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CONFOCAL LASER SCANNING MICROSCOPE ANALYSIS ON POST-BIOFILM ASSESSMENT OF BIOFILM-PRODUCING OSTEOMYELITIC *STAPHYLOCOCCUS AUREUS* TREATED WITH NEW GENTAMICIN-*NIGELLA SATIVA* FUSION EMULSION (GNFE)

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Biofilm complicates osteomyelitis as there is antibiotic resistance and toxicity involved. In order to overcome the challenges of current treatment, gentamicin, the current antibiotic of choice for osteomyelitis, is fused with Nigella sativa oil to form an emulsion. Its efficacy as anti-biofilm agent is assessed using confocal laser scanning microscope (CLSM) against different strains of biofilm-producing Staphylococcus aureus. These strains of S.aureus were first allowed to express biofilm before being exposed for 24 hours to the emulsion with (0.1% (w/v) gentamicin; 40.2% (v/v) N.sativa. Later, the emulsion was removed and the biofilm was stained with fluorescence staining. The slides were viewed under CLSM at 100 times resolution. 3D images of biofilm were reconstructed, using Image J software, to measure the thickness of biofilm and viability of bacteria cells. Results revealed that the emulsion significantly reduced biofilm thickness compared to gentamicin and N.sativa alone in all strains of S.aureus (Tukey's test $p < 0.05$). The emulsion was also able to produce more than 80% and 15% surface percentage (%) of non-viable (dead) bacteria in the sensitive and resistant strain, respectively, at a significant level when compared to gentamicin and N.sativa (Tukey's test $p < 0.05$). As a conclusion, this new fusion of gentamicin-N.sativa may be effective towards the biofilm of S.aureus, and can be developed further as a new promising anti-biofilm agent in osteomyelitis.

Keywords: biofilm, gentamicin-*N.sativa*, *S.aureus*, CLSM

INTRODUCTION

Osteomyelitis is an inflammation of the bone resulting from bacterial infection [1]. Without proper treatment, this disease may progress into the chronic stage in which the infection is difficult to treat. These days, prosthetic implantation is associated with most serious cases, arising from open surgery [2, 3]. Specific pathogens that are commonly found in osteomyelitis are *Staphylococcus aureus* (60%), *Enterobacteriaceae* spp. (23%), *Pseudomonas* spp. (9%), and *Streptococcus* spp. (9%), with 90% of the infection related to prosthetic implant involve *Staphylococcus epidermidis* [4]. Currently, osteomyelitis, in particular, when involving prosthetic implant, is managed by impregnating gentamicin in beads form during a surgery [5]. However, antibiotic-resistance, biofilm-producing bacteria which have become embedded within a matrix of extracellular polymeric substance (EPS) and

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are able to alter gene expression, phenotype and protein production hence making it remarkably different from its planktonic form have also emerged [6]. This has now complicates the current treatment as higher dose of gentamicin is needed, which inadvertently increases the risk of gentamicin toxicity locally and systemically. The use of non-biodegradable beads also means that bacteria have new area to adhere to, and this further prolongs the infection.

To overcome these biofilm-associated problems, a new fusion emulsion is formed aiming at reducing the amount of gentamicin, while at the same time increasing the anti-biofilm and anti-bacterial effect of the new emulsion. Gentamicin is a type of aminoglycoside that is effective against a wide range of bacteria such as *Staphylococcus* spp., *Pseudomon*as spp., and *Streptoccocus* spp. Gentamicin is here combined with *N.sativa* oil which has been shown to possess antimicrobial properties against *S.aureus*, *Pseudomonas* spp., and *Enterobacteriaceae* spp. It is thought to be a suitable combination with gentamicin as it is also able to suppress gentamicin-induced nephrotoxicity in rabbit [7, 8]. Hence, the fusion of Gentamicin-*N.sativa* was tested against different strains of *S.aureus* in anticipation of anti-biofilm properties.

