ORIGINAL ARTICLE

Quantification Biofilm Formation by *Staphylococcus aureus* Isolated from Iraqi Meat

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ABSTRACT

S.aureus is generally present in the skin and nasal passages of the most human and enter into the food during production from sneezes or wound workers hands. Biofilms are one of the factors that promote adhesion and colonization, which leads to repeated or recurrent infections. Five hundred meat samples collected from different supermarkets and puncher shops. The well flat micro titer polystyrene plates and congo red agar method used to investigate the ability of the *Staphylococcus aureus* to form biofilms. The result of the current study found the percentage of *S.aureus* isolates was 30 (6%). The result of the current study presents the ability of all *S.aureus* isolates to form biofilm in 3 degrees, strong biofilm formation in a prevalence of 20 (66.7%), moderate biofilm formation in a prevalence 8(26.6%), and weak biofilm formation in a prevalence 2(6.7%).
Keywords: Biofilms, *Staphylococcus aureus*, Crystal violet, Congo red

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium, coccus shape. It's negative to oxidase, blood hemolysis, catalase and coagulase-positive, non-motile, non-spore-forming bacteria, facultative anaerobic[1]. *S. aureus* is present on the skin, nasopharynx, and mucous membranes as normal flora in humans and animals[2,3]. *S. aureus* influences public health through its interaction with animal's products. Animal food may be contaminated with *S. aureus* that causes human diseases[4]. The virulence depended on various factors, including extracellular proteins, like enzymes and toxins[3]. *Staphylococcus* species have many virulence factors that influence their species' efficacy and pathogenicity in the event of infection[5].

Meat products from infected food handlers that may become asymptomatic carriers during slaughter and processing of animals or cross-contamination during food preparation due to their ability to bind surfaces and form biofilms may be contaminated with *S. aureus*[6].

S. aureus can biofilms formed on both biotic and abiotic surfaces in the food chain and enhance biofilms growth by different processing methods in the food industry such as sub-optimal temperature, insufficient sanitization the composition of salts and sugar[7].

Staphylococcus ability to develop biofilms is among the virulence factors promoting adhesion and colonization, leading to recurring or persistent infections[8].

This study aimed to isolate and identify *S.aureus* from meat and study the ability of *S.aureus* isolates to biofilm formation.

MATERIALS AND METHODS

Isolation and identification: Five hundred meat samples collected randomly from different local supermarkets and butcher shops in Babylon Governorate, Iraq, between November 2019 to April 2020 by using the sterile container. Five grams of meat samples were grinding and suspended in 10 ml of Brain Heart Infusion Broth and incubated under the aerobic condition 24 hours at 37 °C. A loop full of meat suspension was streaking on the Mannitol Salt Agar and incubated for 24 hours at 37 °C. Positive culture samples were re-cultured on *Staphylococcus* chromogenic agar, followed by a confirmative coagulase test by rabbit plasma coagulase kit[9].

Biofilm Formation Test

A-Micro titer plate assay method: The capacity of strains to form biofilms was investigated in polystyrene plates with a flat bottom of micro titer. The methods performed according to the method of [10]. Inoculation a loopful of bacteria into sterile test tubes of brain heart infusion broth for 24 hours at 37 ° C. The growth media removed from the tube and washed with deionized (DI) water. Wash the tubes with distilled water 2-3 times to wash off the planktonic cells. This step helps to remove unattached cells and media components that can be stained in the next step. Adds 2 ml of 0.1% crystal violet dye and incubated at room temperature for 15 minutes. Pour the dye and wash the tubes with distilled water two to three times to remove the remaining dye. Turn the tube upside down and dry in the oven at 50° C for 1 hour.

Add 30% acetic acid to each tube and incubated at room temperature for 15 minutes. Transfer 250 microns from each tube to well flat-bottomed micro titer polystyrene plates. Measured the optical density on 570 nm by using an absorbance microplate reader. The 30% acetic acid in water used as blank (O.D.= 0.04). According to their biofilm formation ability, the isolates were classified into four categories proposed by[11].

B-Congo Red Agar (CRA)Method: Detection of biofilm formation was carried out by Congo Red Agar as described by[12]. The medium was prepared using Brain Heart Infusion Broth 38 g/L, 0.08% of Congo red dye, and supplemented with 2% sucrose and 15 g/L Agar. Congo red dye was prepared separately and added when agar cooled to 55ºC. Inoculated plates were incubated at 37ºC for 24 hours. Black colonies on media indicate a positive test for strong biofilm formation. Black to deep red considered as moderate biofilm formation. Red colonies are considered as weak biofilm formation. White colonies considered as no biofilm formation.

RESULTS AND DISCUSSION

Isolation and Identification of *S.aureus*: The *S.aureus* isolates form a yellow colony with mannitol fermentation on mannitol salt agar. The *S.aureus* form a small purple colony on Chromogenic Agar. All the *S.aureus* isolates give a positive result to the coagulase test. The result showed the percentage of *S.aureus* isolates was 30(6%) from 500 meat samples. Major meat product contamination occurs during handling, processing, and distribution. Therefore, it can use to promote personal health care. Hence, strict control and monitoring programs suggested reducing the risk of transferring animal-associated *S. aureus* to humans[13]. Other studies conducted in many countries of the world by another researcher found the prevalence of *S.aureus* isolated from meat samples was 66.67 (80/120)[14]. Another researcher, found forty-eight out of 485 (9.89%) raw retail meat samples contaminated with *S. aureus*[15].

