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Quercetin mediated inhibition of *Staphylococcus aureus* biofilms and the impact of the isolate phenotype and genotype

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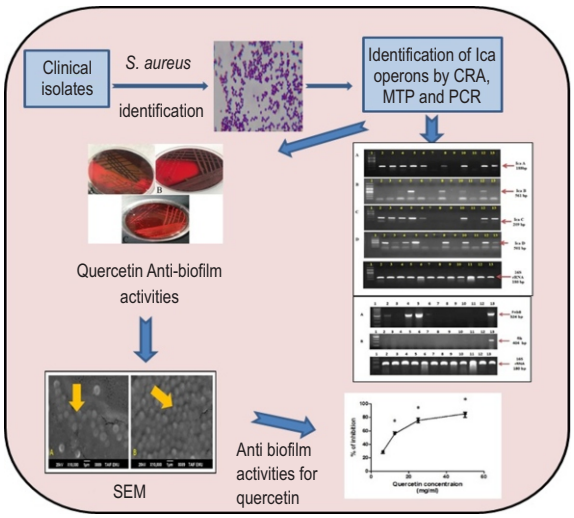
Abstract

Aim: This study was designed to assess the antibiofilm activity of quercetin on characterized *S. aureus* isolates.

Methodology: This study evaluated 36 *S. aureus* isolates, each of which was identified using Gram staining, culture, biochemical, and PCR assays. Isolates were cultured and their biofilm production was evaluated using Congo red agar (CRA) plates, microtiter plate tests and PCR, and the effects of quercetin were examined.



Results: The CRA results revealed that eight (22.3%) *S. aureus* isolates were strongly positive for biofilm production and an additional 18 isolates (50%) showed moderate biofilm capacity. The remaining 10 isolates were negative (27.7%) for biofilm production. *S. aureus* isolates were divided into strong positive, intermediate, and negative groups, 27.8%, 44.5%, and 27.7%, respectively. Scanning electron microscopy showed that the biofilm-producing isolates appeared as aggregates of cells within a heavy matrix. In addition, PCR assay identified IcaA and IcaD (66.6% for both) biofilm production genes in most isolates and *IcaC* (61.1%), *IcaB*, *FnbB* (33.3% for both), and Fib (22.2%) in several other strains. Quercetin significantly inhibited biofilm activity in biofilm producing *S. aureus* isolates in a dose-dependent manner, with an inhibition rate of 29.6-87.7%.

Interpretation: Biofilm production is dependent on Ica gene phenotype and strains with an IcaABCD or IcaABD phenotype produce more biofilm than strains with IcaAD phenotype. Quercetin significantly inhibited *S. aureus* biofilm production, irrespective of Ica phenotype.

Key words: Biofilm, Congo red agar, Ica operon, PCR, Quercetin, *S. aureus*

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Introduction

Staphylococcus spp. are common skin microbes known to inhabit the human skin, sweat glands, and mucous membranes, especially in the nasal cavity of healthy individuals (Costa *et al.,* 2011; Plata *et al.,* 2009). *S. aureus* is also present as a commensal in several other niches inside human body and can act as an opportunistic pathogen with its aberrant growth linked to infection of skin and soft tissues. These infections can even lead to more severe diseases such asosteomyelitis, endocarditis, pneumonia, septicemia and toxic shock (Lowy, 1998; Peacock and Paterson, 2015; Rao *et al.,* 2015). Moreover, *S. aureus* has been implicated in food poisoning and scalded skin syndrome (Haasnoot and De Vries, 2018; Vitale *et al.,* 2015). Several environmental bacteria have also been implicated in both severe acute and chronic infections in humans, with the severity and complexity of these diseases largely dependent on their ability to produce multilayered cellular matrices, known as biofilms (Tsuneda *et al.,* 2003). Biofilms are layers of bacteria within a glycocalyx composed of polysaccharides, DNA and proteins. These films are also implicated in increasing resistance to antibiotics and immune defenses (Chung and Toh, 2014).

Phenotypic identification of biofilm-producing *S. aureus* strains can be completed using Congo red agar, microtiter plate assay and tube methods. Recently, polymerase chain reaction has also been used to assay for bacterial surface components, which may encode adhesive matrix molecules (MSCRAMMs) and Ica operon (Gerke *et al.,* 1998). Biofilm production is dependent on two properties: adherence of bacterial cells to the surface facilitating the production of multi-layered cell clusters (Ghasemian *et al.,* 2015) and biosynthesis of polysaccharide intercellular adhesion (PIA) compounds (Ikonomidis et al, 2009). These intercellular adhesion (Ica) molecules are produced from four open reading frames (ORFs), *IcaA*, *IcaB IcaC*, and *IcaD* (Cucarella *et al.,* 2002), which encode the proteins needed to synthesize the adhesion molecules and are followed by the *IcaR* gene which acts as irregulator (Arciola *et al.,* 2001). IcaR protein negatively regulates the expression of IcaADBC by binding to the promoter and restricting transcription (Parsek and Fuqua, 2004). This repression can be released by various other regulatory factors including SarA and stress-induced sigma factor SigB which act in a positive feedback loop to increase biofilm synthesis (Beenken *et al.,* 2003; Jefferson *et al.,* 2003).

