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Protective Effect of Hamamelitannin against Biofilm Production by Methicillin-resistant Staphylococci Isolated from Blood of Patients at Intensive Care Units

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Authors' contributions

This work was carried out in collaboration between all authors. All authors are equally contributed to the work. All authors read and approved the final manuscript.

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ABSTRACT

S. aureus and *S. epidermidis* are common pathogens in biofilm related infections of indwelling medical devices.
Aim: The aim of this study was to assess the efficacy of vancomycin and clindamycin alone and in-combination with hamamelitannin as a quorum sensing inhibitor in preventing biofilm formation by *S. aureus* and *S. epidermidis*.
Methods: The frequency of biofilm formation and its strength of 21 *S. aureus* and 26 *S. epidermidis* isolated by blood culture from patients admitted to intensive care units of Fayoum and Cairo

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University Hospitals was assessed by modified microtitre plate method. The minimum inhibitory concentrations (MICs) of vancomycin and clindamycin against 22 strains (11 Methicillin-Resistant *S. aureus* (MRSA) and 11 Methicillin-Resistant *S. epidermidis* (MRSE) were assessed by micro-dilution method in concentrations ranging from 0.25 µg/ml to 512 µg/ml. The ability of vancomycin and clindamycin alone and in combination with hamamelitannin as a quorum sensing inhibitor to prevent biofilm formation was detected. The presence of *icaA* and *icaD* genes was determined by polymerase chain reaction.

Results: 63.8% were strong biofilm producers, 25.5% were moderate and 10.6% were non biofilm producers. The MIC_{50} and MIC_{90} of vancomycin were 2 µg/ml and 4 µg/ml respectively against planktonic and sessile cells while those of clindamycin were 0.5 µg/ml and 8 µg/ml respectively against planktonic cells and 4 µg/ml and 32 µg/ml respectively against sessile cells. Hamamelitannin when combined with vancomycin and clindamycin in a concentration of 20 µg/ml succeeded to inhibit biofilm formation in all tested concentrations of both antibiotics.

Conclusion: Hamamelitannin could play a promising role in preventing biofilm formation in association with antibiotics. Lining of indwelling medical devices with a quorum sensing inhibitor may be a new prospect which requires future assessment.

Keywords: MRSA; MRSE; hamamelitannin; vancomycin; clindamycin.

1. INTRODUCTION

Staphylococci are considered a common cause of hospital acquired infections due to their ability to form biofilm on implanted devices. Central venous catheters, dialysis catheters, urinary catheters, orthopedic implants and prosthetic heart valves are most common targets of these infections [1].

Staphylococcus aureus and *Staphylococcus epidermidis* are often the predominant species in biofilms of these devices. Treating chronic biofilm infections is one of the most problematic issues of hospitalized patient, as when bacteria reside in biofilms they are extremely resistant to all types of chemotherapeutic agents as well as to the host immune defense mechanisms leading to high morbidity and mortality [2,3]. Biofilm formation is regulated by expression of polysaccharide intracellular adhesion (PIA), which mediates cell to cell adhesion and is the gene product of icaABCD. Among *ica* genes*, icaA* and *icaD* have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis* [4]. The main function of *ica A* is the synthesis of the N – acetyl –D – glucosamine polymer structure and the co-expression of *ica D* gene provides a significant increase in enzymatic activity and polysaccharide intracellular adhesion (PIA) [5].

In Staphylococci, pathogenesis due to biofilm formation is achieved by a complex regulatory process termed "quorum sensing'' [6]. Quorum sensing refers to regulation of gene expression in response to fluctuations in cell density. Bacteria produce and release quorum sensing signaling molecules called "autoinducers". The concentration of the autoinducers increases as a function of cell density, leading to distinct patterns of gene expression usually regulated by phosphorylation [7].

Many attempts have been developed to overcome biofilm formation and development of antibiotic resistance mechanisms [2,3,6]. One of these trends is preventing virulence by inhibition of bacterial cell-to-cell communication using the quorum-sensing inhibitor (QSI) RNAIII-inhibiting peptide [1,3]. A natural non-peptide analog of the RNAIII inhibiting peptide; hamamelitannin (HAM), have been discovered with good antibiofilm activity against standard strains of *S. aureus* when tested alone and in-combination with antibiotics [7,8]. However, little is known about relationship between the antibiofilm effect of quorum-sensing inhibitors and the susceptibility of biofilms to antibiotics against clinical isolates of *S. aureus* and *S. epidermidis*.