MATERIALS AND METHODS

Test Strain

The *S.aureus* strains were isolated from patients diagnosed with osteomyelitis at Hospital Tengku Ampuan Afzan (HTAA), Kuantan. Isolated *S.aureus* was identified by colony by API Identification System (Bioemerieux, France). A total of 3 biofilm-producing *S.aureus* including 2 clinical isolate (sensitive and resistant to gentamicin) and 1 control strain (ATCC 29213) were used in this study.

Fusion Formulation

Gentamicin sulphate and *N.sativa* oil (Hemani Trading, Pakistan) were formulated in emulsion form, containing a combination of 0.1% w/v gentamicin solution and 40.6% *N.sativa* in a surfactants blends (Tween 20 and Span 20), using a homogeniser for 5 minutes. Once the milky brown emulsion colour was formed, it was subjected for stability evaluation by storing at different condition (8°C, 25°C, and 50°C) for certain period of time (7, 14 and 30 days) followed by centrifugation test at 5000 rpm for 5 minutes.

Biofilm Formation

Thermanox coverslips (10.5 mm × 22 mm) (Nunc, USA) were placed horizontally at the bottom of 6 well plates and sterilised under UV light for at least 1 hour. Then, bacterial suspension in tryptic soy broth (TSB) with 0.5 McFarland was added into each well and incubated for 24 hours at 37°C. Medium was replaced with fresh TSB every 12 hours. After incubation, each well was decanted and washed 3 times with 0.85% saline for 5 minutes.

Treatments

Biofilm that formed on the coverslip was exposed to (i) 50% emulsion, (ii) *N.sativa* oil alone, and (iii) gentamicin alone, for 24 hours at 37°C. Untreated biofilm served as positive control. Biofilm that was exposed to 95% ethanol served as negative control. After 24 hours, the content of each well was aspirated by gentle pipetting, and wells were washed 3 times with saline 0.85% for 5 minutes each.

Fluorescence Staining

Biofilm (treated, untreated, and control) were stained with LIVE/DEAD Baclight L7007 kit (Invitrogen, Canada) according to manufacturer's instruction. Briefly, the stain was prepared by mixing 3 µL of component

A (1.67 mM Syto 9/ 1.67 mM Propidium iodide) and component B (1.67 mM Syto 9, 18.3 mM Propidium iodide) in 994 µL of 0.85% saline (final volume 1 mL). Biofilm was then stained and incubated in the dark for 15 minutes at room temperature (27°C). The stained was removed and biofilm was fixed with Bouin's solution for 10 minutes. After fixation, each coverslip was washed with 0.85% saline for 5 minutes. The coverslip were mounted on the slide with 50 µm spacer.

CLSM Analysis

Slide was viewed under confocal laser scanning microscope (CLSM) (Leica TCS SPE, German) within 48 hours after preparation. The excitation wavelength for Syto 9 was at 488 nm whereas Propidium Iodide was at 532 nm. The emitting light of fluorescent dye was detected between 480-560 for Syto 9 and 590-750 for Propidium Iodide. The setting for laser power and gain were optimized using positive control (Syto 9, green, viable bacteria) and negative control (Propidium Iodide, red, non-viable bacteria). Z-stack images were taken and at least 4 regions (n=4) on each slide were viewed. The series of Z-stack images were analysed using Image J software version 1.48 (National Institute of Health, USA). 3D images of biofilm were reconstructed and the thickness of biofilm (bottom to top) was measured. Images of biofilm (top, middle and bottom) were selected and the surface percentage (%) (total area of 753 ×753 µm) of viable and non-viable bacteria on different depth of biofilm were analysed using colour threshold method in Image J.

RESULT AND DISCUSSION

Lowest biofilm thickness was seen in all strains of *S.aureus* after exposure to emulsion. The value was significant in comparison with gentamicin and *N.sativa* (Tukey's test $p < 0.05$) (Figure 1). The effectiveness of emulsion in reducing biofilm may be due to synergistic effects of combining gentamicin with *N.sativa*. Although gentamicin and *N.sativa* also showed low biofilm thickness towards gentamicin sensitive *S.aureus* and *S.aureus* ATCC 29213, no significant different was seen against gentamicin resistant *S.aureus* (Figure 1).