Quantification Biofilm Formation

A-Microtiter plate assay method: The multi-step testing of the microtiter plate is relatively straightforward, reproducible, and enables researchers to simultaneously analyze multiple samples rapidly. It is very affordable because it does not require the procurement of specialized machines, and crystal coloring is cheap it is safe from pollutants. The crystal violet test can also be changed in biofilms generated in various reactors[16].

Figure 1 showed the degree of biofilm (no, weak, moderate, strong) formation by *S.aureus* isolates. Biofilms are surface-attached microbial communities. Embedded in an extracellular polymeric material creating defense, stabilization and nutrition for the numerous bacteria species inhabited. These populations can build up and causes destruction, the decline of efficiency and infection in a wide range of environments, from manufacturing machinery to medical devices[16].

As the result shown in Figure (2), 29 (96.7%) of S.*aureus* isolates able to biofilm formation divided in three degree (non, weak, moderate and strong) depending on optical density measured by micro titer reader . The optical density for 30 *S.aureus* isolates was 0.187, 0.182, 0.161, 0.279, 0.145, 0.343, 0.425, 0.3, 0.507, 0.04, 0.342, 0.134, 0.118, 0.353, 0.412, 0.423, 0.313, 0.707, 0.261, 0.088, 0.242, 0.261, 0.427, 0.145, 0.102,0.721, 0.238, 0.105, 0.129 and 0.432 showed in Figure (3).

Figure (1): Tube biofilm formation stained with 0.1 % crystal violet after drying by oven at 50°C for 1hr. A-Strong biofilm. B-Moderate biofilm. C-weak biofilm D- No biofilm

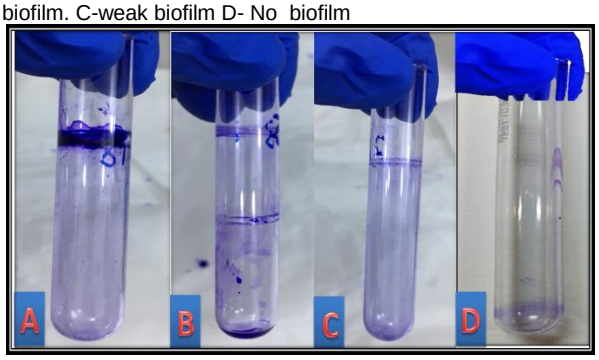


Figure (2): Well flat bottomed microtiter polystyrene plates biofilm formation by *Staphylococcus aureus* (n=30). A- Strong biofilm, B-Moderate biofilm, C-Weak biofilm, D-No biofilm

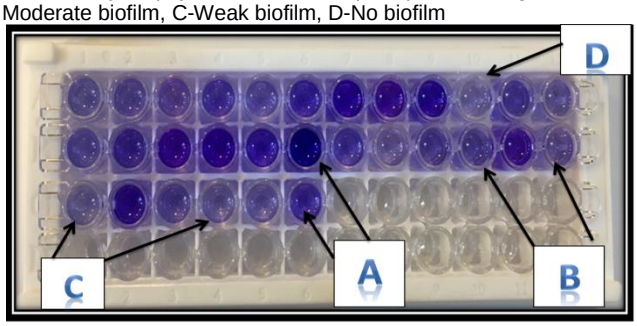
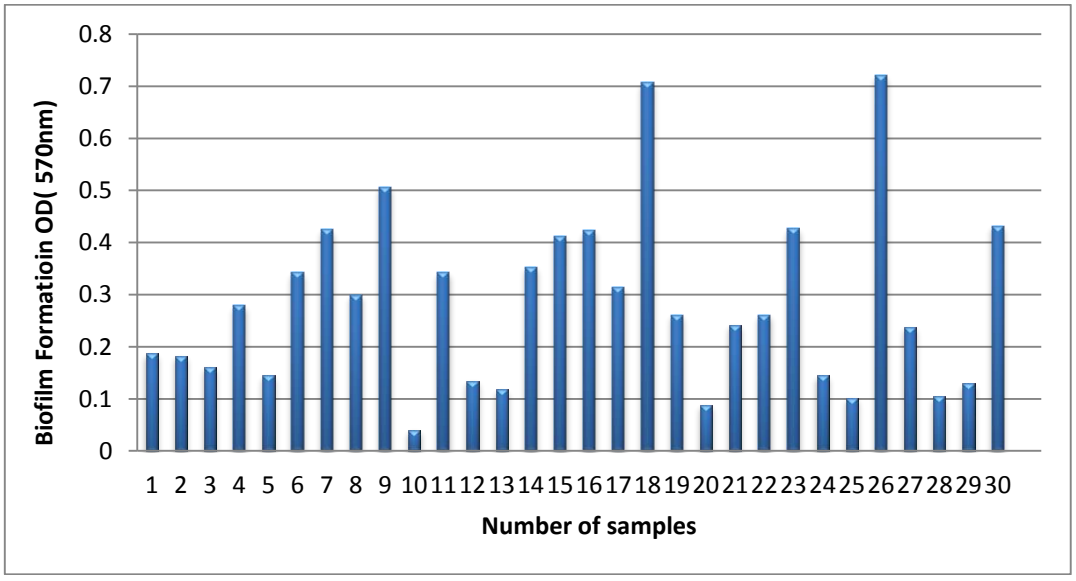


