# Isolation, Identification and Detection of Some Virulence Factors of Staphylococci in milk and cheese in Baghdad

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**Abstract**

During 2011; 300 milk and white cheese samples were collected from Baghdad markets. Out of 200 staphylococcal isolates isolated from milk and white cheese samples, the predominant species was *Staphylococcus aureus* 97 isolates (48%), followed by *S.chromogenes* 82 (41%) and 21 (11%) *S.epidermidis* isolates. *S. aureus* isolates were DNase, coagulase, protease, urease, lipase, gelatinase and slime layer producers, other species were variable in the production of such virulence factors. *S. chromogenes* was the most prevalent isolated staphylococcal species from milk samples; while cheese samples contaminated mainly by *S. aureus*.

**Keyword**: Staphylococci, Identification, Virulence factors, Slime layer production, Milk and cheese.

# عزل وتشخيص والتحري عن بعض عوامل إمراضية المكورات العنقودية في الحليب و الجبن في مدينة بغداد

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**الخلاصة:**

جمعت 300 عينة من الحليب والجبن الأبيض من الأسواق المحلية في مدينة بغداد خلال العام 2011، وتم عزل 200 عزلة من المكورات العنقودية من العينات التي تم جمعها، وكانت المكورات العنقودية الذهبية *Staphylococcus aureus* هي الأكثر شيوعا في العينات إذ بلغ مجموع العزلات 97 عزلة (48%)، تلاها النوع *S.chromogenes* 82 عزلة (41%) ثم النوع *S.epidermidis* 21 عزلة (11%). كانت عزلات المكورات العنقودية الذهبية منتجة لعوامل الفوعة DNase و coagulase وprotease وurease و lipase و gelatinase وslime layer بينما تغايرت الأنواع الأخرى من المكورات العنقودية في إنتاجها لهذه العوامل. كان النوع *S.chromogenes* هو النوع الأكثر انتشارا من المكورات العنقودية المعزولة من الحليب، بينما تلوثت عينات الجبن الابيض بشكل أساسي بالمكورات العنقودية الذهبية *S. aureus*.

## Introduction:

Bacteria are the main cause of foodborne diseases (FBD) in the majority of the countries, causing 2/3 of food poisoning outbreaks [1].

Among the microorganisms associated to foodborne diseases, *Staphylococcus aureus*, men, women and animals are the main *S. aureus* reservoirs. Nasal carriers and food manipulators

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who have hands or arms with wounds infected by the microorganism are important sources of food contamination [2]. The high frequency of *S. aureus* as an infection agent of the mammary glands of milk-producing cows is another important factor in the epidemiology of this pathogen [3]. The main food involved in staphylococci food poisoning outbreaks are milk and dairy product [1,4]. Milk and its products can harbor a variety of microorganisms and can be important sources of food borne pathogens. The presence of food borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment or with excretions from the udder of infected animals [5].

The genus *Staphylococcus* is a member of the Staphylococcaceae family; they are gram-positive, cocci arranged in a grape like cluster, facultative anaerobic, chemoorganotrophic cocci with a respiratory and fermentative metabolism at an optimal temperature of 37°C, also, they are non-motile, nonsporulated, catalase positive and found as pathogens or commensal organisms in both humans and animals [6].

Staphylococci are ubiquitous bacteria found on the skin and mucous membranes of humans and worm-blooded animals; they also can be recovered from different environmental sources such as soil and water and from wide range of foodstuffs [7]. *Staphylococcus aureus* is an extraordinarily versatile pathogen, and it can cause a large spectrum of infections, from mild to severe and fatal. It is important in humans and also economically important when infecting animals, able to cause superficial lesions and systemic infections, *S. aureus* is responsible for toxin-mediated diseases, such as the Toxic Shock Syndrome (TSS), Kawasaki's Syndrome and staphylococcal food poisoning [8]. Coagulase-Negative Staphylococci (CONS), has increased due to their increasing importance in hospital infection, particularly in nosocomial bacteremias [9].

