

Contribution of Protease, Lipase, Hemolysin and Biofilm in Persist of *Staphylococcus aureus* Isolated from Food Sources

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Abstract

The main objectives of this research are: detect the contamination of some local food product by *Staphylococcus aureus* and their ability to excrete protease, lipase, hemolysin enzyme and formation of biofilm, all in three temperatures (35,20 and 8) Centigrade. Thirty samples from each of Meat containing product, yogurts, cheese were randomly chosen from local markets in Baghdad city during February and March 2018. Also hospitalist samples were included in this study. Each sample was diluted to 10% peptone water, incubated at 37°C for 2 hour then streaked on Mannitol salt agar and bed- Barker tullerite agar to isolate *S. aureus*. Sixty out isolate of *S. aureus* were isolated from samples, biofilm as well as, protease, lipase and hemolysin were secreted at 35°C after 48 hour while seven day was needed for production at 20°C comparing with negligible growth or enzymatic activity at 8°C even after three weeks of incubation. Results showed that 25 isolates of *S. aureus* was from meat containing food products, while ten out of *S. epidermidis* was from yogurt, two from cheeses and five isolate from hospitalist samples. Environmental isolates were susceptible to penicillin G, tetracycline, chloramphenicol and naledix acid while tetracycline, chloramphenicol and naledix acid resistance were appeared in hospitalist isolates.

Keywords: Food Product; *Staphylococcus aureus*; Biofilm Formation

Introduction

Low hygiene practices in food processing and manufacturing plant may lead to the contamination of foods and their products with different type of microorganisms that cause either food deterioration or a serious health risk for consumers. Moreover, the most challenge in food industry is the complete elimination of such microorganisms from food processing environments because bacteria can attach to food surfaces or the equipment in plant and form biofilm, where they survive and withstand for long time even after cleaning and disinfection [1,2]. The most harmful contaminant for humans and economic animals is the *Staphylococcus*. Species of Staphylococci are ubiquitous in the environment and can be found in the air, dust, sewage, water, environmental surfaces as well as mammals and birds [3].

The bacterial genus *Staphylococcus* has many members with high health risk for humans and many other animals. The most virulence species is *S. aureus* that require high viable number 10^5 - 10^6 cfu gm⁻¹ of food to produce many toxins and enzymes which play a critical role in virulence and bacterial maintenance in their environment niches. It contaminates many food, food products, milk and diary products. The transfer of *S. aureus* to food and their products through two main sources: human carriage during

food processing, infected animals and contaminated equipment in the factory. This contamination depends on many factors including food quality, processing methods, temperature and duration of storage, season and general sanitation in manufacture area [2-4]. Thus in the last decades, staphylococci and their toxins had also emerged as important and potential pathogen for human and animal [3,5]. The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors such as extracellular enzymes and biofilm formation beside toxins [6,7].

lipases produced from *S. aureus* could increase the rate of food deterioration through their action on lipids causing accumulation of intermediate and end products that change the flavor of foods [8,9]. Also, staphylococcal lipase has relevance on the pathogenicity of *S. aureus* it degrades other substrate like plasma and fats that accumulate on the surface of the body.

Alpha hemolysin play a critical role in staphylococcal ability to cause infection through leucocidal activity that permit a rapid multiplication of bacteria and allowing for production of a large amount of other toxin [10]. On the other hand, *S. aureus* excrete three types of proteases; serine, cysteine and metalloprotease

which may responsible for their virulence and persistence in environment [11-13].

Another virulence factors is the capability of bacterial community to adhere to biotic or abiotic surfaces to form biofilm that aid many advantages to bacteria. Such advantages include higher resistance to harsh environmental conditions, resist to antimicrobial agents and enhance protection from the immune system. Since biofilms are known to be a source of bacterial persistence many approaches will help to combat biofilm formation [14,15].

The aims of this research were screening of protease, lipases and hemolysin activity at 8, 20 and 37°C from *S. aureus* that isolated from different foods as well as hospitalized isolates. Biofilm formation from isolates was screened to predict their ability to cause epidemic prevalence.

Material and Methods

Samples collection and isolation of *Staphylococcus* spp

Thirty samples from each of Meat containing product, yogurts, cheeses and spices were randomly chosen from local markets in Baghdad city during February and March 2018. Also hospitalist samples were included in this study. Each sample was diluted to 10% peptone water, incubated at 37°C for 2 hour then streaked on Mannitol salt agar to isolate *S. aureus* and *S. epidermidis* and on Baird - Packer tellurite agar to identify coagulase positive *S. aureus*.

Screening of extracellular enzymes

The production of Lipase, protease and hemolysin were all screened at different temperature that includes 8, 20 and 35°C for at least 7 day period of incubation.