In the present study, we assessed the efficacy of vancomycin and clindamycin alone and in combination with hamamelitannin as a quorum sensing inhibitor in preventing biofilm formation by clinical isolates of methicillin-resistant *S.aureus* (MRSA) and methicillin-resistant *S. epidermidis* (MRSE).

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

A total 47 Staphylococci isolates from 47 patients (21 *S. aureus* and 26 *S. epidermidis*) were isolated by blood culture from patients admitted

to intensive care units of Fayoum and Cairo University Hospitals from July 2013 till May 2014. The study was approved by Fayoum University ethics committee. Blood cultures were performed when there was a clinical suspicion of bloodstream infection; if two or more of the following clinical signs are present: body temperature greater than 38℃ or less than 36℃, heart rate greater than 90 beats/min, high respiratory rate (more than 20 breaths/min) or, on blood gas, a $PaCO_2$ less than 32 mmHg for spontaneously breathing patients; or requirement for mechanical ventilation in established critical illness, white blood cell count <4000 cells/mm^3 or >12 000 cells/mm^3 or the presence of more than 10% immature neutrophils [9]. Two blood samples (8-10 ml each) from every patient were collected using sterile technique at separate sites, before administering antibiotics and inoculated into blood culture bottles (Oxoid Signal® Blood Culture System), mixed with the medium and incubated for 2 weeks at 35℃ with periodic inspection for any evidence of growth [10]. Aerobic subculture on Columbia Blood and Chocolate agar was performed (Oxoid, UK). Identification of the isolates was carried out using Gram staining and standard biochemical tests including catalase, coagulase, DNase production, growth and fermentation of mannitol on mannitol salt agar. Further identification was carried out by using API Staph (BioMerieux, Marcy-l'Etoile, France). All isolates were tested for antibiotic susceptibility by oxacillin (1 µg), cefoxitin (30 µg), cefazolin (30 µg), doxycylin (5 µg), gentamycin (10 µg), clindamycin (2 µg), erythromycin (15 µg), chloramphenicol (30 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), vancomycin (30 µg) and linezolid (30 µg) (Oxoid, UK) by Kirby- Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) [11]. Clindamycin and erythromycin discs were placed approximately 15 mm apart for detection of any inducible clindamycin resistance (D test) [12]. For confirmation of oxacillin resistance, subculture of isolates on Oxacillin Resistance Screening Agar Base (ORSAB) medium with ORSAB medium supplement SR195 (Oxoid, UK) were performed.

2.2 Biofilm Assay

The ability of the 47 isolates to form biofilm was assessed by modified tissue culture plate method described by Stepanovic et al. [13] using sterile 96-well flat-bottomed tissue culture plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). The optical density (OD) of each well was measured at 570 nm (Stat Fax 2100 Microplate Reader). The strains were classified into the following categories: non biofilm forming (OD ≤ ODc), weak biofilm forming (ODc < OD ≤2 ODc), moderate biofilm forming (2 ODc < OD ≤ 4 ODc), strong biofilm forming (4 ODc < OD), where ODc is the mean OD of the negative control and OD is the mean OD of the isolate.

2.3 Detection of of icaA and icaD Genes by Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from an overnight culture using Gene JET Genomic DNA Purification Kit (Thermo Scientific, USA) with the addition of Lysostaphin (Sigma-Aldrich) at final concentration of 100 µg/ml and incubation at 37℃ for 1 h in the initial step. The presence of *icaA and icaD* were detected by polymerase chain reaction (PCR) as described by Arciola et al. [14]. Detection of *icaA was performed using (*5′-TCTCTTGCAGGAGCAATCAA*) and (*5′-TCAGGCACTAACATCCAGCA*) as forward and reverse primers respectively. For detection of icaD,* (5′-ATGGTCAAGCCCAGACAGAG) and (5′-CGTGTTTTCAACATTTAATGCAA) *were used as forward and reverse primers respectively.* The PCR was performed in a total volume of 25 µl containing 2.5 µl 10X DreamTaq buffer, 150 ng genomic DNA template, 1 µM of each primer, 0.2 mM of each dNTP, one unit DreamTaq DNA polymerase (*Thermo Scientific,* USA) and finally water was added to make volume up to 25 µl. The PCR conditions included initial denaturation for 5 min at 94℃, denaturation for 30 s at 94℃, annealing for 30 s at 55.5℃, extension at 72℃ for 30 s for 40 cycles, and a final extension at 72℃ for 2 min. After amplification, 5 µl of the PCR product was analyzed on 2% agarose gel stained with ethidium bromide (0.5 µg/ml), and visualized under ultra violet light. The Gene Ruler 100 bp DNA ladder was used as a DNA size marker (Detection of *icaA and icaD* were detected with positive bands at 188 bp and 198 bp respectively).

