

Research article

## Biofilm formation by *Staphylococcus* spp. isolated from local food markets at Baghdad city

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### ABSTRACT

Formation and presence of biofilm leads to rise concerning of human towards food industries, because Biofilm formation impact human health and his economic negatively *Staphylococcus* spp. is a pathogenic bacteria, can form biofilm when conditions are adequate, also its able to transition from human to the food and food-contact surfaces due to the nature and habit of these type of bacteria. *Staphylococcus* spp. was Isolated from different food samples in processing plant of food preparation, and identified based on 16SrRNA using T stag primer, also we used forward primer Sa442-1 and revers primer Sa442-2 to confirm the identification of *Staphylococcus aureus*, from 36 isolates, 18 isolates was identified as *S. aureus*. The ability of bacteria to form biofilm was verified by the use of polystyrene microtiter plate assay with crystal violet staining. There were clear differences among *Staphylococcus* spp. isolates in biofilm formation, the ability was measured by determination of adhesion to polystyrene microtiter plates and compared with *Bacillus* spp. as a control positive and control negative. Isolates of *Staphylococcus* spp. showed different ability to form biofilm and adhere on polystyrene plates.

**Keywords:** Biofilm formation, Congo red agar, Microtiter plate, *Staphylococcus* spp., 16SrRNA.

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## INTRODUCTION

Biofilm is a phenomenon, refers to the aggregation of bacteria or that mean it's a collection of these bacteria which surrounded by exopolysaccharide matrix, were they secrete exopolysaccharide for attachment to either an inert or living surface after adhering to surfaces [1]. In recent years, the Biofilm is more popular research topic including food safety [2]. Biofilm is may include spoilage and pathogenic bacteria [3]. There are several conditions can help to form biofilm, were biofilm formation is influenced by the nature of substrate, electric charge of cell surface, presence of flagella and microbial growth phase [4]. The presence of bacteria on the production surfaces or food pr-

products can leads to serious problems, where they impacts human health also cause an economic losses due to food spoilage [5]. For example, for the population of *Staphylococcus* spp. the type *S. aureus* usually exists on the skin of human and his nasal mucus, it's often isolated in the foods directly that handled by human. *S. aureus* bacteria are distinguished with a salt-tolerance at range (10-15%), the outbreaks of food borne are usually associated with salty foods, such as dried fermented sausages and rather raw foods [6]. Bacteria that form biofilm are known to possess greater resistances to stress conditions than their planktonic counterparts that dispersed



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in the environment, including the susceptibility to sanitizers and other antimicrobials [6]. Bacteria in a biofilm community may use polymer production as a weapon to push themselves into the more nutrient and oxygen rich region at the air liquid interface [7,8]. *Staphylococcus* spp. is widespread micro-organisms that may play an important role as agent of food-borne diseases [9,10]. *S. aureus* and coagulase-negative *Staphylococcus* (CNS) are frequent bacteria on the human skin and mucous membranes, mainly as commensal flora [11].

*Staphylococcus* spp are the relevant biofilm-forming organisms. Biofilm well studied in dairy and meat plants process [12, 13], cross-contamination [14] and mastitis [15-18]. There are few studies regarding the biofilm production ability of *Staphylococcus* spp., which are isolated from ready- to-eat, snacks and sandwiches. Snacks are fast foods usually derived from one or more basic food items and they are often eaten between meals [19]. This study aimed to isolate and identifying *Staphylococcus* spp. in foods, and evaluate the ability of these isolates in producing biofilm.

## MATERIALS and METHODS

### Sample collection and preparation

Thirty six isolates of *Staphylococcus* spp. were isolated, and collected along study period from January 2014 to May 2014, from different types of food which include, cooked meat, fresh meat, cooked rice, different vegetable, salads and dairy products. Samples were collected in disposable plastic petri dishes from local Iraqi markets at different markets at Baghdad city, then after collection samples were transferred to the laboratories of Food Contamination Researches Center at Ministry of Science and Technology-Iraq.