Autoinducer 2 (AI-2) is also known to control this system and has been previously described as a universal language for interspecies communication, especially in *S. aureus*. AI-2 precursor molecule regulates rbf transcription and decreases PIA-dependent biofilm production in S. aureus (Ma *et al.,* 2017). *Ica* operons were first reported in *S. epidermidis* (Heinrichs *et al.,* 1996) and then in *S. aureus* (Cramton *et al.,* 1999). *S. aureus* attaches to various surface materials, including host tissues and surgical devices, which is mediated by its ability to produce a variety of adhesion molecules in addition to those produced by the Ica operons (Beenken *et al.,* 2004). Most adhesion molecules recognize glycoproteins found in the extracellular matrix and plasma of patients. Diversity of these adhesion molecules can be attributed to the ability of *S. aureus* to adapt to a wide range of growth environments, including connective tissues, bone, blood stream and vascular tissues. These surface-uncovered proteins are recognized and communicated within *S. aureus* communities via microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which are capable of initial attachment to native tissues and medical devices (Christensen *et al.,* 1985).

Inhibition of these biofilms has obvious clinical benefits, highlighting the need to develop novel therapeutics targeting these structures. Given this, it is not surprising that this remains a focus of research for many international groups and has given rise to a wide variety of anti-biofilm compounds with unique structures, including herbal compounds, chelating agents, antibiotics, antimicrobial peptides and synthetic chemicals (Sadekuzzaman *et al.,* 2015). Quercetin is a plant-derived flavonol present in several food products, including capers, onions, peppers, cranberries, tomatoes, apples, and grapes (Nabavi and Silva, 2018). Quercetin has a documented antibiofilm effect against several Gram-positive and Gram-negative bacterias (Earl *et al.,* 2008). Quercetin affect anti-biofilm activities by decreasing the total protein and viable cells within the biofilm (Zeng *et al.,* 2019). Thus, the present study examined and summarized the phenotypic and genotypic isolation and characterization of *S. aureus* biofilms. Moreover, the possible treatment of biofilms formed by these isolates was also checked.

Materials and Methods

Isolation of clinical samples: *S. aureus* was isolated from infected patients. A total of 36 samples were collected from King Faisal Hospital, Taif, Saudi Arabia. These samples were isolated from wound swabs (28/36), blood (4/36), sputum (2/36) and catheter (2/36) and collected between September 2019 and February 2020.

Identification of isolates: All isolates were initially identified using conventional bacterial identification tests such as Gram stain, catalase, coagulase and mannitol salt agar specific for *S. aureus* (Kot *et al.,* 2018). Isolates were stored at -20°C for further study.

Congo Red Agar method (CRA): Bacterial isolates were cultured following the method of (Freeman *et al.,* 1989) with minor modifications. The medium consisted of brain heart infusion broth (BHI) supplemented with 1% glucose. Congo red was set up as an independent aqueous solution and autoclaved before it was added to agar plates, inoculated and incubated at 37°C for 24 hr. Strong biofilm producers showed intense black colonies with dry crystalline consistency, while intermediate producers created colonies with darkened center or black colonies without a dry crystalline consistency. Pink colonies indicated weak slime CRA.

Microtiter plate assays: Adherence assays on microtiter plates were used as the *in-vitro* measure of biofilm activity as described

by (Cafiso *et al.,* 2007) with minor modification. Briefly, *S. aureus* isolates (36 strains) were inoculated in TSB media supplemented with 0.5% glucose and incubated at 37°C for 18 hr. After that, a cell suspension containing 10^8 CFU ml^{-1} was prepared by transferring the incubated culture to new tryptic soy broth with 0.5% glucose. A 200 µl of this cell suspension was then loaded onto a 96 well microtiter plate in triplicate and incubated for 48 hr at 37°C to evaluate biofilm productions. The plates were then washed twice with sterile phosphate-buffered saline (PBS) and fixed in 250 µl of methanol for 15 min. The plates were then removed and stained with crystal violet (1%, 200 µl) for 5min, rinsed with running tap water and then air-dried. The colorant was dissolved in 95% ethanol to measure absorbance at 492 nm and any values of ≥ 0.12, were considered positive for biofilm production. Any samples producing values of <0.2 were regarded as weak biofilm producers, 0.2-0.4 as moderate producers and >0.4 as strong producers, respectively.