It was also found that treatment with the emulsion produced highest surface percentages (%) of non-viable *S.aureus*

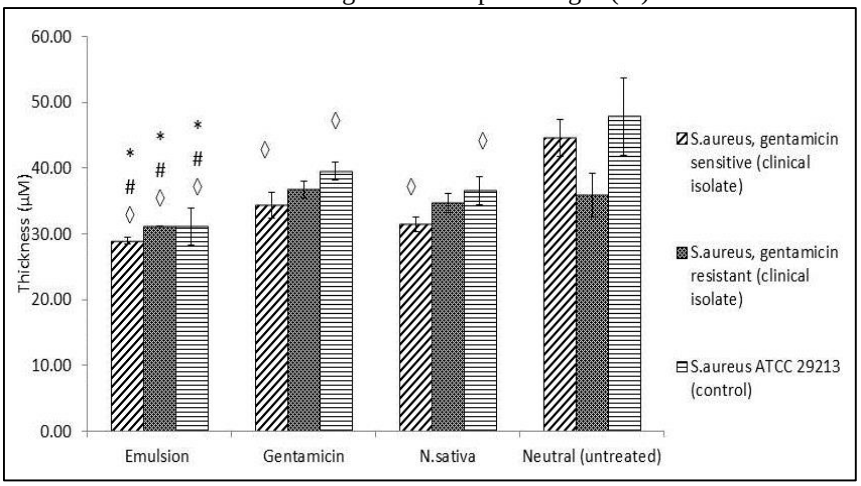
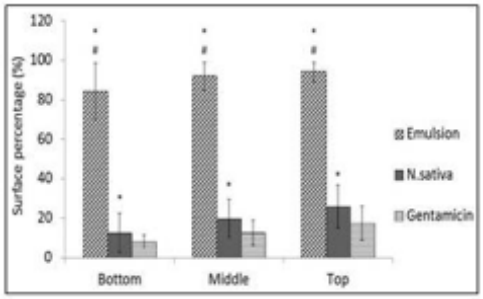
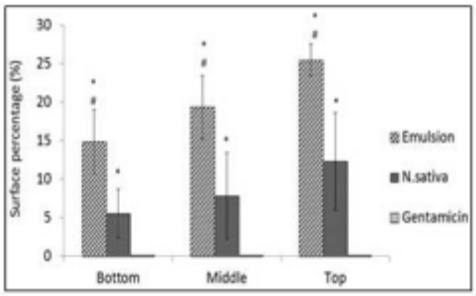


Figure 1: *The mean (n=4) thickness of S.aureus biofilm in different treatment group. (*) Denotes (Tukey's test $p < 0.05$) compared to gentamicin; (#) denotes (Tukey's test $p < 0.05$) compared to N.sativa; (◊) denotes (Tukey's test $p < 0.05$) compared to untreated.*

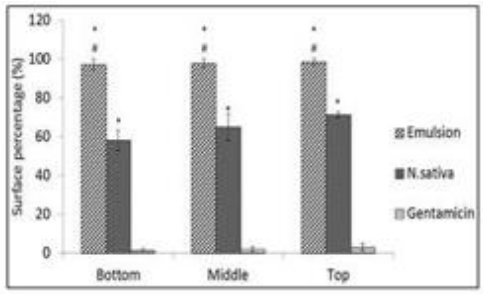
regardless of biofilm depth (Figure 2; A, B, and C) with significant difference to the gentamicin- and N.sativa oil-only groups (Tukey's test $p < 0.05$). More than 80% of non-viable bacteria were seen in S.aureus gentamicin sensitive and more than 98% in S.aureus ATCC 29213 (Figure 2; A and C).



