75.0%). Statistical analysis showed no significant difference ($P > 0.01$) with treatment concentrations at 1xMIC and 10xMIC, but a significant reduction effect ($p < 0.01$) with treatment concentration at 40xMIC compared with 1xMIC (Fig. 5). EVOS fluorescent microscopic study revealed direct evidence of decrease in viable Saur06 cells in 40xMIC linezolid treated coverslip, compared to the untreated. Cells were however not completely inactivated at 40xMIC (Fig. 6).

Staphylococcal enterotoxin A (*sea*) and toxic shock toxin (*tst*) genes and proteins are expressed by the *S. aureus* clinical isolate

Quantitative and qualitative integrity of the *S. aureus* clinical isolate DNA extract showed DNA quantity as 121.79ng/µl and quality ratio as 2.01. Further analysis revealed the presence of *S. aureus* protein A (spa gene), staphylococcal enterotoxin A gene, and TSST gene (Fig. 7), while *seb*, *sec*, *sed*, and *see* genes were absent. Reversed passive latex agglutination assay (SET- RPLA and TST-RPLA), revealed the expression of Sea and Tst proteins in Saur06 culture supernatants and an absence of *seb*, *sec*, *sed*, *see* proteins.

Quantitative and qualitative integrity of *S. aureus* clinical isolate RNA extract

Quantitative and qualitative integrity of *S. aureus* clinical isolate RNA extract revealed RNA quantity as 117.05ng/µl in linezolid treated RNA extract with a quality ratio of 2.15. For the untreated (control), RNA extract quality ratio was 2.14 and quantification was 121ng/µL.

Expression of the toxin proteins of *S. aureus* clinical isolate is regulated with treatment at sub-lethal linezolid concentration

Phenotypic SET- RPLA and TST-RPLA assays, revealed downregulation in *S. aureus* clinical isolate toxins proteins expression in linezolid treated Saur06 culture at

sub-lethal concentration (0.25×MIC). By double dilution, TSST and staphylococcal enterotoxin A proteins were observed to be two-fold less expressed in the linezolid treated setup, compared to the untreated control.

Table 1: Phenotypic characterization of the experimental strains.

S. aureus strains	Gram reaction	Catalase test	Staph latex agglutination
Saur06	+	+	+
RP62a	+	+	-
ATCC 29213	+	+	ND

Table 1: all experimental staphylococcal strains by Gram staining reactions and catalase test results are positive. Staph latex agglutination test indicates RP62a as negative. Abbreviations: ATCC. American Type Culture Collection, + (positive), - (negative), ND. Not Determined.

Table 2: Susceptibility pattern of planktonic *S. aureus* experimental strains to linezolid.

S. aureus strains	MIC (mg/L)	MBC (mg/L)	Ratio of mbc/mic
Saur06	4	32	8
ATCC 29213	4	32	8

Table 2: *in vitro* antibiotic susceptibility with linezolid, indicating MIC, MBC, and MBC/MIC ratio. Abbreviations: ATCC. American Type Culture Collection, MBC. Minimum Bactericidal Concentration, MIC. Minimum Inhibitory Concentration.

Figure 1: API STAPH identification of Saur06.

