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Evaluation of biofilm formation and associated slime encoding determinants in *Staphylococcus aureus* isolated from clinically diseased pets

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Abstract---*Staphylococcus aureus* (*S. aureus*) is an important zoonotic pathogen implemented in various hospital, community as well as livestock infections. Pets as dogs and cats have increased their close social relation with human leading to significant elevation in transmission of zoonotic multidrug resistant virulent pathogens. Diplex polymerase chain reaction was applied on ten clinical *Staphylococcus aureus* isolates obtained from wound and nasal samples from dogs and cats for detection of two slime formation encoding genes; *icaA* and *icaD*. The positive genes carrying isolates were encouraged to produce biofilm and evaluated phenotypically by cultivation onto Congo red medium. Quantitative assessment was done using a microtiter plate assay, also the formed biofilm examined by fluorescent and scanning electron microscopy. Four out of tested ten *Staphylococcus aureus* isolates were found to harbor the two genes separately. The four isolates displayed positive biofilm production onto Congo red medium while only three isolates produced biofilm in sterile polystyrene 96-well microtiter plate. One isolate associated dog infection developed strong biofilm formation which examined by both fluorescent and scanning electron microscopy. *Staphylococcus aureus* isolates obtained from diseased dogs and cats can produce biofilm that increasing their virulence and pathogenicity as well as public health concern.

Keywords---biofilm, *ica* genes, dog and cat, *Staphylococcus aureus*, fluorescence, SEM.

Introduction

Staphylococcus aureus is an over-present organism that has the potency to colonize and infect humans as well as a wide scope of other mammals and birds (Bruce et al., 2022). *S. aureus* can induce hospital and community associated infections in humans which can extent from skin and soft tissue infections to life-threatening infections such as bacteremia and endocarditis (Olaniyi et al., 2016). Recently, the one health concept was emerged especially that concerned with strains which acquired the multidrug resistance merit and transmitted from animals as pets: dog and cat. Companion animals have developed a public health importance because of close relation to their owners which facilitate the transmission of zoonotic organisms as *S. aureus* via direct contact (Overgaauw et al., 2020).

The ability of *Staphylococcus* strains to produce biofilm on polymeric surfaces triggers them more pathogenic. Biofilm composed of multilayered bunches of cells entrenched in an extracellular polysaccharide matrix 'slime' that eases adhesion and guards versus the host immune response and antimicrobials (Szcuka et al., 2016). The intercellular adhesion '*ica*' operon is accountable for the formation of slime, encoded by the linked genetic determinants of *icaADBC*. The production of N-acetylglucosaminyltransferase and its maximum expression are encoded by *icaA* and *icaD* genes respectively. The mentioned enzyme is implemented in the

formation of polysaccharide intercellular adhesion (PIA), so these genes play important roles among *ica* genes in the production of biofilms (Arciola et al., 2001). Because of the turn of biofilm formation in *S. aureus* of animal origin “including dog and cat” not well realized, since biofilm surveys in animals are uncommonly accounted, as most studies performed so far focus primarily on bovine mastitis (Kern et al., 2018; Pedersen et al., 2021). The study targeted the investigation of biofilm formation by *S. aureus* strains obtained from pets as a virulence character through phenotypic, genotypic and microscopical assessments.

Material and Methods

Sampling

The current study was conducted on formerly identified ten *Staphylococcus aureus* strains obtained from nasal and wound in clinically ill dogs and cats (Elshabrawy et al., 2020).

Genotypic characterization of *Staphylococcus aureus* isolates

Chromosomal DNA of the freshly cultured ten *S. aureus* strains was extracted using Qiagen Genomic Purification Kit (Germany, GmbH). The isolates were tested for two biofilm encoding determinants; *icaA* and *icaD* by diplex polymerase chain reaction technique (PCR), using specific primers (Metabion, Germany) as previously described (Cramton et al., 1999).