Rhodamine B and Tween 80 method were analyzed to screen Lipase enzyme activity. Briefly, Rhodamine B method was achieved for screening of true lipases according to Kouker and Erich [16]. Briefly, nutrient agar was supplemented with 2.5% olive oil, 3% of Arabic gum and sterilized, 1% solution of 1 mg ml⁻¹ Rhodamine B stain was added, mixed and poured in petri dishes. Lipase producing isolates gave florescence orange color.

Also tween 80 method was used for screening of both lipases and esterases. The appearance of a halo zone around growth of *S. aureus* isolates on the top of 1% tween 80 agar was indicated for esterases activity while development of obscure region around growth indicated for lipases activity [17].

Baird

Packer agar augmented with potassium tellurite and egg yolk was chosen for the detection of Phospholipases (licithinase). Opaque region around growth was indicated for licithinase enzyme. Also, nutrient agar supplemented with 1% egg yolk was used and clear zone indicated for licithinase enzyme [18].

Skim milk agar was prepared for the detection of protease enzyme; 5% skimmed milk was dissolved in sterilized distilled water, mixed with sterilized solution of agar and poured in petri dishes.

The appearance of clear zone around bacterial culture indicated as a positive result.

Alpha, beta and gamma hemolysis of blood was screened on 7% blood agar.

Detection of biofilm and slime formation

Two different methods were used in detection of biofilm formation, tube method and congo red method which described by Christensen, *et al.* [19] Bose, *et al.* [20], Gundogan, *et al.* [21]. Brain heart infusion medium containing 1% sucrose was divided into 5 ml portion in glass vials, sterilized, cooled and inoculated by test bacteria, incubated statically at 8, 20 and 37°C for 72 h. growths were withdrawn, glass vials were air dried. Biofilm formation recorded as +++ (heavily positive), ++ (moderate positive), + (weak positive) and – for negative biofilm formation.

The second method was based on Congo red stain 0.8% that incorporated into brain heart infusion agar containing 1% sucrose. Bacterial isolates were streaked and incubated at different temperature for 72 h, black colonies with a dry crystalline consistency indicated for biofilm formation; while, bright red to black colonies were established as slime formation.

Antibiotic susceptibility test

Antibiotic susceptibility test was done by agar diffusion method using standard antibiotic discs [22]. Discs of Penicillin G (30), tetracycline (10), endoxan (10) and chloramphenicol (10) were applied at experiment. Development of clear zone around bacterial growth was an indicative for bacterial susceptibility while growth around disc indicated for bacterial resistance.

Results and Discussions

At present work Many *Staphylococcus* spp contaminated different food grade and food products; *S. aureus* differentiated from meat containing medium and cheese samples. These isolates developed on Baird - Packer tellurite medium and Mannitol salt agar while *S. epidermidis* isolated from yogurt and cheese samples figure 1. Spices containing each *S. aureus*, *S. epidermidis* and *Bacillus* spp. While, hospitalized samples contained *S. aureus* (17 isolates) and *S. epidermidis* (5 isolates) and table 1 outline the type of samples and *Staphylococcus* isolates.

Figure 1: Identification of *Staphylococcus* spp on Mannitol salt agar

A= mannitol ferment isolates; B= non mannitol ferment isolates.

Sample type	No of isolates	MSA	BPT agar	Coagulase test	Characterization
Meat containing food	25	Yellow color	Black color with pricipitate	+	<i>Staphylococcus aureus</i>
spices	7	Yellow	Black precipitate	+	<i>Staphylococcus aureus</i>
		Pink	No- precipitate	-	<i>Staphylococcus epidermidis</i> <i>Bacillus</i>
yogurt	20	yellow	No- precipitate	-	<i>Staphylococcus epidermidis</i> <i>Bacillus</i>
cheese	7	Yellow	Black precipitate	+	<i>Staphylococcus aureus</i>
		Pink	No- precipitate	-	<i>Staphylococcus epidermidis</i>
Hospitalized	22	Yellow	Black precipitate	+	<i>Staphylococcus aureus</i>
		Pink	No- precipitate	-	<i>Staphylococcus epidermidis</i>

Table 1: Isolation and characterization of *Staphylococcus* spp from different samples.

Food borne bacteria are the most dangerous among bacterial community and many bacterial species are produce one or more of toxins that results of food poisoning. Coagulase positive strains of *S. aureus* and other related *Staphylococcus* spp are the most common food related pathogenic bacteria [23]. Different foods and their products may become contaminated during or after processing in plant field or at markets; workers as Tamp (2001) and Gundogan [21] referred to isolate *S. aureus* strains from meat and meat products, chicken, milk and dairy products.