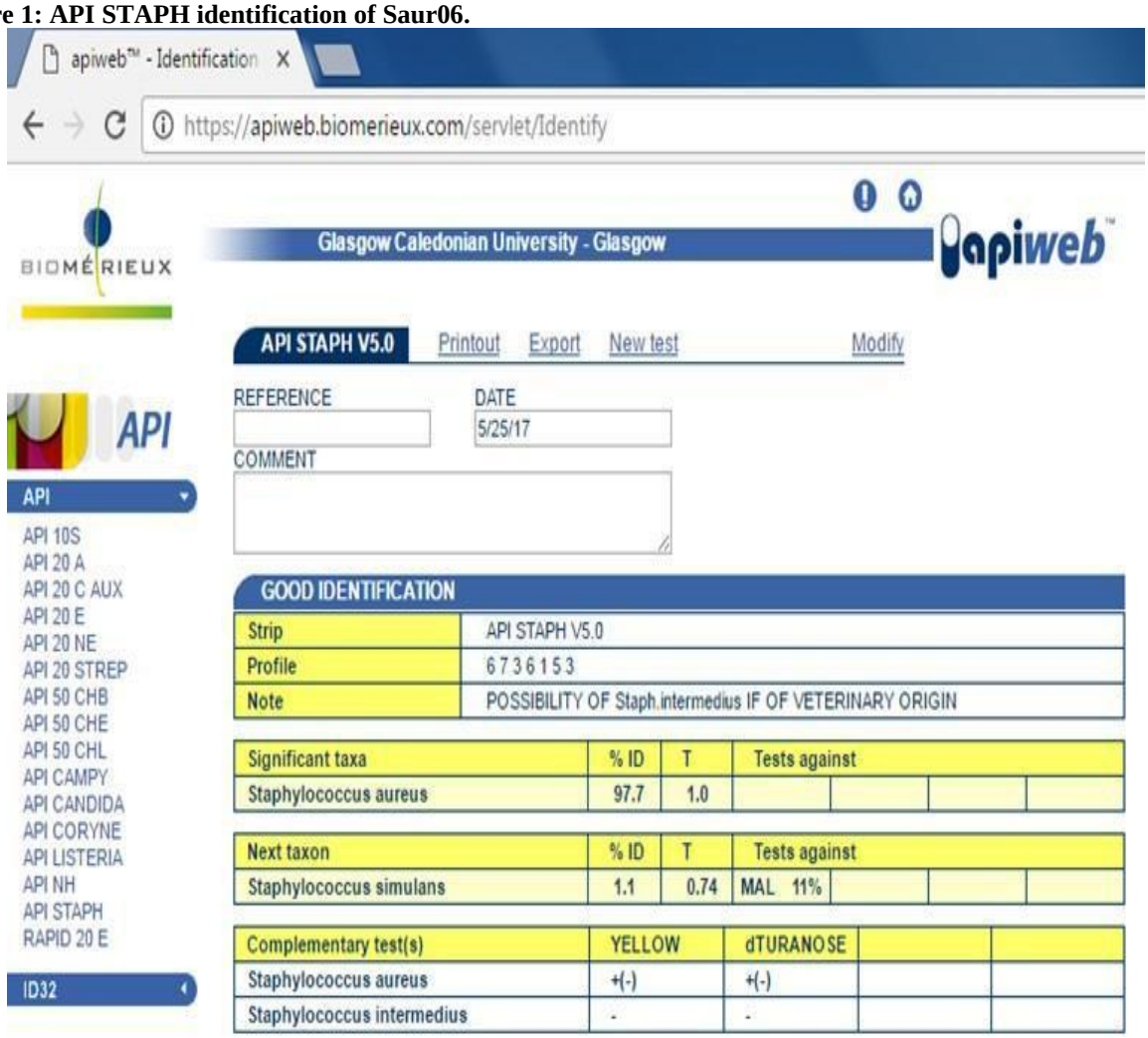

Fig. 1: Similarity of the *S. aureus* clinical isolate to *S. aureus*, using numerical profile indicates a 97.7% specie similarity (apiWeb).

Figure 2: Growth graphs of the *S. aureus* clinical isolate.