Scanning electron microscope study of produced biofilm: Biofilms were visualized using SEM following incubation on glass slides (1 cm × 1 cm). Plates were incubated at 37°C for 48 hr and then, the glass slides were washed twice with PBS (PH7.4) and fixed at 4°C for 2 hr in 3% glutaraldehyde. The samples were dehydrated in an ethanol gradient (30%, 50%, 70%, 90%, and 100%, 10 min each) and then dried and sputter-coated with gold before being examined under a high-resolution scanning microscope (Priester *et al.,* 2007). These biofilms were photographed under SEMatthe Electron Microscope Unit of Taif University (model JEOLJSM-6390 LA serial number PM14400099).

DNA extraction of *S. aureus* strains: *S. aureus* colonies were purified and cultivated in 1 ml of tryptic soy broth for 24 hr at 37 °C. DNA was then extracted from 50-100 bacterial colonies suspended in 400 µl DEPC water and boiled for 10 min at 100 °C. The tubes were then centrifuged at 14000 rpm for 7 min and a clear supernatant was used for PCR amplification after measuring the concentration of DNA on a BIO-RAD spectrophotometer.

PCR amplification: Specific primers were used to facilitate species specific identification of the *Ica* genes (Table 1). These primers were designed using a TaqMan primer designer program and the primers were purchased from Macrogen (GAsa-dong, Geumcheon-gu. Korea). PCR was performed in a final volume of 25µl and consisted of 5 µl DNA template, 1 µl of 10 pM forward and reverse primers, and 12.5 µl master mix, and the volume was adjusted with sterilized deionized water. PCR was then performed using the following cycle conditions: 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing as described in Table 1, and extension at 72°C for 60 sec, with a final extension for 7 min at 72°C. 16S rRNA gene was used as an amplification control and PCR products were visualized using a 2% agarose gel stained with ethidium bromide in Tris-Borate-EDTA (TBE) buffe rand UV light and photographed using a gel documentation system (SynGen, USA).

Quercetin antibiofilm activity: To assess the efficiency of quercetin mediated biofilm inhibition, strains were treated with various doses of quercetin, 50, 25, 12.5, and 6.25 mg ml^{-1} in DMSO. Quercetin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and biofilm inhibition was assayed as follows. Mature biofilms were established by inoculating wells with heavy bacterial suspension (1×10^8 CFU ml^{-1}) prepared in TSB/0.5% glucose, and then aerobically incubated with shaking at 37°C for 48 hrs. The media were discarded, and the wells were washed with sterile PBS (pH 7.2). These biofilms were then treated with various concentrations of quercetin under aerobic conditions at 37°C for 24 hrs and then stained and quantified as described above. All tests were performed in triplicate for three different experiments.

Statistical analysis: Data obtained from the study were expressed as mean ± S.E. for all 36 *S. aureus* isolates. Data were analyzed using analysis of variance (ANOVA) with Bonferroni test in SPSS software version 11.5, for Windows (SPSS, IBM, Chicago, IL, USA) and statistical significance was set at $P< 0.05$.

Results and Discussion

CRA assay (Table 2) revealed that eight (22.3%) isolates showing intense black color were strongly positive for the production of biofilms, while 18 other isolates (50%) were intermediate producers and the remaining 10 isolates (27.7%) were negative for biofilm production (Fig.1). In addition, these results showed that isolates from sputum tended to be the strongest biofilm producers (100%), with blood (25%) and wound swab isolates (17.8%) demonstrating a much smaller capacity for biofilm production. In contrast, none of the catheter isolates were biofilm producers (100%). Quantitative MPT was then conducted based on the CRA test results. After reading the absorbance at 492 nm, the strains were classified as strong, moderate and weak biofilm producers.

The results of all 36 human clinical *S. aureus* isolates were identified as 10 (27.7%) strong biofilm producers, 16 (44.4%) intermediate producer strains and 10 (27.7%, Table 2) non-producer strains. *S. aureus*, which is commonly found in the nasal cavity of healthy individuals (30%), is both a genetic carrier for and producer of biofilms (Kluytmans and Wertheim, 2005). *S. aureus* biofilm production is enhanced by increasing glucose concentrations up to 0.5% in TSB media (O'Neill *et al.,* 2007). Biofilm-producing isolates were detected using CRA containing 0.5% glucose and this assay is the most reproducible, fastest and easiest initial indicator of biofilm production currently available, relying on a simple color based determination of activity (Arciola *et al.,* 2002). The results of CRA test identified more positive isolates than MTP assay which is consistent with the previous data (Gowrishankar *et al.,* 2016b). Of all the *S. aureus* strains examined, eight showed a clear strong positive phenotype (22.2%) for biofilm production and these results are similar to the previous reports where 88.9% of *S. aureus* isolates analyzed produced some form of biofilm (Ammendolia *et al.,* 1999). It is well