2.4 Determination of Minimal Inhibitory Concentration (MIC)

The MICs of vancomycin and clindamycin against 22 Methicillin-Resistant strains with strong or moderate biofilm forming ability (11 MRSA and 11 MRSE) were determined by microdillution method in a concentrations ranging from 512 µg /ml to 0.25 µg /ml. MIC of planktonic cells were determined according to CLSI [15]

and MIC of the drugs that prevent biofilm formation (sessile cells) was assessed by the method described by Nuryastuti et al. [16], Briefly, wells of a 96-well tissue culture polystyrene microtiter plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) were filled with 100 µl of the antibiotic solution (twofold dilution) and subsequently inoculated with a 1:100 dilution of an overnight culture after adjustment to 0.5 *McFarland*. After incubation for 24 h at 37℃, the plates were gently washed twice with phosphate-buffered saline and stained with 0.4% (wt/ vol) crystal violet solution for 30 min at room temperature in order to determine the biofilm mass. Excess stain was washed off with dH2O. The biofilms were re-suspended in acid isopropanol (5% [vol/vol] 1 M HCl in isopropanol) and, finally, the OD of each well was measured at 570 nm (Stat Fax 2100 Microplate Reader). MIC is the minimum concentration of antibiotic that prevent biofilm formation (OD ≤ ODc). Also the MIC_{50} (the minimum concentration that inhibit 50% of the isolates) and MIC_{90} (the minimum concentration that inhibit 90% of the isolates) of each antibiotic were reported.

2.5 Determination of Minimum Inhibitory Concentration of Vancomycin and Clindamycin in Combination with Hamamelitannin

The method described by Nuryastuti et al. [16] was used; vancomycin and clindamycin were prepared in sub MIC concentrations with addition of hamamelitannin (Sigma-Aldrich) in a concentration of 20 µg/ml.

2.6 Statistical Analysis

All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Sciences; International Business Machines Corporation, Armonk, NY, USA) version 15 for Microsoft Windows. Data were statistically described in terms of frequencies and percentages.

3. RESULTS

Of 47 tested Staphylococci isolates, 82.97% were resistant to oxacillin, 89.36% were resistant to cefoxitin and 89.36%, 78.7%, 72.34%, 70.2%, 61.7%, 59.57%, 48.94% were resistant to cefazolin, erythromycin, levofloxacin, doxycylin, ciprofloxacin, gentamycin, and chloramphenicol respectively and 70.2% were resistant to clindamycin (3 isolates showed inducible clindamycin resistance). All isolates were susceptible to vancomycin and linezolid.

Forty two isolates (42/47 (89.36%)) had the ability to form biofilm; 30/47 (63.8%) were strong biofilm producers, 12/47 (25.5%) were moderate and 5/47 (10.6%) were non biofilm producers (Table 1).

All biofilm producing staphylococci isolates were positive for *icaA* and *icaD* genes except for one *S. epidermidis* isolate with positive *icaA* and *icaD* genes and negative biofilm phenotype (Fig. 1).

The MIC_{50} and MIC_{90} of vancomycin were 2 µg/ml and 4 µg/ml respectively against planktonic and sessile cells while those of clindamycin were 0.5 µg/ml and 8 µg/ml respectively against planktonic cells and 4 µg/ml and 32 µg/ml respectively against sessile cells (Table 2).

Biofilm formation was inhibited at all tested dilutions of vancomycin and clindamycin when combined with hamamelitannin in a concentration of 20 µg /ml.

Table 1. Biofilm production by isolated staphylococci

Isolates (no)	Strong biofilm		Moderate biofilm		Non-biofilm	
	no.	%	no.	%	no.	%
S. aureus (21)	16	76.2%	3	14.28%	2	9.52%
MRSA (18)	13	61.9%	3	14.28%	2	9.52%
MSSA (3)	3	14.28%	0	0%	0	0%
S. epidermidis (26)	14	53.85%	9	34.61%	3	11.54%
MRSE (24)	14	53.85%	7	26.92%	3	11.54%
MSSE (2)	0	0%	2	7.69%	0	0%
Total (47)	30	63.83%	12	25.53%	5	10.64%