Regarding the facts above this research aimed:

- Investigate the spread of staphylococci in milk and white cheese.
- Determine the most frequent *Staphylococcus* species in these samples using both cultural and immunological methods.
- Assessment of the microbial load of ready to consumption milk and cheese and determination of the prevalent species of each sample.
- Detection of some staphylococcal virulence factors.

## Methods

### Samples'Collection

Three hundred milk and white cheese samples were purchased from Baghdad markets. These samples included raw, pasteurized, flavored, condensed, evaporated and dried milk. Raw salty, processed, soft, hard and semi-hard white cheese.

The samples were collected randomly according to the instructions of the Iraqi Standard Criterion No.2/2270 in Sampling, afferent by the Iraqi Central Organization for Standardization and Quality Control (C.O.S.Q.C) [10].

### Staphylococcal Isolation

The Iraqi Standard Criterion No.3/2270 in Isolation, Enumeration and Identification of Microbiological Groups in Foods, [11] was depended in Staphylococcal Isolation.

### I-Staphylococcal Isolation from Milk Samples

One milliliter of each milk sample was transferred (in triplets) to a sterilized Petri dish, then 15-18 ml of the agar medium Staph No.110 were poured (the medium should be maintained melted at 44-46$^{o}$C), the plates left to solidify at room temperature, and thereafter they were incubated at 37$^{o}$C for 24-48 hours. Because of its constituent of 7.5% NaCl; Staph No.110 is a selective medium so the isolates which can tolerate this salt concentration strictly could be isolated. Then the grown colonies were further investigated.

### II- Staphylococcal Isolation from Cheese Samples

Ten grams of each cheese sample was suspended in 90 ml distilled water, stomached for 1 minute, three 10-fold dilutions were made and 0.1ml of each step was inoculated on the agar medium Staph No.110, the plates left to solidify at room temperature, and thereafter they were incubated at 37$^{o}$C for 24-48 hours. Then the grown colonies were further investigated.

### Staphylococcal Identification

Staphylococcal Identification was performed according to: [9, 12, 13, 14, 15, 16, 17] in addition to the Iraqi Standard Criterion No.3/2270 in Isolation, Enumeration and Identification of Microbiological Groups in Foods [11].

### Detection of the bacterial ability for the slime layer production [18]

The congo red agar method was used to detect the bacterial ability to produce the slim layer; this was performed by culturing the bacterial colony on the congo red agar plate, which then

incubated at 37°C for 24 h. Black colonies indicates highly production of slim layer, red colonies indicates moderately production of slim layer, while light colonies means negative result.

**HiStaph Latex Test (Rapid slide agglutination test)**

The HiStaph Latex reagent was allowed to equilibrate to room temperature before it was used, then it was shake well, and one drop of the latex reagent was added into a circle of the test card, 2-3 average sized isolated colonies from a fresh overnight culture plate was picked up by a clean mixing stick and emulsified in the latex drop, the card was then rotated slowly and read within 1 minute. A positive result was indicated by visible aggregation of the latex particles with a clear background.

In latex agglutination test (slide test), latex particles covered with fibrinogen and IgG were used. IgG antibodies bind with coagulase on *S.aureus*, which results in latex particles clumping in about 20 seconds. This slide test may be negative in a small percent; in this case a tube test, which detects both free and bound coagulase, has to be performed. About 97% of human *S. aureus* isolates possess both forms of coagulase.

**Results and discussion**

The collected milk and cheese samples were cultured on the agar medium Staph No.110; because of its constituent of sodium chloride (7.5%) Staph No.110 is a selective medium, so that only staphylococci and any bacteria which can tolerate this salt concentration will grow.

Out of 234 bacterial isolates grown on Staph No.110 Figure -1 were more purified by ABC streaking method, and then all of them were examined microscopically for gram stain ability, shape and cluster arrangement.