Table1: Sequence details of PCR primers used to detectthe Ica genes in the PCR assays

Target Gene	Annealing conditions	Amplicon size	Strand	Sequence(5'-3')
IcaA	50°C, 45 sec	188 bp	Sense	CAACCTCAACTAACGAAAGGTAG
			Antisense	GTCTAAGAAGTTTGCTGTTATG
IcaB	48°C, 45 sec	561 bp	Sense	GTGTTAGTCAATCACAATTTGAATC
			Antisense	CATTGGAGTTCGGAGTGACTG
IcaC	50°C, 45 sec	209bp	Sense	GTCACAGTTACTGACAACCTTG
			Antisense	CAATGAGTCTAGAATGATTGGATG
IcaD	50°C, 45 sec	501 bp	Sense	GTTGGTATCCGACAGTATACTG
			Antisense	CGTGAATCGTCATCTGCATTTG
fib	55°C, 60 sec	404 bp	Sense	CTACAACTACAATTGCCGTCAACAG
			Antisense	GCTCTTGTAAGACCATTTTCTTCAC
FnbB	55°C, 60 sec	524 bp	Sense	GTAACAGCTAATGGTCGAATTGATACT
			Antisense	CAAGTTCGATAGGAGTACTATGTTC
16S rRNA	50°C, 45 sec	180 bp	Sense	GTTGGGCAGTCTAAGTTGACT
			Antisense	CTTCATGTAGTCGAGTTGCAG

Table 2: Distribution of Ica genes in biofilm producing isolates identified using Congo red agar (CRT) and Microtitre plate test (MPT) assays

Isolate origin	No. of samples	SPI N	CRA		SPIN	MPT	
Wound swab	28	5/28	15/28	8	6/28	14/28	8/28
Catheter	2	-	-	2	-	-	2
Blood	4	1/4	3/4	-	2/4	2/4	-
Sputum	2	2	-	-	2/2	-	-
Total samples	36	22.2%	50%	27.7%	27.7%	44.4%	27.7%

Values represent experiments completed in triplicate. SP: strong positive; I: intermediate; N: negative; CRA: Congo red agar and MPT: microtitre plate test.

established that the microtiter plate test is a more quantitative and accurate test for biofilm detection in *S. aureus* with high accuracy, specificity and positive predictive values (Mathur *et al.,* 2006). The microtiter plate test results of 36 *S. aureus* isolates identified in this study revealed 26 isolates as biofilm producers, 10 of which were strong producers (27.7%), 16 were intermediate (44.4%), and 10 produced nobiofilms, which is in agreement with the results of CRA assay. SEM was used to visualize these biofilms on glass slides and the presence of heavy clump biofilms, composed of aggregates of bacterial cells embedded in a matrix, confirmed strong biofilm producer label applied to several *S. aureus* isolates of this study (arrowheads in Fig. 2B).

The negative biofilm producers appeared round with the characteristic grape shape without a matrix (Fig. 2). The SEM results also confirmed *S. aureus* aggregation in response to biofilm production confirming our earlier assays. Biofilm production is dependent on the expression of polysaccharide intercellular adhesion (PIA) compounds (Mack *et al.,* 1996) and previous reports have linked the presence of *IcaADBC* genes with PIA production and the occurrence of biofilms as genes in this operon mediate intercellular adherence and production of multilayer biofilms (Lappin-Scott and Bass, 2001). Given this data, further study was conducted to determine the prevalence of

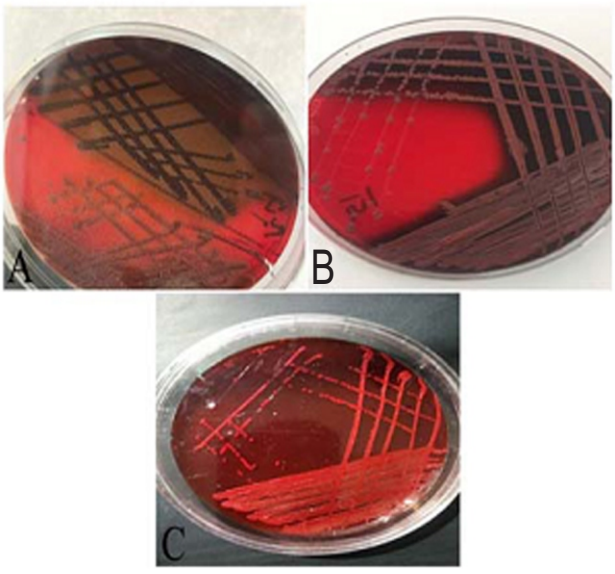

Fig. 1: Congo red agar based detection of *S. aureus* biofilm producers: (A) Black colonies with dry crystalline consistency (strong positive); (B) Dark colonies without the dry crystalline phenotype and blackening of centers (intermediate producers) and (C) Smooth pink colonies (none producers).