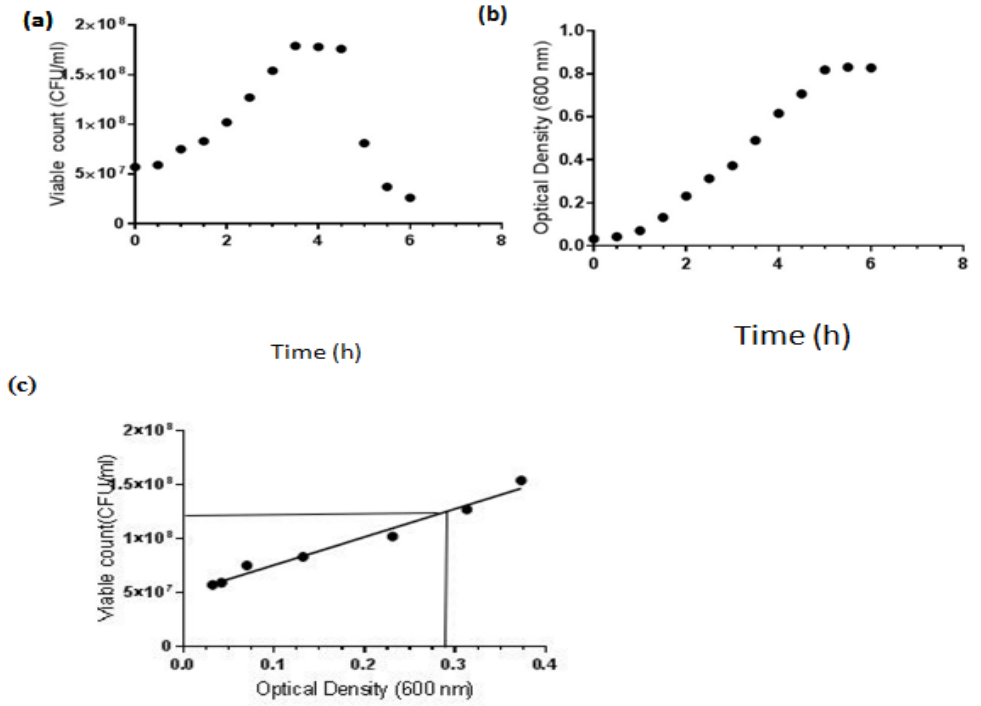

Fig. 2: (a) planktonic *S. aureus* clinical isolate growth graph by viable count (CFU/ml), at 30mins interval for 6h indicates the lag phase, exponential phase, stationary phase and death phase (b) growth graph determined by optical density (OD600) measurements (c) calibration graph indicates a linear relationship between viable count and OD, at OD600: 0.3 (approximately 1 x 10^8 CFU/ml). Abbreviations: CFU/ml. Colony Forming Unit per millilitre, h. hour.

Figure 3: Antibiotic time kill graphs of Saur06.