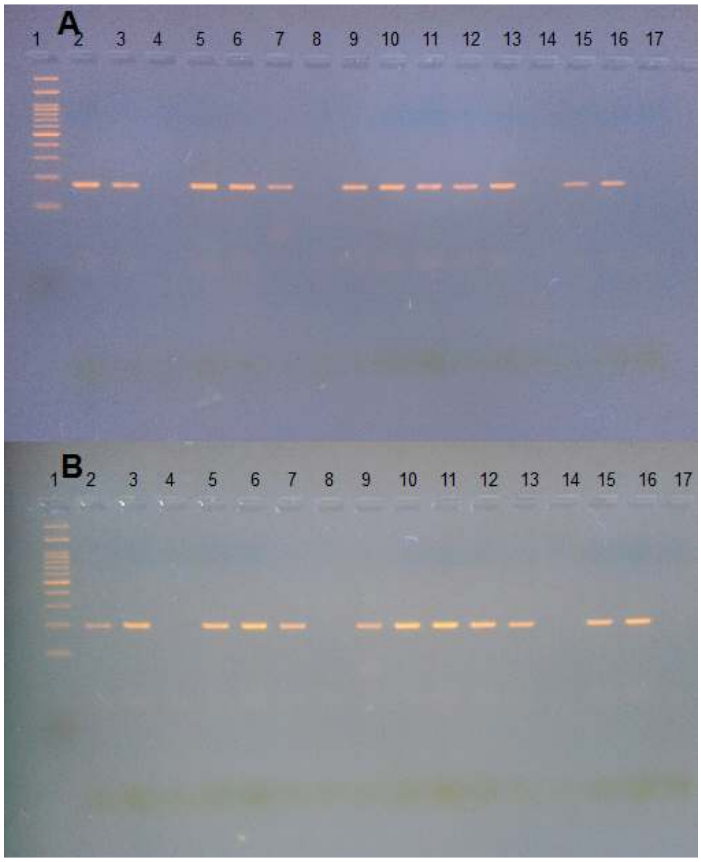

Fig. 1. Agarose gel electrophoresis of PCR products A: The PCR product of *icaA* gene (*188 bp)*; lane 1: DNA ladder, lane 2,3,5,6,7,9,10,11,12,13 positive samples, lane 4,8, 14 negative samples, lane 17 negative control. B: The PCR product of *icaD* gene (*198 bp)*; lane 1: DNA ladder, lane 2,3,5,6,7,9,10,11,12,13 positive samples, lane 4,8, 14 negative samples, lane 17 negative control

Table 2. MIC_{50} and MIC_{90} of tested antibiotics with and without hamamelitannin

	Vancomycin		Clindamycin	
	Planktonic	**Sessile**	**Planktonic**	**Sessile**
MIC_{50} antibiotic	2 µg/ml	4 µg/ml	0.5 µg/ml	4 µg/ml
MIC_{50} antibiotic+ hamamelitannin	NA*	< 0.25 µg/ml	NA*	< 0.25 µg/ml
MIC_{90} antibiotic	2 µg/ml	4 µg/ml	8 µg/ml	32 µg/ml
MIC_{90} antibiotic+ hamamelitannin	NA*	< 0.25 µg/ml	NA*	< 0.25 µg/ml

**NA: not applicable*

4. DISCUSSION

Colonization of medical indwelling devices with resistant bacterial strains and subsequent biofilm formation may precede bacteremia and sepsis in critically ill patients, catheter related blood-stream infections have been reported to result in longer hospital stays as well as increased mortality rate [17]. Moreover, tissues in proximity to the implanted device may sustain permanent damage from immune complexes and neutrophils, leading to an extensive inflammation and necrosis [18]. Finding an effective antibiotic treatment for infections caused by biofilm forming organisms has been difficult as the antibiotic concentration required to kill bacteria in the biofilm is 100–1000 times higher than that needed to kill the same species in planktonic state [2,18].

In the present study we assessed the efficacy of vancomycin and clindamycin alone and in combination with hamamelitannin as a quorum sensing inhibitor in preventing biofilm formation by clinical isolates of MRSA and MRSE.

We classified the isolated *S. aureus* and *S. epidermidis* strains according to their ability to form biofilm by modified tissue culture plate method which reported to be an accurate method for quantification of biofilm [19] and we found that 42/47 (89.36%) can form biofilm; 30/47 (63.8%) were strong biofilm producers, 12/47 (25.5%) were moderate and 5/47 (10.6%) were non biofilm producers. These results are in agreement with that of Ziebuhr et al. [20] who studied the biofilm forming ability of 51 *S. epidermidis* strains isolated from blood cultures and found that 87% are biofilm producer. Also Gad et al. reported that out of 18 clinical *S. aureus* strains, 83.3% were biofilm producers and out of 35 clinical *S. epidermidis* strains, 88.6% were biofilm producers, further classification of Staphylococcal strains revealed that 56.6% were strong biofilm producer, 30.2% moderate producer and 13.2% non-biofilm producers [4].