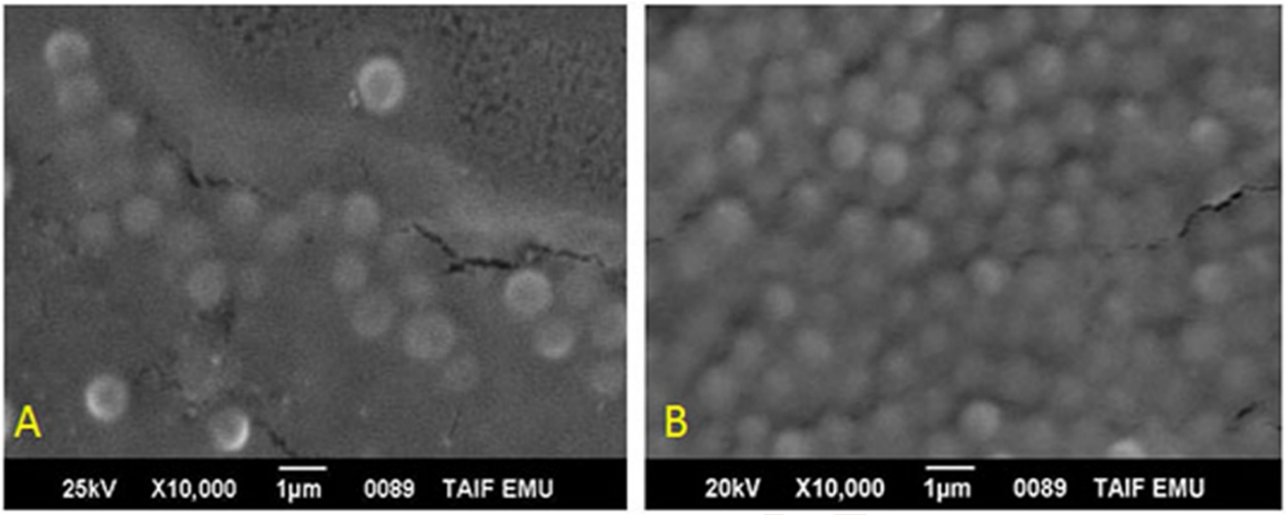

Fig. 2: SEM micrographs showing: (A) biofilm negative *S. aureus* isolates (Grape-like cells without matrix) and (B) biofilm producing *S. aureus* isolates (Cell aggregates embedded in a matrix).

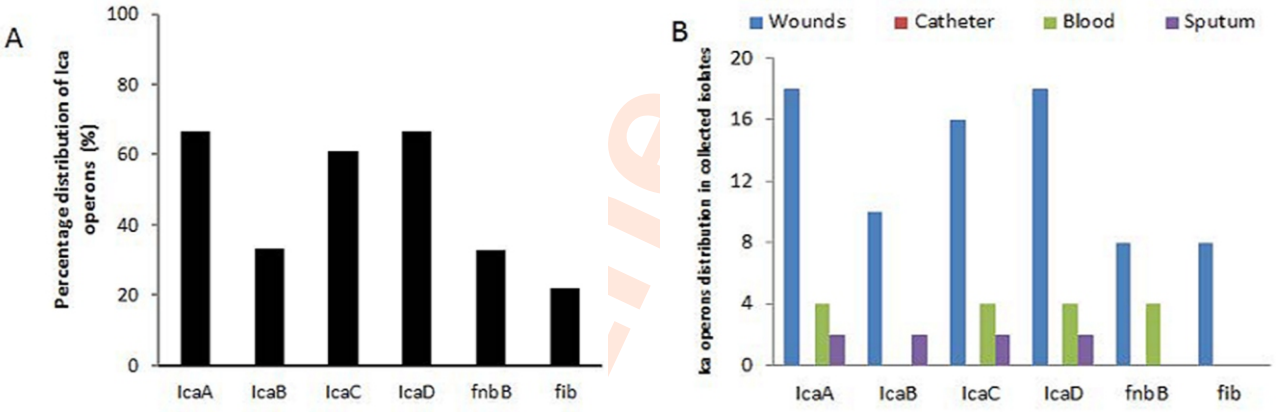

Fig. 3: (A) Percentage of samples positive for IcaABCD operon genes: *IcaA* and *IcaD*: 66%, *IcaC*, 61.1%, *IcaB* and *fnbB*, 33.3% and *fib*, 22.2%; (B) Distribution of *IcaABCD* positive samples. All data are expressed as mean from three different experiments.