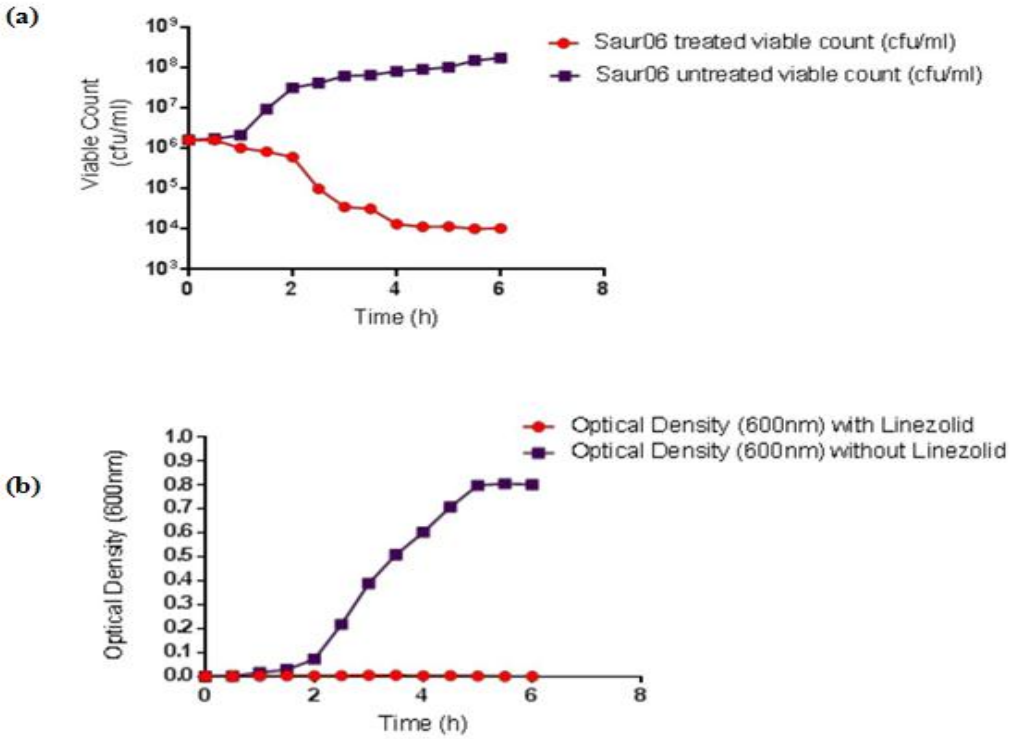

Fig. 3(a): Treatment effect with linezolid (1xMIC) indicates a bacteriostatic effect (< 3log10 CFU/ml). Each data point represents the average viable count from two technical replicates at every 30min interval for 6 h. (b) Saur06 graph determined by optical density (OD600) measurements in treated and untreated cultures. Abbreviations: CFU/ml. Colony Forming Unit per millilitre, h. hour.

Figure 4: Comparative graph of biomass and metabolic activity of Saur06.

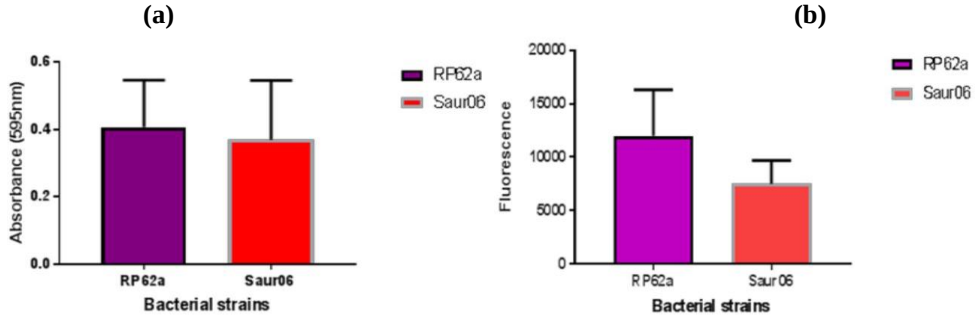

Fig. 4: (a) Biomass of Saur06 shown to be lesser, compared to RP62a although not significantly different (p > 0.01) (b) indication of lesser metabolic reduction activity of Saur06 biofilms compared to RP62a, but difference not significant (p > 0.01). Each data point represents the average from twelve technical replicates, with error bars representing the Standard deviation.

Figure 5: Linezolid treatment effects on Saur06 biofilms.

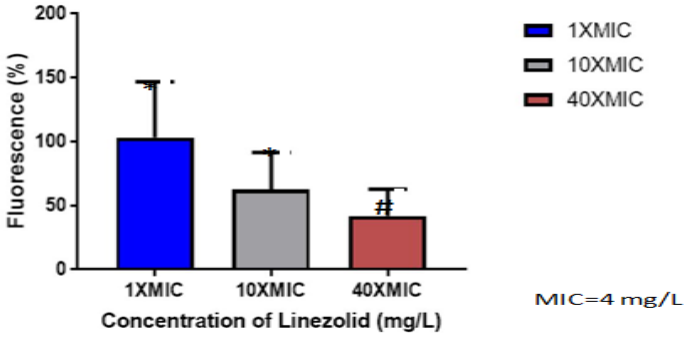

Fig. 5: Treatment effect of different linezolid concentrations on Saur06 viability in biofilm. * indicates no significant difference on reduction in viability (P > 0.01) at 10xMIC compared to 1xMIC. # indicates significantly greater reduction effect (p < 0.01) at 40xMIC compared to 1xMIC. Error bars represents the standard deviation.