Twenty five μ l of PCR mixture included 1 mM forward and reverse primers, dNTP mix and one unit Taq DNA polymerase, five μ l Taq buffer (5X), and fifty ng DNA template. PCR conditions were applied using Biometra thermal cycler and contained a primary denaturation (94°C for five minutes), followed by thirty cycles of denaturation (94°C for thirty seconds), annealing (55°C for thirty seconds), and extension (72°C for forty five seconds), then finally an extension (72°C for ten minutes). Five μ l of each PCR product was analyzed on two percent (wt/v) agarose gel stained with ethidium bromide (0.5 μ g/ μ l), and seen beneath ultraviolet trans-illumination and photographed using gel documentation system (Alpha Innotech, Biometra). *S. aureus* ATCC® 25923 was included in all plates as a positive control (Arciola et al., 2001).

Phenotypic biofilm formation assay

Biofilm producer strains were determined qualitatively by culturing the isolates on one liter of brain heart infusion agar (Himedia, India) mixed with 0.8 gm Congo red agar (Sigma-Aldrich, St. Louis, MO), and 36 g sucrose as previously described (Mathuret et al., 2006). The strains were incubated at 37°C for 1 to 2 days at 37 °C, under aerobic conditions.

Quantitative evaluation of slime formation

The four positive biofilm encoding genes *S. aureus* strains were cultured overnight in trypticase soy broth ‘TSB’ (Himedia, India) supplemented with 0.5% dextrose and 3.0% NaCl. The overnight cultures were adjusted to 1 McFarland scale and diluted 1:10 in TSB with glucose and then 20 microliters were inoculated on

wells of microtiter plate (Thermofisher) 'triplicate for each strain'. Positive control wells were inoculated with aliquots of biofilm producer '*S. aureus* ATCC® 25923' while negative one contained sterile biofilm medium alone, then incubated at 37°C without shaking for 24 hours. Bacterial cultures were removed from each well and gently washed 3 times with 200 µL of sterile PBS, followed by fixation with 200 µL of 100% ethanol. After that immediate getting rid of the ethanol, and the plates were dried for 10 min in a sterile hood. Twenty µL of crystal violet was added to each well for just 2 minutes, and then was aspirated gently from each well. The wells were washed 3 times with sterile PBS and let to dry overnight. After that, the stain was eluted by addition of 100 µL of 100% ethanol and left to dry. The optical density was read at 490 nm using microtiter plate reader ELx 800 UV (Bio-Tek, USA) and evaluated as mentioned (Peeters et al., 2008).

Detection of biofilm formation using Fluorescence Microscope

Bacterial cell culture of the strong biofilm producer *S. aureus* isolates was suspended in three percent TSB and agitated at 180 rpm and incubated at 37 °C for 18 h. A bacterial suspension adjusted at 10⁷ CFU/ml in 1.5% TSB provided with 0.3% glucose. Biofilms were initiated by adding one ml aliquots of the bacterial suspension to sterile Cellview™ cell culture plates (Greiner Bio-One, Germany), and incubated at 37 °C. The medium was discarded and the plates were gently washed using PBS solution. The biofilms were stained with dyes (SYTO9® BacLight™) for sixty min and then washed with PBS to free the stains. After that, fluorescent images were taken out using fluorescence microscopy (Olympus, CKX41) (Gorokhova et al., 2012).

Detection of biofilm formation using Scanning Electron Microscope

The planktonic cells free formed bacterial strong biofilm producer were examined by scanning electron microscopy 'SEM' as following; the specimen was fixed in 3% phosphate-buffered (v/v) glutaraldehyde (Himedia, India), pH 7.4 overnight at 4 °C. Secondly, the samples were post-fixed in one percent osmium tetroxide and 0.1 M PB for 1 hour. After that, the fixed samples were dehydrated in graded cold ethanol /water series (Cheville and Stasko, 2014). Ultra-thin dried slime sections were coated with gold and inspected under SEM (QUANTA FEG 250, Japan), at a voltage of 20 kV and magnifications x 3000 and x 6000.

Statistical analysis

The statistics of data was carried out by applying one-way ANOVA, and the results were presented as means ± SD.

Results

Among the tested ten *Staphylococcus aureus* strains, the assessed two genes were present as sole within four strains; the amplification of 1315 bp which represent *icaA* gene was found in two strains while the other two strains harbored the other gene '*icaD*' represented by 381 bp amplification. The four *S. aureus* strains identified as positive biofilm producers as they have developed black colonies, whereas the non-producing ones appeared as red colonies.