Ica operon in these isolates using PCR against the *IcaA* (188 bp), *IcaB* (561 bp), *IcaC* (209 bp), *IcaD* (501 bp), *FibA* (404bp) and *FnbB* (524bp) genes using 16S rRNA gene as a control (180 bp). Analysis of 36 isolates revealed that *IcaA* and *IcaD* were present in 24 isolates (66.6%), and their distribution was abundant in samples isolated from sputum, blood and wound swabs (Fig. 3A-B; Fig. 4). The *IcaC* gene was detected in 22 isolates (61.1%) distributed evenly between the sputum, blood, and wound swabs (Fig. 4), while *IcaB* and *FnbB* were detected in 12 (33.3%) samples of sputum and wound swabs (Fig. 4, 5A).

Finally, the *fib* gene was only detected in 8 isolates (22.2%) (Fig. 5 B). PCR data also revealed that five out of 36 isolates did not harbor any *IcaADBC* operons (13.8%), which was consistent with the results of Gowrishankar *et al.* (2016a). It is worth noting that all of the strong biofilm-producing strains harbored both *IcaADBC* and *IcaADB*. *IcaD* was also present in both the intermediate and weak biofilm-producing isolates. Our data is in agreement with other studies that have shown that all strong biofilm-producing *S. aureus* isolates contain *IcaABCD* and *IcaABD*, while *IcaAD* is more common in weak to moderate producers (Piechota *et al.,* 2018). The *IcaA* protein plays a crucial role in the synthesis of PIA, while *IcaD* does not possess any exclusive transferase activity (Gupta *et al.,* 2017).

Current data suggests that the expression of *IcaA* is associated with massive biofilm production. Distribution of *IcaA* among the present isolates studied was 66.6% and was