Figure 6: Fluorescence microscopic study of Saur06 biofilms.

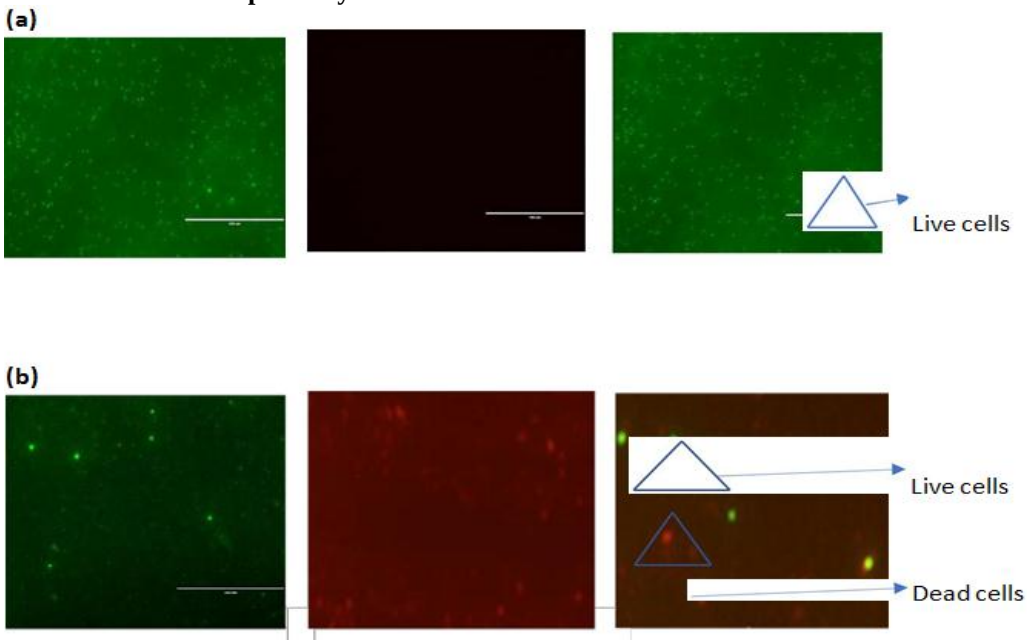

Fig. 6: (a) EVOS fluorescence microscopic examination of untreated Saur06 biofilms on cover slip under GFP, TEXAS RED and OVERLAY imaging respectively, indicates proportion of live cells higher in untreated coverslip (b) linezolid treated coverslip indicates decrease in live cells but tolerance to linezolid at 40×MIC.

Figure 7: Staphylococcal toxin genes detection in Saur06 DNA extract.

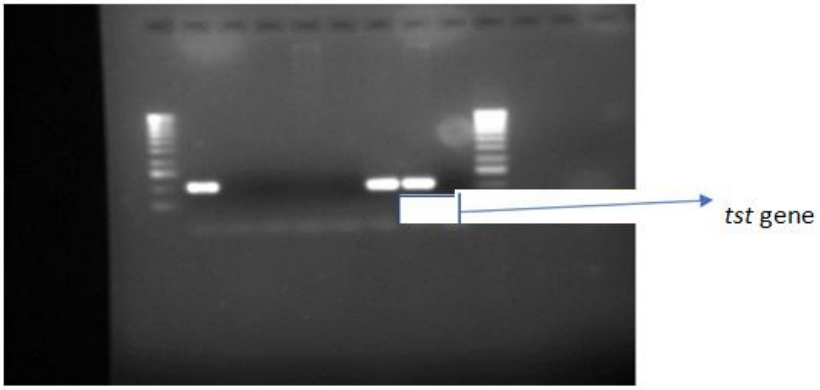

Fig. 7: Presence of *tst* gene encoding staphylococcal toxic shock toxin, in Saur06 DNA extract.