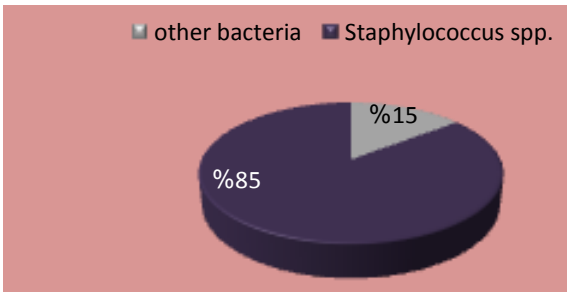


**Figure 1-** The percentage of *Staphylococcus spp*. isolated from milk and cheese samples

Two hundred colonies were identified morphologically as gram positive cocci arranged in grape-like irregular clusters this clusters occur because the bacterial cells divide in three planes in an irregular pattern producing branches of cocci which are characteristics to *Staphylococcus spp*. [9,19].

Table -1 illustrates the results of the biochemical tests applied to identify the staphylococcal isolates.

**Table 1-** The biochemical tests and their results for the staphylococcal isolates

| Biochemical tests | Results | | |
|---|---|---|---|
| | *S.aureus* | *S.chromogenes* | *S.epidermidis* |
| Gram stain | Positive | Positive | Positive |
| Motility | Negative | Negative | Negative |
| Oxidase | Negative | Negative | Negative |
| Catalase | Positive | Positive | Positive |
| Growth on high salt containing media | Positive | Positive | Positive |
| Nitrat Reduction | Positive | Positive | Positive (weak) |
| Acetoin production | Positive | Negative | Positive (50-80%) |
| Mannitol fermentation | Positive | Variable (Positive 11-89%) | Negative |
| Growth on BPA | Black shiny colonies surrounded by zone of clearing | Not shiny orange-brown may produce clearing | Not shiny black and seldom produces clearing |
| Staphyloxanthin production | Positive | Positive | Negative |
| DNase | Positive | Negative | Negative |
| Coagulase & Clumping factor | Positive | Negative | Negative |

All isolates gave the negative result of the **motility test** as well as the **oxidase tes**t; the latter was performed to differentiate *Staphylococcus* from *Micrococcus* genus that usually gives the positive result as a purple color [13, 20].

For further identification, the **catalase** test was performed for all the isolates and all of them produced catalase enzyme that differentiates *Staphylococcus* from the genus *Streptococcus* which gives negative result of the catalase test. The isolates were unique in growing on high salt medium containing (10%, 15% NaCl) [9,14].

Additionally **nitrate reduction** test was performed for further identification because *Staphylococcus spp*. often reduce nitrate to nitrite [13, 20]. Also some isolates showed the ability to produce **acetoin** and other did not [13, 20, 21].

All the isolates had the ability to grow on **mannitol salt agar** which considered selective and differential medium for the genus *Staphylococcus* [14]. Some isolates had the ability to ferment mannitol sugar and form large golden colonies surrounded by wide yellow zones and turned the color of the medium from pink to yellow, others mannitol non fermentor which appeared as small pink colonies as *S.epidermidis* or large deep yellow to deep orange colonies as *S. chromogenes* and in both cases no color change was observed in the medium [9] Figure -2.


**Figure 2-** Mannitol salt agar cultured with *Staphylococcus spp.* mannitol non fermentor colonies of *S.epidermidis* (to the left) and mannitol fermentor colonies of *S.aureus* (to the right)

All the isolates grown on **Baird-Parker Egg Yolk Tellurite Medium** which is selective and differential medium used for the isolation and identification of *Staphylococcus* species from foods, colonial morphology was detected according to [22] as shown in table -2