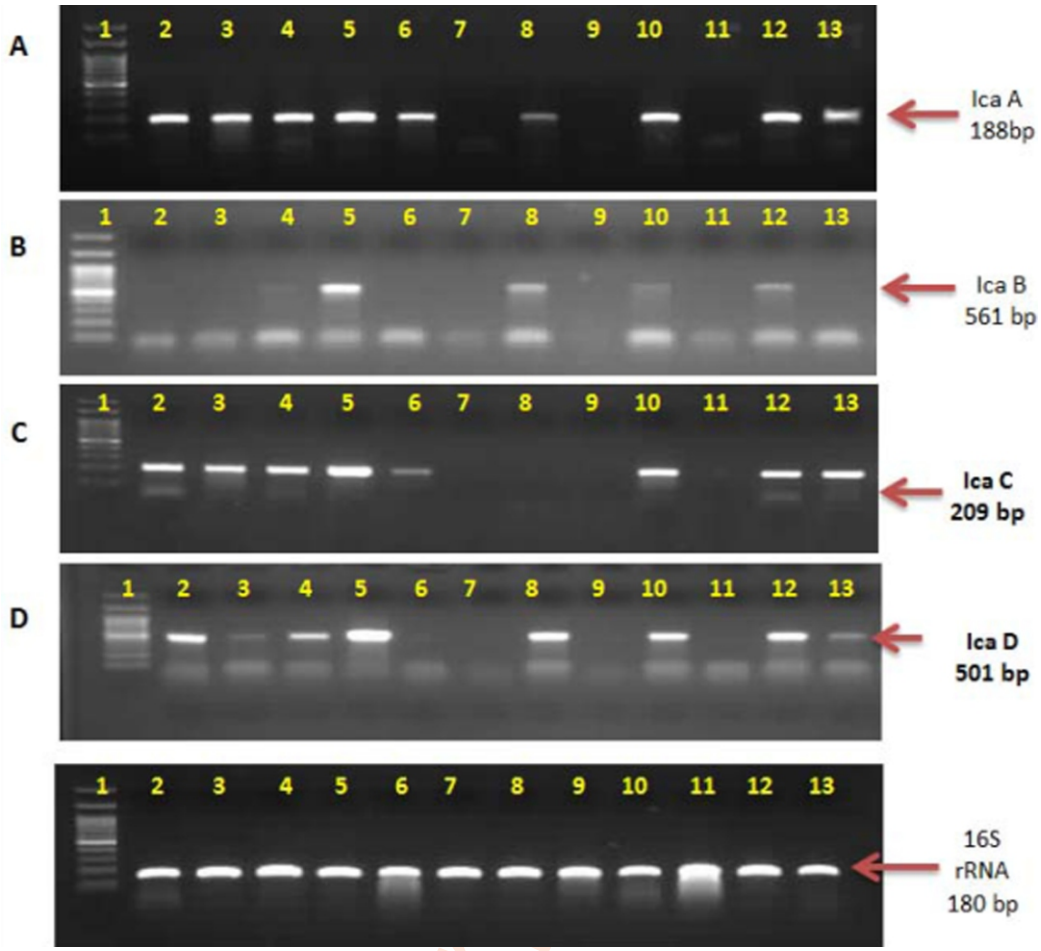

Fig. 4: Amplification of Ica *A, B, C* and *D* genes from *S. aureus* isolates using species specific PCR. Upper panel describes the visualization of these results; lane 1: 100 bp DNA ladder and lanes 2-13 are identified *S. aureus* isolates. Lower panel shows amplification of 16S rRNA gene in these sample samples.

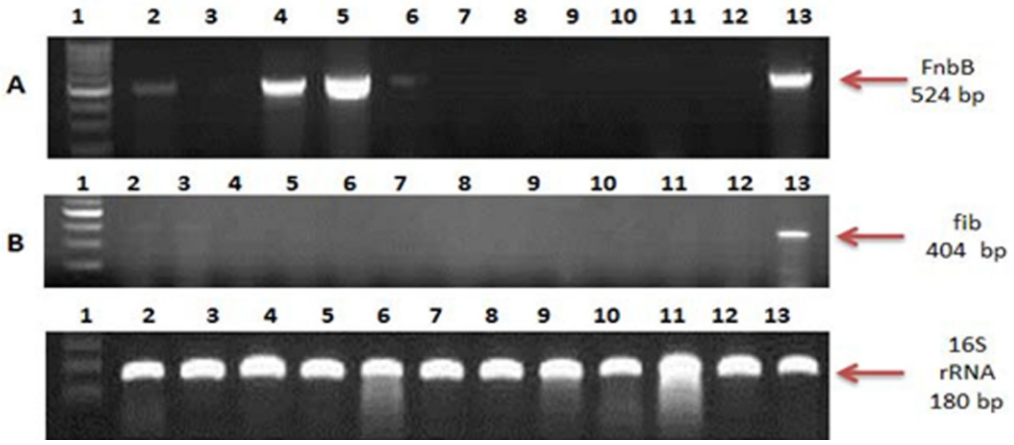

Fig. 5: Amplification of *FnbB* and *fib* genes in *S. aureus* isolates using species specific PCR. Upper panel shows target gene amplification in the *S. aureus* isolates; lane 1 is 100 bp DNA ladder and lanes 2-13 are *S. aureus* isolates. Lower panel shows 16S rRNA amplification in these samples.

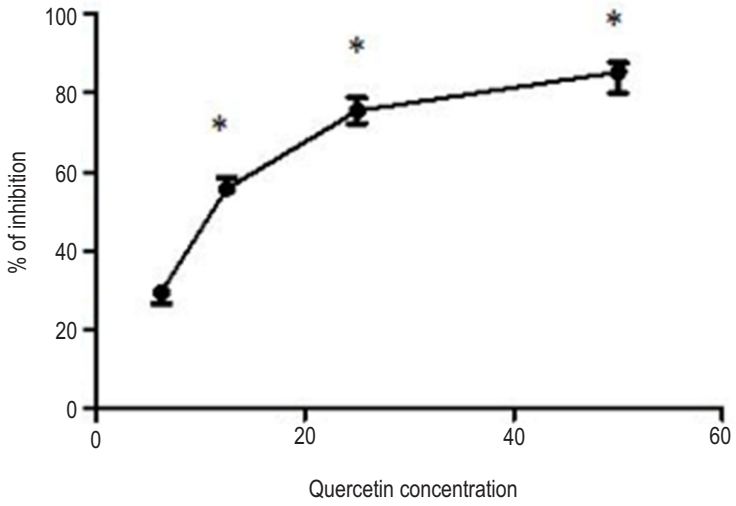

Fig. 6: Anti- biofilm effect of quercetin against *S. aureus* isolates. Maximum effected was achieved using 50 mg ml^{-1} which totally inhibited biofilm production, reducing it by 87.7%.

expressed in all of our strong biofilm-producing strains. Other previous studies (de Silva *et al*., 2002; Ninin *et al*., 2006) described the relationship between the presence of *IcaAD* operon and massive biofilm production, while others did not find this association, which is consistent with our findings. Another study reported that the prevalence of *IcaA* and *IcaD* was 66% and 58.4%, in *S. aureus* isolates (Kroning *et al.,* 2016), which was also consistent with our results. *IcaB* possess deacetylase, which deacetylates poly-N-acetylglucosamine. Deacetylation is critical for biofilm formation and has been linked to biofilm development in MRSA MSSA. MRSA MSSA biofilm production has also been shown to be *IcaADBC* dependent and responsive to specific environmental signals (Pokrovskaya *et al.,* 2013). Furthermore, the absence of *IcaB* leads to a defect in the synthesis of poly-N-acetyl glucosamine and less efficient binding to bacterial cell surface decreasing biofilm production (Vuong *et al.,* 2004).