(a)



(b)



(c)

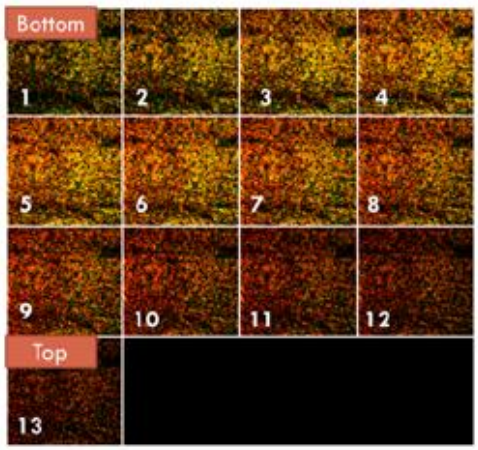
Figure 2: *The surface percentage (%) of non-viable (A) S.aureus, gentamicin sensitive (clinical isolate), (B) S.aureus, gentamicin resistant (clinical isolate) and (C) S.aureus ATCC 29213. (*) Denotes (Tukey's test $p < 0.05$) compared to gentamicin; (#) denotes (Tukey's test $p < 0.05$) compared to N.sativa.*

Furthermore, in gentamicin resistant *S.aureus*, treatment with emulsion produced 15% to 30% (top, middle, and bottom) of non-viable bacteria. Both Gentamicin and *N.sativa* produced lower percentage of non-viable bacteria than emulsion in all *S.aureus* strains.

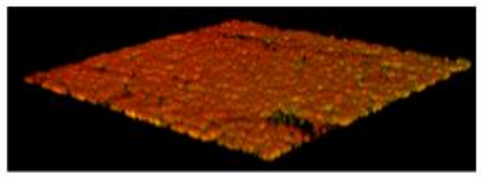
The ability of an antimicrobial agent to penetrate biofilm without losing its efficacy is really important in combating biofilm. Inability to kill bacteria at the bottom of biofilm will cause viable bacteria that remain undisturbed at the bottom of biofilm to growth [7]. In this study, it was shown that the formulated emulsion could produce anti-biofilm activities by reducing biofilm thickness and percentage of viable surface *S.aureus*. In comparison to the gentamicin- and *N.Sativa*-only groups, the emulsion appears to be able to effectively kill bacteria within biofilm. Nevertheless, the percentage of death bacteria at the bottom was less than at the top surface of biofilm (Figure 2). The overlapping fluorescence staining of syto 9 (green) and propidium iodide (red) within bacteria cells indicates that the damage of bacteria cell wall is variable according to biofilm depths (Figure 3; A and B). Three colours (yellow, orange, and red) showed different degree of severity of the cell wall damage. In the Z – stack images (Figure 3; A), the colour changed from the bottom to the top, from yellow (slight damage) to orange (severe damage) and to red (complete damage).

As far as it concern, this is the first study on the combination of antibiotic and natural product, especially on the fusion of gentamicin and *N.sativa*. Most of the previous studies were only focusing against planktonic bacteria such as by Braga et al, (2005) that reported combination of pomegranate with gentamicin, chloramphenicol, ciprofloxacin, ampicillin, tetracycline and oxacillin were effective towards methicillin resistant *S.aureus* (MRSA) [8]. Another study by Mossa et al,

(2004) showed that combination of totarol, ferulenol and plumbagin increase isoniazid potency against *Mycobacterium* sp. [9].
Such studies have showed that the fusion of antibiotic and natural product might be the future in the development of new antimicrobial agents.



(a)



(b)

Figure 3: *CLSM images of S.aureus gentamicin sensitive treated with gentamicin-N.sativa fusion emulsion (A) Montage of Z-stack images and (B) 3D-reconstructed image of biofilm.*

CLSM is a powerful tool in biofilm analysis [10]. It has the ability to reconstruct 3D structure of biofilm and quantified the numbers of bacteria within the matrix using proper fluorescence staining method. Furthermore, CLSM can be used on hydrated biological structure without fixation. Hence, it is a non-destructive technique and suitable for live cell viewing [11]. Many studies on biofilm have been using CLSM and it has become a standard tool for biofilm investigation [12].

CONCLUSION

Newly formulated gentamicin-*N.sativa* fusion emulsion (GNFE) is effective towards biofilm of *S.aureus*, thus can be developed further as a new promising anti-biofilm agent for osteomyelitis.

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