### Isolation and identification of isolates

To isolate *Staphylococcus* spp., 25 gm of food samples were taken and homogenized with 225 mL of buffered peptone water 0.1% in a stomacher for one min, after that, homogenate was diluted serially, then 0.1 mL of each dilution were plated onto Baird-Parker agar supplemented with egg yolk solution (12.5 mL egg yolk in 25 mL 0.85 % saline solution and 1% potassium tellurite). After incubating plates at 37 °C for 48 h, colonies were grown typically and gave a jet black to dark grey with an entire margin and without a halo, then the colonies of *Staphylococcus* spp. from the appropriate dilutions are counted. The purified *Staphylococcus* spp. colonies tentatively are identified with gram staining and catalase activity [20].

### DNA Extraction and PCR Condition

*Staphylococcus* spp. isolates were confirmed by molecular detection of *Staphylococcus* specific genes encoding 16S rRNA, bacterial DNA was extracted using a wizard Genomic DNA Purification Kit (Promega, Madison, WI). PCR amplification was performed using green master mix (Promega). The amplified products were stained with ethidium bromide after electrophoresis in a 1.5 % agarose gel. PCR primers and conditions of PCR assay are shown in **Table 1**.

### Reference strains

Reference *B. subtilis*, strain PY79 as negative control was obtained from Laboratory of molecular Bacteriology intercollegiate Faculty of Biotechnology Poland, and for positive control *B. amyloliquefaciens* was obtained from culture maintained in the microbial culture collection of department of structure and functional biology, University of Naples, Federico II, detection of slime formation by Congo red agar method (CRA).

CRA method was used to estimate the ability of producing slime, CRA medium [50 gm sucrose (Sigma, St. Louis, Mo), brain heart in infusion broth 37 gm (Oxoid Basing stoke Hampshire UK) 10 gm agar, 0.8 g Congo red (Sigma, St. Louis, Mo) and distilled water 1000 mL] was incubated at 37 °C for 24 h, after incubation, bright black colonies were presented as slime positive, while non-producing isolates developed as a red colonies [21].

### Detection of quantitative biofilm formation by the microtiter plate method (MP)

Biofilm-forming ability was estimated by determination of adhesion to polystyrene microtiter plates [22]. Briefly, isolates were inoculated in Luria-Bertani (LB) and incubated for 18 h at 37 °C. 300 µL of LB broth were distributed in flat-bottom and inoculated with 10 µL of bacterial broth, after that, plates were incubated for 18 h at 37 °C, then they were washed with adjusted Phosphate Buffer Saline (PBS) to pH 7.0, after that plates were air-dried for 1 h at 60 °C and stained with a volume of 300 µL from 0.25% crystal violet for 1 min after washing, 300 µL of 33 % acetic acid was used to destain the samples, an optical density (OD) of each well content was measured at 570 nm by using spectrophotometer (Bio TEK). Cut-off OD (ODc) was defined for microtiter- plate test as three values of standard deviation above the mean of OD for negative control, an adherence ability of tested isolates was classified based on the OD. All tested were carried out three times and the results were averaged.

**Table 1.** PCR primers and conditions used in PCR assays

Gene	Gene Description	Sequence (5'-3')	Size of amplified product (bp)	PCR conditions cycling
16SrRNA	Forward	GGCCTGTTGAACGTGGTCAAATCA	370	94°C 5 min; 37 × (94°C 40 s, 55°C 60 s, 72°C 75 s); 2°C 10 min.
A	Reverse	ACCATTCAGTACCTTCTGGTAA		
Sa442-1	Forward	AATCTTTGTCCGGTACACGATATTCTTCACG	108	94°C 5 min; 35 × (94°C 1 min, 55°C, 2 min; 72°C 33 S
Sa442-2	Reverse	CGTAATGAGATTTTCAGTTAGATAATACAACA		

## RESULTS and DISCUSSION

### Bacterial identification

Isolates of *Staphylococcus* spp. were isolated from different types of food (Table 2), all of (36) isolates were identified as *Staphylococcus* spp. due to morphological characteristics were the colonial characters was white to golden colonies with clear zone around the colonies on Baird-parker agar with egg yolk tellurite (Fig 1).