Our results are higher than that reported by Mathur et al. [19] who found that from 152 isolated Staphylococcus spp. 57.8% displayed a biofilm positive phenotype under the optimized conditions in the tissue culture plate method and strains were further classified as high (14.47%) and moderate (39.3%) while in 46% isolates weak or no biofilm was detected.

In this work, all Staphylococci isolates (*S. aureus and S. epidermidis*) were subjected to PCR for detection of *icaA* and *icaD* genes. We found that all biofilm producing Staphylococci isolates were positive for *icaA* and *icaD* genes except for one isolate. These results are in agreement with those of Gad et al. [4], De Silva et al. [21] and Arciola et al. [14] who reported that all *S. aureus* and *S. epidermdis* biofilm positive strains isolated mainly from intravenous catheters were positive for *icaA* and *icaD* genes indicating that these genes are required for full slime synthesis which confirm the fact reported by Gad et al. that the two genes are part of one operon, so that the entire operon is either present or absent [4].

Expression of the icaABCD operon was shown to be a variable factor modulated by genome rearrangements and phase variation. It was suggested that the variability in biofilm expression contributes to adaptation of the bacteria in changing the environmental conditions of incubation [22,23], this may explain that one of our isolates was with biofilm negative phenotype in spite of the detection of both *icaA* and *icaD* genes by PCR. Chaieb et al. [23] reported that mutation in three unlinked gene loci abolished PIA synthesis and led to a biofilm negative phenotype by genetic inactivation of the *ica* locus.

Because of the increase in methicillin-resistant strains, many prophylactic strategies have included pre-coating of devices with glycolpeptide antibiotics had been developed, but the recent appearance of *S. epidermidis* strains resistant to vancomycin and teicoplanin has created the need for alternative strategies [3]. Cell to cell communication via quorum-sensing systems affects the expression of virulence factors in many bacteria, thus, targeting of quorum-sensing systems with so-called quorum sensing blockers has been suggested as an alternative means of treating bacterial infections. Also quorum-sensing blockers act only by suppressing biofilm formation and do not kill bacteria so the development of resistant strains by natural selection is minimized [18].

Hamamelitannin (2,5-di-O-galloyl-Dhamamelose) is a natural polyphenol extracted from the bark of Hamamelis virginiana that belongs to tannins family [17], it was discovered by Kiran et al. as a nonpeptide analog of RNAIII-inhibiting peptide (RIP); a well-known QSI; which can prevent device-associated infections, including infections caused by methicillin-resistant *S. aureus* and *S. epidermidis* strains [7]. Previous reports have highlighted the role of hamamelitannin in preventing biofilm formation but little is known about its effect in combination with antibiotics.

Glycopeptide antibiotics have long been considered the gold standard for treatment of documented or suspected life-threatening multiresistant Gram-positive bacterial infections, with vancomycin is the widely used glycopeptides [24]. Also clindamycin is considered an attractive agent for empirical therapy for suspected *S. aureus* infections because of its excellent pharmacokinetic and pharmacodynamics properties [25]. In this study we have assessed the ability of vancomycin, clindamycin alone and in combination with hamamelitannin as a quorum sensing inhibitor to prevent biofilm formation against 22 isolates (11 *S. aureus* and 11 *S. epidermidis*) in a

concentrations ranging from 512 µg/ ml to 0.25 µg /ml. The strains were chosen to be methicillin-resistant and biofilm producer. Surprising to us hamamelitannin when combined with vancomycin and clindamycin in a concentration of 20 µg/ ml was able to inhibit biofilm formation at all tested concentrations of both antibiotics. Our results were supported with that obtained by Christensen et al. [2] who inserted silicone tube implants pre-colonized with wild-type *P. aeruginosa* into the peritoneal cavity of BALB/c mice. Treating mice with intraperitoneal or subcutaneous injections of the QSIs; ajoene, furanone C-30, or horseradish juice extract; in association with tobramycin resulted in a significantly lower colony forming units per implant as compared with the placebo groups for all QSIs tested.

5. CONCLUSION

Hamamelitannin could play a promising role in preventing biofilm formation in association with antibiotics. Lining of indwelling medical devices with a quorum sensing inhibitor may be a new prospect which requires future assessment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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