Table A.1: PCR primer pairs for DNA amplification. Abbreviations:(F). Forward primer, (R). reverse primer, RT-PCR. Real Time – Polymerase Chain Reaction.

Appendixa		
Table A.1		
Toxin genes	**Primers**	**Oligonucleotide sequence (5'– 3')**
sea	*sea* (F)	TTTGGAAACGGTTAAAACGAATAAG
sea	*sea* (R)	TTTCCTGTAAATAACGTCTTGCTTGA
seb	*seb* (F)	AGGTGACTGCTCAAGAATTAGATTACC
seb	*seb* (R)	AAGGCGAGTTGTTAAATTCATAGAGTT
sec	*sec* (F)	GGCGATAAGTTTGACCAATCTAAATAT
sec	*sec* (R)	AAGGTCGACTTCTATCTTCACACTTTT
sed	*sed* (F)	CACAAGCAAGGCGCTATTTG
sed	*sed* (R)	TCGGGAAAATCACCCTTAACA
see	*see* (F)	CTTTGGCGGTAAGGTGCAA
see	*see* (R)	ACCGTGGACCCTTCAGAAGA
tst	*tst* (F)	GTAAGCCCTTTGTTGCTTGC
tst	*tst* (R)	CTGATGCTGCCATCTGTGTT
spa	*spa*(F)	TTAGCATCTGCATGGTTTGC
spa	*spa*(R)	AAGAAGACGGCAACGGAGTA

DISCUSSION

The virulence determinants of *S. aureus* enhances its pathogenic potentials, and limits antibiotic treatment efficacy for some of its associated infections, as observed in some clinical isolates.[19]

In the present study, the *S. aureus* clinical isolate was shown to be Gram positive, catalase positive, Staph latex agglutination positive, with a 97.7% similarity to *S. aureus*. Additionally, growth studies revealed comparable growth pattern between the isolate and the normal bacterial growth curve; including a linear correlation between viable count and OD, at OD600: 0.3 (approximately 1 x 10^8 CFU/ml). This agrees with a report by Wang *et al*.,[20] on the phenotypic characteristics of *S. aureus* and correlates with a report by Biesta-Peters *et al*.[21] that the plate count and optical density methods, produce comparable estimations for vital bacterial growth parameters. Furthermore, Robassa *et al*.,[22] reported that the growth of microbes under both isothermal and non-isothermal temperature, follows the Gaussian distribution as a time-dependent function, and varies in relation with factors such as temperature, nutrient availability, and pH.

In vitro antibiotic susceptibility testing, revealed the MIC of linezolid on the *S. aureus* clinical isolate as 4 mg/L; which correlates with the EUCAST accuracy range of 1-4 mg/L.[14] Additionally, the MBC was 32 mg/L, and the ratio of MBC/MIC was 8. The MBC/MIC ratio of 8, suggests linezolid as bacteriostatic against planktonic cells of the *S. aureus* clinical isolate, and corroborates the report of Pankey *et al*., (2004) of bacteriostatic activity being a ratio of MBC/MIC of > 4 and < 32. However, while valuable information can be obtained from the MBC/MIC ratio on the impact of an antimicrobial agent *in vitro*, pharmacokinetic and pharmacodynamics data aids more precise prediction of *in vivo* efficacy.[23,24]

Excellent bacteriostatic effect was obtained at 1×MIC linezolid concentration against the *S. aureus* clinical isolate using *in vitro* time kill assay, as reduction of viable cells (CFU/ml) from the initial inoculum was not

>2log10 thus, suggesting that linezolid impaired the clinical isolate growth and may disrupt expression of its virulence factors. This agrees with the findings of previous studies, that linezolid is bacteriostatic against staphylococci, and acts early in protein synthesis, by inhibiting translation initiation.[25]