Genetically, there were 18 isolates of *Staphylococcus* spp were confirmed as *S. aureus* which were isolated from minced meat, chicken meat, fried food, cocked rice, dairy products and pastry (Table 3).

Table 2. Source of *Staphylococcus* spp. isolated from different types of food.

Isolates number	Samples	Isolates type
1 -3	Minced Meat	<i>Staphylococcus</i> spp.
4 -8	Chicken meat	<i>Staphylococcus</i> spp.
9-13	Fried food	<i>Staphylococcus</i> spp.
14-19	Cocked Rice	<i>Staphylococcus</i> spp.
20-25	Salads	<i>Staphylococcus</i> spp.
26-30	Dairy products	<i>Staphylococcus</i> spp.
31-36	Pastry	<i>Staphylococcus</i> spp.

Table 3. Source of *Staphylococcus aureus* isolated from different types of food, which confirmed genetically.

Isolates number	Samples	Isolates type
1 -3	Minced Meat	<i>Staphylococcus aureus</i>
4 -8	Chicken meat	<i>Staphylococcus aureus</i>
9-13	Fried food	<i>Staphylococcus aureus</i>
3	Cocked Rice	<i>Staphylococcus aureus</i>
1	Dairy products	<i>Staphylococcus aureus</i>
1	Pastry	<i>Staphylococcus aureus</i>



Fig 1. *Staphylococcus* spp. colonies on Baird- parker medium, 137 typical hallow clear zone around the colonies.

### PCR Reaction

PCR- based detection of 16SrRNA gene was fully reproducible, results of the PCR products in lanes (1-18), which obtained with primer pairs for T stag (*Staphylococci*-specific 16SrRNA) (Fig. 2).

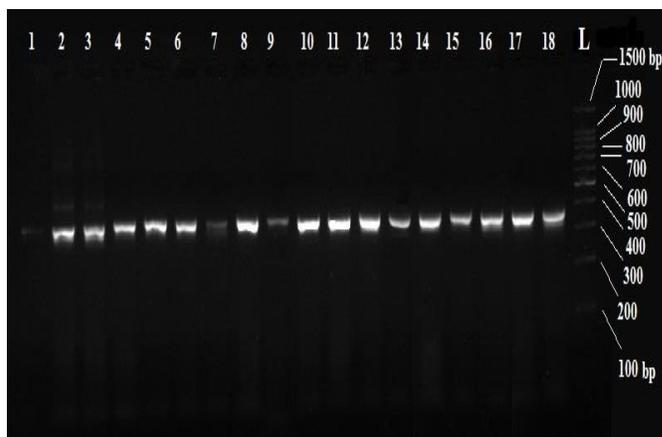


Fig 2. PCR based detection of 16SrRNA gene for *Staphylococcus* spp.

PCR reaction of forward primer Sa442-1 and reverse primer Sa442-2 to confirm the species of *S. aureus*, results of the PCR products in lanes (1-16) (Fig. 3).

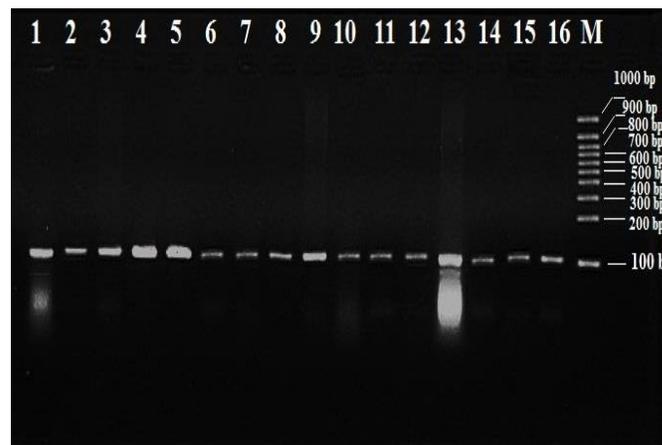


Fig 3. PCR reaction of forward primer Sa442-1 and reverse primer Sa442-2 gene for *S. aureus*.