**Table 2-** Colony characteristics of typical organisms on Baird-Parker Egg Yolk Tellurite Medium [22]

| Organism | Growth | Colonial morphology |
|---|---|---|
| *Staphylococcus aureus* | Good | Dark grey-black shiny convex 1-1.5 mm diameter (18hrs) up to 3 mm (48hrs) narrow white entire margin surrounded by zone of clearing 2-5mm |
| *Staphylococcus epidermidis* | Variable | Not shiny black and seldom produces clearing |
| *Staphylococcus saprophyticus* | Variable | Irregular and may produce clearing. Wide opaque zones may be produced in 24 hrs |
| *Staphylococcus chromogenes* | Good | Not shiny orange-brown average size colonies, may produce clearing |
| *Micrococcus species* | Variable | Very small in shades of brown and black No clearing |
| *Bacillus species* | Variable | Dark brown matt with occasional clearing after 48hrs |
| *Escherichia coli* | Variable | Large brown-black |
| *Proteus species* | Variable | Brown-black with no clearing |
| *Yeasts* | Variable | White, no clearing |

Three types of colonies appeared: dark grey-black shiny convex 1-1.5 mm diameter (18hrs) up to 3 mm (48hrs) narrow white entire margin surrounded by zone of clearing 2-5mm colonies identified as *S. aureus* Figure -3, black not shiny colonies and seldom produces clearing as *S. epidermidis* and orange-brown not shiny average size colonies, may produce clearing as *S. chromogenes* [22].

**Figure 3-** Baird-Parker Egg Yolk Tellurite agar cultured with *S.aureus* which appeared as black shiny convex colonies with lipase activity (shown as hydrolyzed clear zone surrounding the colonies)

On **milk agar** plates a number of pigmented colonies appeared from white to deep orange, *S.epidermidis* appeared as white colonies, golden-yellow colonies expected to be *S.aureus*, while deep yellow and deep orange colonies expected to be *S. chromogenes* Figure -4.

**Figure 4-** Skim milk agar cultured with *S.aureus* which appeared as glistening orange convex colonies (due to Staphyloxanthin production) with protease activity (shown as hydrolyzed clear zone surrounding the colonies)

Among the best-recognized bacterial pigments are the carotenoids that impart the eponymous golden color to the major human pathogen, *S. aureus*. This organism produces multiple carotenoid pigments via a well described biosynthetic pathway that culminates with golden staphyloxanthin as the major product and yellow 4040-diaponeurosporene as a minor product. Staphyloxanthin also acts as a virulence factor. It has an antioxidant action that helps the microbe evade death by reactive oxygen species produced by the host immune system [23].

**DNase** production was detected by culturing the isolates on DNase agar, the appearance of yellow zone surrounding the colonies considered as a positive result. The results showed that 97 *S. aureus* isolates (100%) were DNase producers. Most of pathogenic isolates of *S.aureus* produce DNase enzyme. DNase degrades the host DNA and that increases the invasiveness and pathogenecity of staphylococci that possess it [9].

After isolates identification at the generic level, the **coagulase** test was performed to identify the bacterial isolates at the species level, ninety seven isolates (48.5%) showed the ability to produce coagulase enzyme (coagulase positive) and 103 isolates (51.5%) were coagulase negative as shown in figure -5, this result belong to the fact that coagulase enzyme is the most important identification agent that recognizes coagulase positive from coagulase negative Staphylococci. Coagulase is an important virulence factor; it's acting by deposit fibrin on the surface of Staphylococci, thereby altering their destruction inside the phagocytes.

**Clumping factor** is a surface *S. aureus* compound that is responsible for adherence of the organisms to fibrin and fibrinogen [9].

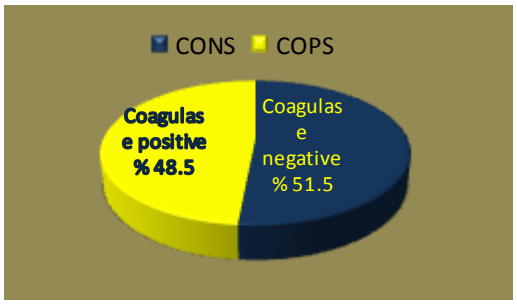


**Figure 5-** The percentage of each Coagulase-Positive and Cogulase-Negative among isolated *Staphylococcus spp.*

**API Staph system** was used to confirm the identification of the most prevalent *Staphylococcus spp*. isolated from milk and cheese samples *S.aureus* which previously identified by conventional biochemical tests. The test was applied only for five isolates, and the results obtained from API Staph system were in agreement with those obtained from biochemical identifications. Table -3 and figure-6.