Regarding the quantitative evaluation of biofilm formation; only three isolates displayed biofilm formation with two pictures; one isolate showed strong biofilm while the other two isolates manifested weak one (Fig. 1). Green viable cells were represented by fluorescence microscopy (Fig. 2) and coccal masses in SEM images (Fig. 3a and b).

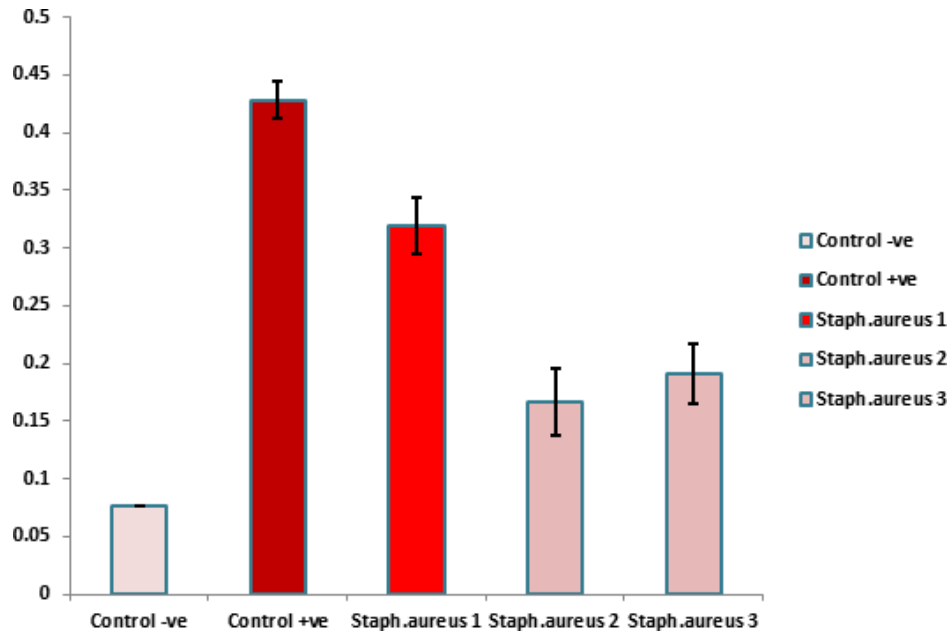


Figure (1): the OD values of tested 3 *S.aureus* isolates showing the strong biofilm producer (≥ 0.3), and the weak producers (≥ 0.1 and < 0.2), beside positive and negative controls < 0.1 .

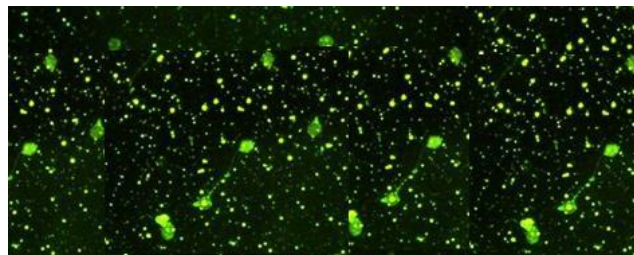


Figure (2). Fluorescent microscopy criteria of SYTO 9 stained *S. aureus* biofilms formed after 1 h incubation.