Different environment consider as a natural niches for *S. aureus* to survive and multiplication; foods and their products provide a convenient and enrich source for their growth and subsequently causing outbreaks. Foods that involved in outbreaks differ from country to another due to food habits [24]. On the other hand, many factors may influence the growth, multiplication and production of virulence factors of *S. aureus* on foods such as the nutritional value of food, temperature, pH and competition with other microflora of food. Such assumption is agree with present results that *S. aureus* didn't contaminate yogurt product which might due to the presence of *Lactobacillus* spp that reduce pH value as well as the production of extracellular inhibitor substances as H₂O₂, volatile compounds, nutritional competitions and antibiotics [25]. In addition, the most effective way for inactivation of *S. aureus* in milk is heat treatment that caused complete destruction of bacterial cell.

Results showed that twenty five isolates of *S. aureus* came from meat containing products; they excrete different hydrolytic enzymes at different temperatures as presented in table 2. All isolates produced alpha hemolysin at 37°C and 20°C while little growth appeared at blood agar without hemolysis of blood when incubated at 8°C. Lipase enzyme produced at 8°C after prolonged incubation that reached to 7 days. While, protease excretes from 72% of isolates at 37 and 20°C with slightly growth at 8°C without detectable protease enzyme activity. The excretion of extracellular hydrolytic enzymes influenced with temperature, time period, food grade and

strain isolate as discussed by Hennekinne [3] and outlined that at low temperature around 7°C there are excretion of toxin after 3 - 4 days of incubation.

Despite that the action of hemolysin, protease and lipase enzyme can change the quality of food rich with lipid, fat, fatty acid and proteins resulting in decreased the shelf life of foods and their product, *S. aureus* hydrolytic enzymes collectively can act to damage the immune system and skin barrier causing several infections [26,27].

Many reports deal with the extracellular hydrolytic enzymes (Hemolysin, protease, phospholipase and lipase) production by *S. aureus* and *S. epidermidis* due to their importance in persistence and pathogenicity [12,21]. Beside hemolytic activity of Alpha hemolysin, it may related with cytotoxic, dermonecrotic and lethal activity to cell line and the toxin of interest in cow associated mastitis (Halpin - Dohnalek and Marth 1989).

The consequence of lipase production from *S. aureus* that isolated from food results from their importance in lipid metabolism as well as their role in pathogenicity of this bacterium (Cadielux 2014).

The isolates with high protease activity may be cause many infections in human and animals and such suggestion is in accordance with Gundogan., et al. [21] who reported that protease from *S. aureus* caused dermatitis in chickens.

Screening of *S. aureus* from milk containing products showed that yogurt samples (11 samples) contaminated with either *Bacillus* spp (4 isolates) or *S. epidermidis* (16 isolates). All *S. epidermidis* isolates produced alpha hemolysin; whereas, only one isolate produced protease and lipase at 37 and 20°C. Cheese samples (4 samples) contaminated with *S. aureus* and *S. epidermidis*; five distinct isolate characterized as *S. aureus* and two other as *S. epidermidis* and table 3 summarized their enzymatic activity at corresponding temperature.

Isolate no	Hemolysin			lipase			Protease		
	37C	20C	8C	37C	20C	8C	37C	20C	8C
1	+++	+++	-	-	-	+	-	-	-
2	+++	+++	-	-	-	+	-	-	-
3	+++	++	-	-	-	+	+++	++	+
4	++++	++	-	-	-	+	++++	++	+
5	+++	+++	-	-	-	+	-	-	-
6	+++	+++	-	-	-	+	-	-	-
7	+++	+++	-	-	-	+	-	-	-
8	+++	+++	-	-	-	+	-	-	-
9	++	+	-	-	-	+	++	+	-
10	++	+	-	-	-	+	++	+	-
11	++	+	-	-	-	+	++	+	-
12	++	+	-	-	-	+	++	+	-
13	++	+	-	-	-	+	++	+	-
14	++	+	-	-	-	+	++	+	-
15	++	+	-	-	-	+	++	+	-
16	++	+	-	-	-	+	++	+	-
17	++	+	-	-	-	+	++	+	-
18	++	++	-	-	-	+	++	++	-
19	+++	++	-	-	-	+	+++	++	+
20	+++	++	-	-	-	+	+++	++	+
21	++	+	-	-	-	+	++	+	-
22	+++	++	-	-	-	+	+++	++	+
23	++	++	-	-	-	+	++	++	+
24	+	+	-	-	-	+	+	+	-
25	+	+	-	-	-	+	+	+	-

Table 2: The production of hydrolytic enzymes from *Staphylococcus aureus* isolated from meat products
Lipase + at 8°C after 7 days of incubation.