IcaC is an integral membrane protein that may convey N-acetylglucosamine oligomers across the bacterial cell membrane. *IcaB* deacetylates PIA activity (Gotz, 2002). This study demonstrated 61.1% prevalence of *IcaC*. Conversely, *clfA* and *FnbB* (A and B) adhesions are important in host cell binding and encode MSCRAMMs, required for adhesion to abiotic surfaces. Hypothetically, fibronectin-binding proteins (*FnbA* and *FnbB*) act to facilitate invasion by modulating the adhesion and internalization of bacterial cells into the host tissues. In addition, it is well established that fibronectin proteins facilitate primary adherence and intercellular accumulation in biofilms increasing colony aggregation (Heilmann, 2011). However, it was found that only 33.3% and 22.2% of the test isolates were positive for the *FnbB* and *fib* genes, respectively (Table 2). Interestingly, the prevalence of *FnbB* and fib were 17.9% and 71.8% in their isolates (Patel *et al.,* 2009), however, variation in these results may be correlated to variation of bacterial strains in different geographical regions. Grouping of biofilm-producing *S. aureus* was demonstrated by others who grouped *S. aureus* isolates into three clusters based on their potential biofilm production creating a blood isolates, colonizing intravenous devices, and commensal isolates from the skin or nose categories (Agarwal and Jain, 2013). Our findings clarify that the strongest biofilm-producing strains were isolated from sputum, blood and wound swabs, with prevalence rates of 100%, 50%, and 21%, respectively, which may be due to difficulty of host respiratory environment, which causes the bacteria to produce intensive biofilm to resist these adverse conditions. Moreover, blood isolates need to invade blood vessels which may be aided by the secretion of adhesion genes and increased biofilm production. Quercetin had good bacteriostatic effect on *S. aureus* and *E. coli* (Wang *et al.,* 2021). Incubating these biofilms with different concentrations of quercetin resulted in significant inhibition of their biofilm production in a dose-dependent manner (Fig. 6).

Recently, several flavonoids, including quercetin have been identified as strong contenders for the production of antibiofilm agents (Matilla-Cuenca *et al.,* 2020; Memariani *et al.,* 2019). Quercetin was able to eradicate pre-prepared *S. aureus* biofilms at 50 mg ml^{-1}, which completely inhibited biofilm production (87.7%) in a dose-dependent manner. A contemporary study demonstrated the inhibitory effect of quercetin on both clinical and reference isolates of *S. aureus* (250-500µg ml^{-1}) (da Costa Júnior *et al.,* 2018) and another study reported that quercetin crippled biofilm production in *S. aureus* isolates when applied at various concentrations (1-50 mg ml^{-1}) (Kim *et al.,* 2018) which is consistent with our findings. Comparable results show that quercetin also demonstrated reliable antibiofilm activity against *S. mutans*, which may be used for treating dental caries (Zeng *et al.,* 2019).

S. aureus biofilm production differs according to sample type with sputum isolates being the strongest biofilm producers and wound swab isolates the weakest biofilm producers. All strains positive for IcaABCD and IcaABD genes were potent biofilm producers, producing significantly more biofilms than strains positive only for IcaAD. Quercetin significantly inhibited biofilm production and showed good ameliorative effects. Quercetin may be useful in the eradication of biofilm-forming *S. aureus* isolates, which may persist in the hospital environment potentially increasing antibiotic resistance in these environments.

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Add-on Information

Authors' contribution: E.H. Eldrehmy: Designed the experiment and carried out the phenotypic characterization of the bacterial isolates; **S.M. Abdel-Hafez:** carried out the phenotypic characterization of bacterial isolates; **Y.S. Alghamdi:** Interpreted the data and prepared the manuscript; **M.M. Soliman:** Carried out PCR assays and revised the paper; **S.H. Alotaibi:** interpreted data and prepared manuscript; **A. Alkhedaide:** Carried out PCR assays and revised the paper; **M.Y. Hassan:** Examined the antibiofilm activity of quercetin; **H.H. Amer:** Interpreted the data and prepared the manuscript; **Nada Alqadri:** carried out the phenotypic characterization of the bacterial isolates.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: The Scientific Research Ethical Committee of Turabah University College, Taif University, Saudi Arabia signed off all procedures used in this study. The Scientific Deanship of Taif University, Saudi Arabia, along with its Ethical Committee, approved all procedures used in this study for the project #6064-439-1.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not Applicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

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