### Detection of biofilm-forming ability on Congo red agar (CRA)

Biofilm-forming ability of each isolate was detected by CRA plate test following the colorimetric scale proposed by previous study [23]. Colonies colored from very black to almost black have been described as typical of a biofilm-positive phenotype, whereas negative slime producers form colored colonies from dark red to burgundy. Some of the isolates formed black colonies after 24 h, with color changing from red to black in surrounding agar. Thus, all strains showed slime production ability after 24 h. All isolates which maintained as a positive biofilm phenotype during 48 h, most of these isolates were formed as a red colony onto CRA (Fig 4).



Fig 4. *Staphylococcus* spp. on Congo red agar with three different distinct color ranging from red, dark red to black

### Quantification of biofilm

Biofilm formation was increased continuously during incubation period and quantified in terms of biomass by using the crystal violet staining method. Results of isolate of samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 were lower than positive control and higher than negative control strain. In fact the first 11 isolates formed biofilm more than other isolates were isolated from chicken meat, minced meat and fried food, whereas 25 out of 36 were less biofilm-forming ability, the less biofilm forming bacteria, were isolated from pastry, cocked rice, salads and diary product (Fig 5), due to the obtained results, after 18 h there were a significant among isolates to form biofilm for each isolate.

Perhaps this is due to the effect of food types were isolates of *Staphylococcus* spp. isolated, such as protein nature, complex polysaccharides nature and vegetables.

In present study, 16SrRNA gene amplification was used to characterize *Staphylococcus* spp. isolates. Primer set could be utilized for direct and rapid identification of a wide range of Staphylococci isolates at genus level. Until now, most of the PCR identification of the Staphylococcal species were isolated from food production was performed with one or two primer pairs, also in confirming species of *S. aureus* when the isolates were tested genetically. An obtained results of biofilm formation have shown that all isolates of *Staphylococcus* spp. were isolated from different food samples which have been chosen according to Iraqi people food-processing facilities, and 18 isolates was identified as *S. aureus*. In general, there were different ability of biofilm formation on polystyrene microplates was observed. The isolates of food-processing facilities does not only involve an immediate risk to food safety but more important a risk of long-term presence (even persistence) unless appropriate measurements are applied to sterile them.

Biofilm forming ability was assessed in terms of biomass using the crystal violet staining method. Thus, it could be observed that some isolates had a high biofilm-forming ability on polystyrene microplate under experimental conditions stimulating situations normally found in food. Several studies have also detected the presence of *S. aureus* biofilm on food-processing surfaces [24, 25, 26]. Biofilm biomass increased proportionally as biofilm aged. Higher variability in biofilm biomass was found among strains throughout the time course of biofilm formation, which is in accordance with previous studies [21, 3, 27]. The risk of *S. aureus* presence would be highly dependent on both the strain and the age of biofilm, as previously observed by the authors [28].