**Table 3-** Results of the biochemical test for identification of Staphylococcus spp. by API Staph system

| **Biochemical Test** | ***S.aureus*** | ***S.chromogenes*** | ***S.epiderm-idis*** |
|---|---|---|---|
| Negative control 0 | - | - | - |
| Acidification of D-glucose GLU | + | + | + |
| Acidification of D-fructose FRU | + | + | + |
| Acidification of D-mannose MNE | + | + | + |
| Acidification of D-maltose MAL | + | + | + |
| Acidification of D-lactose LAC | + | + | + |
| Acidification of D-trehalose TRE | + | + | - |
| Acidification of D-mannitol MAN | + | - | - |
| Acidification of xylitol XLT | - | - | - |
| Acidification of D-melibiose MEL | - | - | - |
| Reduction of nitrate to nitrite NIT | + | + | + |
| Alkaline phosphatase PAL | + | + | + |
| Acetyl-methyl-carbinol production VP | - | - | - |
| Acidification of raffinose RAF | - | - | - |
| Acidification of xylose XYL | - | - | - |
| Acidification of sucrose SAC | + | + | + |
| Acidification of α-methyl-D-glucoside MDG | - | - | - |
| Acidification of N-acetyl glucoseamine NAG | + | + | - |
| Arginine dihydrolase ADH | + | + | + |
| Urease URE | + | - | - |

**Figure 6-** *S. aureus* results obtained by API Staph system

**Immunological method:**

**HiStaph Latex Test** is a rapid slide agglutination test was performed to all the coagulase positive Staphylococci which were previously identified by biochemical tests, 90 (92.8%) COPS isolates gave the positive result of the test which was indicated by visible aggregation of the latex particles with a clear background as shown in figure -7.

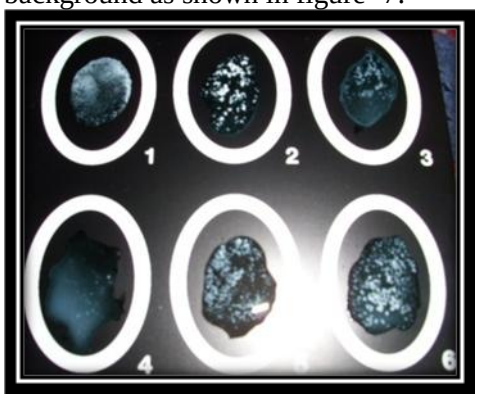


**Figure 7-** The results of HiStaph Latex Test (2 *S.aureus*; 4 *S.chromogenes*; 6 Control positive)

The results showed that 97 (48%) of the staphylococcal isolates isolated from milk and white cheese samples were *S.aureus*, followed by 82 (41%) *S.chromogenes* and 21 (11%) *S.epidermidis* Figure -8.

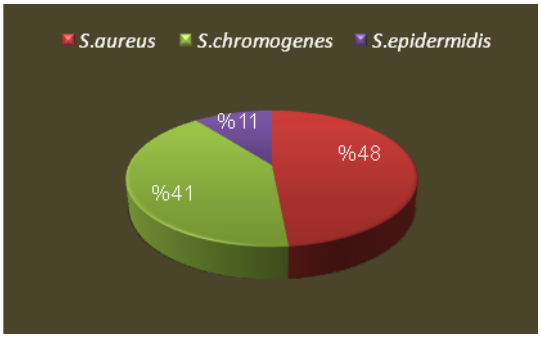


**Figure 8-** The prevalence of *Staphylococcus aureus* according to all the Staphylococcal isolates isolated from milk and cheese samples

Staphylococci are ubiquitous bacteria found on the skin and mucous membranes of humans and worm-blooded animals; they also can be

recovered from different environmental sources such as soil and water and from wide range of foodstuffs [7]. *S. aureus* is the causative agent of mastitis in lactating animals [24]. Other possible sources that contribute to high levels of *S. aureus* in milk are the improper hygiene and poor farm management [25].