Staphylococcus aureus									
Isolate No	Hemolysin			lipase			Protease		
	37°C	20°C	8°C	37°C	20°C	8°C	37°C	20°C	8°C
1	+++	-	-	-	-	-	++	+	-
2	+++	+	-	-	-	-	++	+	-
3	+++	+	-	+++	-	-	+++	++	+
4	++++	-	-	+++	+++	-	++++	++	+
5	+++	+	-	+++	++	-	++	+	-
<i>Staphylococcus epidermidis</i>									
6	+++	-	-	-	-	-	++	-	-
7	+++	+	-	-	-	-	++	-	-

Table 3: The production of hydrolytic enzymes from *Staphylococcus* spp isolated from cheese.

Screening of hydrolytic enzymes from hospitalized isolates indicated that hemolysin enzyme, lipase and protease were all predominant from characterized *S. aureus* and *S. epidermidis*; lipase enzyme recognized by both methods Rhodamine B and tween 80 method (Figure 2). Hemolysin, lipase enzyme produced by 31% of isolates at 37°C and decreased to 18.18% at 20°C in comparison

with protease enzyme which produced by 100% of isolates at 37°C and lessen to 31% at 20°C as summarized in table 4.

The results of Biofilm formation for the 25 isolates of *S. aureus* from meat products indicated that 100% of isolates produced biofilm at 37°C (Figure 2) while, the percentage decreased to 65% at

Staphylococcus aureus									
Isolate No	Hemolysin			lipase			Protease		
	37°C	20°C	8°C	37°C	20°C	8°C	37°C	20°C	8°C
2	+++	-	-	+++	-	-	++	+	-
8	++++	-	-	+	-	-	++	+	-
9	+++	-	-	+	-	-	+++	++	+
13	+++	+	-	++	+++	-	++++	++	+
Staphylococcus epidermidis									
20	+++	+	-	++++	++	-	++	+	-
21	+++	+	-	++	+	-	++	+	-
22	+++	+	-	++	+	-	++	+	-

Table 4: The production of hydrolytic enzyme from hospitalist isolates of *Staphylococcus* spp.

Figure 2: Screening of lipase enzyme from hospitalized isolate of *Staphylococcus aureus*
Left= Rhodamine B method; right= tween 80 method.

20°C and to less than 10% at 8°C because of the low growth of bacterial biomass.

There were observed ability to form slime layer at 37°C from *Staphylococcus* spp isolates from cheese. While, at 20°C only *S. aureus* could form slime layer and none of isolates produced the slime layer at 8°C.

Results obtained for hospitalized isolates revealed that *S. aureus* and *S. epidermidis* isolates could formed only slime layer at 37°C.

Also, all isolates formed slime layer with the appearance of slimy bacterial growth with red color (Figure 3).

Figure 3: Formation of biofilm and slime layer on congo red medium by *Staphylococcus* spp isolate.
A= biofilm formation, B= slime layer formation, C= no- biofilm.

The phenomenon of biofilm formation is due to the aggregation of bacteria either on biotic or abiotic material and embedded in extracellular matrix composed mainly of polysaccharides and to some extent proteins and DNA [28]. The formation of these highly organized multicellular complexes is accomplished as a major and important virulence factor in *S. aureus* [29,30]. On the other hand, biofilm can lead to persistent contamination or infection due to resist sanitation procedures applied at plants and to the action of the host immune system of human and animals beside the resistance to antibiotics. [15]. The surfaces that come in contact with different foods and their products are very important vehicles for *S. aureus* multiplication and transmission of this bacterium and while, *S. aureus* can adhere to many food contact surface, they developed biofilm and affects on the quality and safety of foods [31]. Many factors that influence the formation of biofilms in *S. aureus* that include genotype of bacterial community, temperatures, period time, the composition of medium, static or dynamic conditions for testing biofilm, procedures that applied in biofilm formation [32,33].

Our results for biofilm formation from 100% of *S. aureus* isolates of meat containing products and formation of slime layer from isolates belong to cheese and yogurt is in accordance with other work of Souza [2] and Avila - Novoa (2018) who found that *S. aureus* from meat and dairy industries formed biofilm which resist sanitation, cleaning and chemical removal for biofilm, the explanation of such finding is that meat enriched medium and milk containing products facilitate the formation of biofilm.

The ability of hospitalized *S. aureus* and *S. epidermidis* to form slim layer at 37°C is an indication for their importance at medicinal level. This suggestion is assimilate to [34] who noticed that many non- coagulate species of *Staphylococcus* other than *S. aureus* may be responsible for nosocomial infections and many are involved in animal diseases [35,36].

Conclusion

Recent results of antibiotic susceptibility showed that *S. aureus* isolated from meat containing food products, yogurt and cheese samples were susceptible to penicillin G, tetracycline, chloramphenicol and endoxan while they resist nalidexic acid. The isolates

of *S. epidermidis* isolated from cheese resisted penicillin G, chloramphenicol and nalidixic acid which supports the idea of being responsible for public health concern.

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