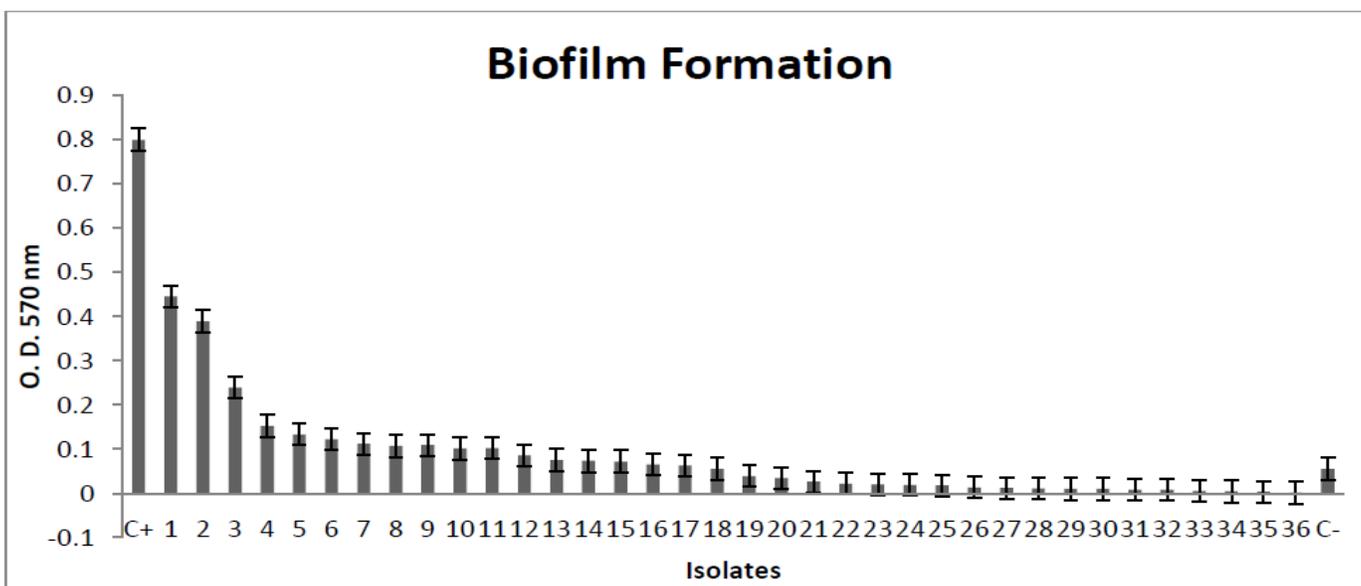


Fig 5. Isolates of *Staphylococcus* spp. with highest biofilm-forming and isolates with lowest biofilm-forming ability that isolated from different types of food. C+, control positive; C-, control negative.

Biofilm formation also was inspected by CRA plate test, which is easier to perform and less time-consuming than staining methods. This method was used successfully for detecting biofilm-forming strains of *S. epidermidis* [29, 30]. In this study, only black-colored colonies after 24 h of incubation were considered to be typical of a biofilm-positive phenotype [22] which recommended that all isolates formed black-colored colonies after 72h and biofilm-positive phenotype was maintained subsequently. These results are in accordance with those of crystal violet staining, and it contrasts with claims that CRA test results do not always correspond to biofilm production determined by staining methods [31].

The phenotypic expression of the biofilm affected conditions cultivation of the microorganism, such as the atmosphere of incubation [32]. In this study, the aerobic incubation of isolates was showed a significant differences in growth and biofilm expression, also the adoption of the chromatic scale for the reading the plates and extension of incubation at 96 h were allowed, at first case, to reduce the possible variability associated with the interpretation of operators, while in the second case allowed for us to estimate blackberries correct color of colonies above the surface of Congo red agar. In fact, the reading carried out to 24 h allowed to estimate in a comprehensive manner color of colonies only in positive isolates and in the case of negative isolates, however, the reading to 24 h allowed a correct explanation only in all of the isolates. All tested isolates in this study showed a significant ability to adhere and form biofilm on polystyrene micro-plate surfaces. In fact, some isolates showed a biofilm-forming ability moderately higher respect to a common reference strain tested, thus food-contact surfaces can be an important reservoir for *S. aureus* in the food. Also Biofilm formation detection with use of CRA plate test, is easier to perform positive results in less time-consuming than staining methods.

#### Conflict of interest

The authors declare that they have no conflict of interests.

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