Biomass measurement using CV staining assay, revealed the S. aureus clinical isolate as a good biofilm producer while resazurin assay, revealed tolerance of the clinical isolate cells in biofilms at 1×MIC, 10×MIC, and 40×MIC treatment concentrations. Statistical analysis, showed no significant difference ($P > 0.01$), with linezolid treatment concentrations at 1xMIC and 10xMIC on reduction of viable cells in biofilm, but a significant difference ($p < 0.01$), with linezolid treatment concentration at 40xMIC, compared with at 1xMIC on the *S. aureus* clinical isolate viable cells decrease in biofilm. El- Azizi *et al*.,[26] reported that linezolid was less efficient in killing bacteria cells in disrupted or intact biofilms, due to factors including; poor penetration, changes in physiology, and biofilm associated repression which results in treatment difficulty.

Fluorescent microscopic evaluation using live-dead assay, revealed direct evidence of decrease in the *S. aureus* clinical isolate viable cells in the 40xMIC treated biofilm coverslip, compared to the untreated coverslip which showed more proportion of live cells. However, linezolid treated coverslips at 40xMIC still revealed the presence of some viable cells thus, implying incomplete viable cells elimination in biofilm. Reffuveille *et al*.[27] reported that poor antibiotic penetration may encourage decreased antibiotic effect despite concentration.

The ability to produce toxins contributes to *S. aureus* virulence. The *S. aureus* clinical isolate expressed SEA, and TSST genes thus, indicating its ability to cause severe infections because, SEA is a challenge with multidrug resistance, and implicated in causing staphylococcal food intoxication syndrome while TSST (produced by 5-25% of *S. aureus* isolates), causes toxic shock syndrome and is implicated during menstruation with producing *Staphylococcus aureus* in the vagina.[28,29] Evaluation of treatment with sublethal linezolid concentration (0.25×MIC) at mid-exponential growth, was shown to downregulate SEA and TSST (proteins). Reverse passive latex agglutination assay (TST-RPLA), revealed downregulation of TSST (proteins) expression by two-folds in the treated clinical isolate culture. Linezolid is likely to have a positive therapeutic impact in severe toxic shock infection cases caused by the *S. aureus* clinical isolate used in this study, by limiting TSST-1 level of expression leading to excellent clinical outcomes in treatment as previously reported.[30]

Stevens *et al*.[31] reported that the impact of antibiotic sublethal treatment on toxin expression is vital for therapeutic consideration. Similarly, Smith *et al*.,[4] showed that sublethal treatment with tigecycline (a bacteriostatic antibiotic), downregulated TSST expression in an MRSA isolate by 10-folds. Additionally, down regulation of SEA toxin expression (SET- RPLA) by 2 folds, was observed in the linezolid treated *S. aureus* clinical isolate culture. This agrees with the findings of a dose dependent study, that linezolid treatment led to a reduction of SEA expression.[31]

CONCLUSION

The present study asserts Saur06 ability to produce virulence factors such as biofilms and potent toxins (SEA and TSST). In biofilms, the clinical isolate was shown to initiate difficult to treat cells, and inhibited complete killing at higher than linezolid MIC, including downregulating toxins expression by 2-folds at sublethal treatment. Taken together, the *S. aureus* clinical isolate possesses pathogenic potentials, but linezolid use is advantageous in treating its associated infections, due to its efficacy in limiting virulence factors expression. Similar studies have reported linezolid preference over beta-lactams, as beta-lactams tend to induce prolonged toxin production, thereby contributing to worse outcomes.[31]

The rare reports of linezolid resistant *S. aureus* strains, informs the need to optimize surveillance. Also, while majority of patients have shown good tolerance to linezolid, adverse reactions including optic neuritis and peripheral neuropathy could occur thus, necessitating its appropriate use based on defined guidelines.[11] Additional studies are required, to determine linezolid effectiveness in treating *S. aureus* associated infections in immunocompromised individuals, and to define linezolid cost effectiveness to encourage its use in low income regions.

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