Milk is a good substrate for *S. aureus* growth, and dairy products are common sources of intoxication [26]. *S. aureus* can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing raw milk [27].

Cheese is a complex and dynamic microbial ecosystem characterized by the presence of a large variety of bacteria, yeasts, and molds, some microorganisms including species of lactobacilli or lactococci, are known to contribute to the organoleptic quality of cheese, whereas the presence of other microorganisms may lead to spoilage or titute a health risk, *S. aureus* is recognized worldwide as an important food-borne pathogen, owing to the production of enterotoxins in food matrices [28].

Fooladi *et al*. found that 32% of the dairy products were contaminated by *S. aureus* [29].
Contamination of food products with *S. aureus* pathogens may result from their presence in the basic raw material - milk, this is of great importance especially in countries with large production of dairy products such as cheeses [30].

Algero *et al*. who analyzed 172 food samples including milk, soft cheese, hard cheese, ice cream, yoghurt, and fast food like sandwiches in a market in Brazil, reported that 26 samples (15.1%) of the food were contaminated with *S. aureus* [31].

*S. aureus* was found in 70.4% in raw and pasteurized bovine milk samples as demonstrated by [32].
Dastmalchi Saee *et al*. evaluated 370 cow milk samples, using culture and PCR methods. They reported that 58 samples (15.67%) were contaminated with coagulase-positive *S. aureus* [33].

The presence of this microorganism (*S. aureus*) after pasteurization can be attributed to inefficacy of the thermal process [32].
The CONS species most commonly isolated from mastitic milk were *S.chromogenes* and *S. simulans*. *S. chromogenes* was the major CONS species in subclinical mastitis, the majority of CONS isolates in milk samples in a research in USA, were *S. chromogenes* [34].

The predominant CONS species of 298 isolates in milk samples from subclinical mastitis in Germany were *S. chromogenes* (33%) and *S. simulans* (23%) [35]. In addition to the predominant species, various other CONS species are less frequently isolated from milk samples. *S. hyicus, S. epidermidis* and *S. haemolyticus* were detected in some samples [35, 36, 37, 38, 39, 40], but *S. warneri, S. sciuri* and *S. xylosus,* and several other CONS species usually only occur seldom [35, 38, 41, 42].

A wide variety of CONS species have been isolated from cows' skin, the predominant CONS species in cows' beddings and environment were reported to be *S.xylosus*, *S.sciuri*, and *S.saprophyticus* [43]. The same species are frequently isolated from cows' haircoat. The predominant species in milk and extramammary samples are mainly different, but *S.chromogenes* is an exception. It seems to be adapted both to conditions on the skin and in the udder. The only CONS species isolated both from mastitis and from milkers' hands were *S.chromogenes* and *S.succinus* subsp. *succinus.* The isolates from the hands were, however, of different pulsotypes than the mastitis isolates. Although we cannot exclude the possibility of elimination and re-infection with the same species, we assume that the CONS infections remained persistent in the udder. *S. simulans* is a more specific mastitis pathogen and adapted to udder conditions. In contrast, *S. chromogenes* is able to live on bovine skin, but can also infect the mammary gland and cause mastitis.

In the study of [44] *S.epidermidis* species occupied 14.3% of the staphylococcal spp. isolated from dairy products in Portugal.

*S.epidermidis* species was detected in food samples in a percentage of 40% in the research of [45].

This species is a common inhabitant is a common inhabitant of human skin and mucous membranes of individuals manipulating food and animals, and is able to contaminate raw products and processed foods [45, 46].