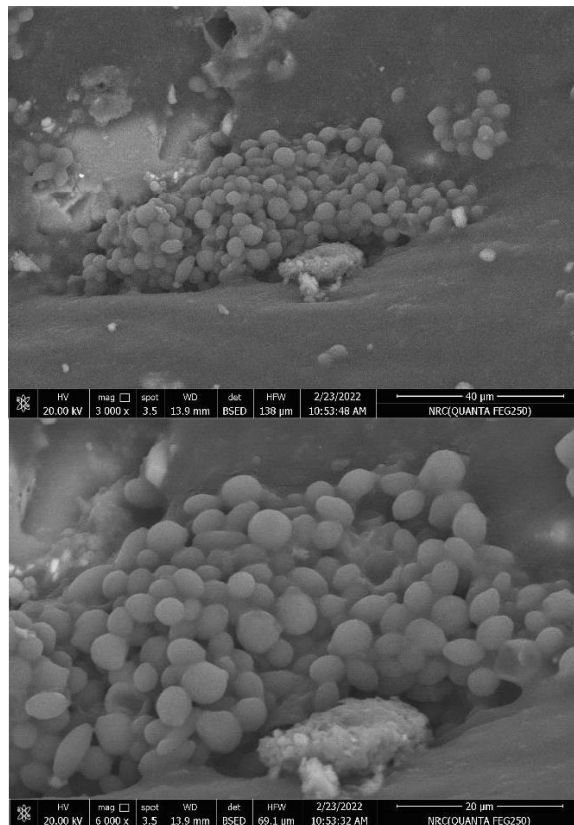


Figure (3a, b): SEM for *S. aureus* biofilm cells grown in sterile polystyrene 96-well microtiter plate 24 (magnification x 3000 and ×6000)

Discussion

The human–animal bond has been altered over time, as the role of pets has transformed from labor to social function, granting companionship. However, the role of companion dogs and cats in one health relations is often under-assessed and the increasing in knowledge about their turn in either direct or indirect transmission of zoonotic pathogens is needed (Schmidt, 2009).

S. aureus is defined as one of the most prevalent causes of biofilm-accompanied infections. Whilst, the role of biofilm synthesis in animal infections is not well estimated, and uncommonly recorded, as most reports have mainly given a focus on bovine mastitis (Pedersen et al., 2021).

Namvar et al., proposed that *S. aureus* could not synthesize biofilm unless strains were positive for the *ica* operon genes. Our study was conducted on ten previously defined *S. aureus* strains isolated from clinically diseased dogs and cats. Molecular PCR assay of two slime encoding genes revealed detection of the genes in 4 isolates (40%), each gene in two separate isolates. These out findings were near with that mentioned by (Ghasemian et al., 2016; Avila-Novoa et al., 2018; Mohammadi Mollaahmadi et al., 2021). On the other hand, many studies

reported high detection of these genes (70 -100.0%)(Gowrishankar et al., 2016;Dai et al., 2019; Chen et al., 2020; Hamel Mohaisen et al.,2021).

The four isolates were wound infections; three obtained from dog and one from cat and displayed phenotypic picture of positive biofilm formation on Congo red medium. Our result was supported with the data mentioned by Marques et al., (2017) who reported that all the biofilm-producing strains harbored either *icaA* or *icaD* or both.

On quantitative microtiter plates; three *S. aureus* isolates were biofilm producers, but one only derived from dog wound sample classified as strong and the other three were considered weak following the scale values. The obtained data was coincided with that mentioned by Silva et al., (2022) who indicated that *S. aureus* strains isolated from dog clinical samples were biofilm producers. It is essential to identify *Staphylococcus* spp. isolates that form biofilms because this virulence merit provides the maintenance and persistent adhesion of the pathogen to host tissues and intense the concern of zoonotic infection.

Concerning the microscopical examination by fluorescent showed green viable cells while at SEM, the tested isolate exposed a dense coccal cell clusters aggregations which was observed also in other studies (Marques et al., 2017; Mlynek et al., 2020; Kranjec et al., 2021).

Conclusion

The importance of the One Health relies on the detection of highly multidrug resistant zoonotic microorganisms which can transmit from animals particularly pets to human. One of the most public health pathogens is *S. aureus* which implemented in different acute and chronic animal and human dangerous infections. The clinical *S. aureus* strains usually harbor slime encoding determinants as virulence merit. Also they have the ability to produce compact cellular and matrix biofilms under harsh conditions.

Conflict of Interest

The authors stated that no conflict of interest.

Authors' Contributions

Sohier M. Syame, Ashraf Hakim and Mona A. Elshabrawy designated the plan of study and supervised all procedures. Gaber, E. S and Engy Farahat performed the cultural and PCR assay, Hanan M. El-Hewairy conducted microscopy, Hussien A. Abouelhag conducted the microtiter assay. Randa Mohamed Ismail has analyzed the data and shared in writing of manuscript with Ashraf Hakim. All authors revised and shared in publication.

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