Figure (3): Determination the *Staphylococcus aureus* biofilm formation

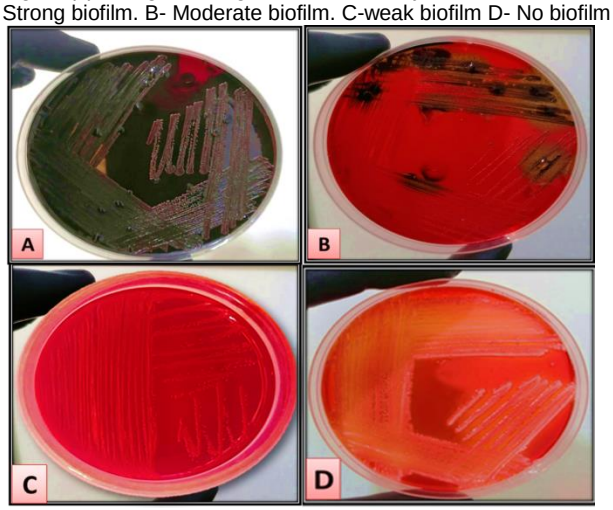


B- Congo Red Agar method: The *S.aureus* isolates cultured on Congo Red Agar for 24 hours at 37° C. The result of the current study showed the *S.aureus* isolates form biofilm in three degrees. Strong (black colony), moderate (black to the red colony), weak (red colony), and no biofilm (white colony) Figure (4).

Food contact surfaces can cause major health problems by forming biofilms. Biofilms decrease efficiency of sanitizers, cause industrial economic losses, pollute meat, and increase resistance to antimicrobials[17].

Biofilms improve bacteria resistance to environmental stress in the food industry, including washing, disinfection and inhibition, so that microorganisms can continue to live on the substrate and industrial processes, comparison to planktonic [18,19, 20]. *S. aureus* can bind and grow biofilms on food surfaces, thus affecting food quality and protection[21].

Figure (4): Congo Red Agar method to study biofilm formation A- Strong biofilm. B- Moderate biofilm. C-weak biofilm D- No biofilm



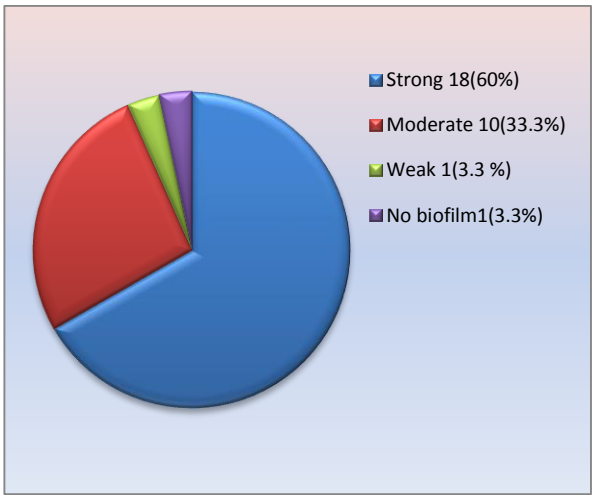


Figure (5): Quantitative of biofilm formation of *Staphylococcus aureus*

As a result of optical density mention in Figure (3), and the result obtains after cultured *S.aureus* isolates on Conge Red Agar Figure(4) present the percentage of strong, moderate, weak. No biofilm formation was 20 (60%), 8(33.3%), 1(3.3%), and, 1(3.3%), respectively Figure (5).

Another researcher found 86.0% could form a biofilm. 69(42.1%) weak biofilm, 38(23.2%) moderate biofilm, and 34(20.7%) strong biofilm[22]. While [23] found 53.5%, 33.2% and 13.2% were moderately positive, strongly positive, and negative, respectively, by the tube method. Also, [24] found 72(38.29%) of *S.aureus* isolates able to biofilm formation, in three degrees strong, moderate, and no biofilm in a percentage 34(18.08%), 38 (20.21%) and 116(61.7%) respectively.

So many hypotheses have suggested explaining why biofilm pathogens are often more virulent than their planktonic equivalents[25]. First, pathogens can initiate the infection in biofilms by seeding or dispersal of large-cell biofilm clumps. Second, virulent phenotypes could survive and spread among morphologically diverse microbes in a biofilm within a biofilm matrix. Eventually, closely linked cells from biofilm might start networks for quorum sensing that control virulence gene expressions. Also, dense aggregated, virulent organisms can contribute to biofilm-related bacterial infections[26,27].

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