It was found that *S. aureus* as 54% in raw milk samples by [47], while [48] isolated *S. aureus* in 75% from bovine milk samples.
The numerous examples of *S. aurues* causing bacteremia were reported in human with predisposing conditions of dairy farms [2, 49]. The presence of *S. aurues* in the milk sample is

a new and appealing as well as an important finding of this study.

Regarding samples' type and the isolated staphylococcal species, there was a relationship between them as demonstrated in table -4.

**Table 4-** Relationship between the samples' type and the isolated staphylococcal species

| Samples' type | No. of the isolated species of staphylococci | | |
|---|---|---|---|
| | COPS | CONS | |
| | *S. aureus* | *S. chromogenes* | *S. epidermidis* |
| Milk | 23 | 57 | 9 |
| Cheese | 74 | 25 | 12 |

From milk samples *S. chromogenes* was the most prevalent isolated staphylococcal species; while cheese samples contaminated mainly by *S.aureus*.

Inadequate pasteurization of initially contaminated milk samples from animal skin or hair contributed in such high number of *S. chromogenes* in these samples, while food handling during cheese processing may be the source of *S. aureus* in these samples.

**Detection of Virulence Factors of Staphylococci**

The production of haemolysin, coagulase and clumping factor, DNase, lipase, protease, urease and gelatinase enzymes and slime layer production were detected in this study.

**Haemolysin:** Haemolysin production was detected by culturing the isolates on blood agar; positive results appeared as a haemolytic zone around the colonies. Seventy eight (80%) *S. aureus* isolates showed haemolytic activity and the lysis was β-haemolysin.

**Protease** production was detected on skim milk agar plates, casein is the predominant protein in milk, and its presence causes milk to have its characteristic white appearance, protease which hydrolyzes casein to produce more soluble peptides, transparent derivatives. The hydrolyzed clear zone around the colonies considered positive result, the results obtained by this study showed that most of the isolates were protease producers.

**Lipase** production was detected by culturing isolates on Baird Parker Egg Yolk Tellurite medium, the hydrolyzed clear zone surrounding the colonies considered as positive result (Figure 3). All the isolates were lipase producers, these enzymes acting by helping bacteria to invade host tissues throughout hydrolyzing cells membranes and spoiling foods such as cheese and milk.

**Urease** production was done on urea agar slants, changing the color of phenol red indicator from yellow to pink represents the positive result. Results showed that all *S.aureus* isolates were urease producers.

**Gelatinase** production test was done by stabbing the isolates in gelatinase medium, liquefaction of the medium is the positive result, results showed that all *S.aureus* isolates were gelatinase producers.

These results evidence on the ability of the staphylococcal isolates to produce a variety of enzymes that helps in invading the host's tissues as well as the foods texture (proteases, lipases and gelatinases).

**Slime layer production detection** for both COPS and isolates, was investigated by using Congo Red Agar method revealed slime production in 74.22% of COPS Figure -9, while CONS isolates did not produce it Figure -10.

**Figure 9-** Slime layer producing *S. aureus* colonies on the congo red agar


**Figure 10-** *S. chromogenes* colonies on the congo red agar (non slime layer producers)

All these results of the detection of some virulence factors produced by Staphylococcal isolates are illustrated in table -5.

**Table 5-** Virulence factors produced by Staphylococcal isolates

| Virulence factor | *S. aureus* | *S. chromogenes* | *S. epidermidis* |
|---|---|---|---|
| Protease | Positive (100%) | Positive (95%) | Positive (78%) |
| Urease | Positive (100%) | Variable | Positive (78%) |
| Lipase | Positive (100%) | Positive (68%) | Positive (100%) |
| Gelatinase | Positive (100%) | Positive (62%) | Negative |
| Haemolysin | Positive (80%)β type | Negative | Negative |
| Slime layer production | Positive (74.2%) | Negative | Negative |

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