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Full Length Research Paper

Phenotypic and Genotypic Detection of Enterotoxigenic *Staphylococcus aureus* in Domestic Dairy Products in Port Said Governorate, Egypt.

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Staphylococcus aureus **is an important food- borne pathogen involved in a variety of invasive diseases. So the current study aimed to detect the frequency of contaminated dairy products by *S. aureus* and the presence of enterotoxins genes in the isolated *S. aureus* strains. One hundred samples of milk and milk products were collected randomly of different areas in Prot Said governorate. Thirty milk samples; twenty raw milk, six pasteurized. milk and four powdered milk. Thirty cheese samples; ten karish cheese, five damitta cheese, five rommy cheese, five cheedar cheese and five cooked cheese. Twenty Yoghurt samples; ten canned yoghurt pasteurized and ten hand-made yoghurt non-pasteurized. *Staphylococcus aureus* strains were isolated by cultivation on two selective media, Baird-Parker agar and Mannitol Salt agar . This study deteted no *S. aureus* growth at pasteurized milk , the organism in pasteurized milk by a percentage of (70%) and attributed that to inefficacy of the thermal process. The current study detected *S. aureus* in 50 % of the examined samples of cheese. *S. aureus* only in 10 % of the cheese samples Karish cheese was the highest in *S. aureus* contamination (70%) among different types included in the present study. Rommy and damitta cheese also show a high rate o contamination (60%). The least percentage was in cheedar and cooked cheese (20%) each. This may be due to unhygienic storage and handling of rommy and damaitta cheese while cooked and cheedar cheese were sold sealed. Yoghurt is the most popular type of fermented milk in Egypt, the present study detected *S. aureus* in 35% of all examined samples. Similar, *S. aureus* detected in 72% of yoghurt samples collected from different dairy shops and supermarkets, in Prot Said governorate. The present study detected *S.aureus* in 40% of the examined samples of both cream and butter, which came after milk (53.5%) and cheese (50%). Percentage of positive samples for *S. aureus* were higher in milk (53.3 %), followed by cheese (50 %), cream and butter (40 %) and lately yoghurt (35 %).Staphylococcal Enterotoxins are classified by serological criteria into types designated SEA to SEE and SEG to SEU and encoded by sea to see and seg to seu genes. One of the goals of this study is to determine the presence of enterotoxin genes among isolated strain of *S. aureus* from dairy Products. To achieve this goal, Multiplex PCR technique was applied on -*S. aureus* colonies from each positive sample isolated from culture on Baird- Parker agar. In the present study enterotoxin genes were detected in 19 isolated strains of *S. aureus* out of 46 strains positive for *S. aureus* in a percent of (41.3 %). This result is higher than that detected by enterotoxin gene in (31.1 %) in isolated *S. .aureus* colonies from milk and milk products. On the other hand SE detected gene in 37 % of raw milk**

samples, SEB is also incriminated in food poisoning. Staphylococcal enterotoxin (A) gene was highly reported (52,6%), while enterotoxin (B) gene was reported in (31.6%). Double enterotoxin genes (sea and seb) were reported in (15.8%). Regarding genotypic findings by PCR, sea gene was isolated with a higher frequency than seb gene as it was detected in 52.6% of all isolated enterotoxin genes, followed by seb (31.6%) and lastly (sea+seb) (15.8%). agarose gel electrophoresis for PCR showing of 270. bp sea gene and 165 bp seb gene. (Lane M) is the (100 bp ladder marker), (Lane 2) is for sea, (Lane 3) is for seb, (Lane 4) is for both genes (sea& seb).

Keywords: *Staphylococcus aureus,* Phenotypic and Genotypic, Domestic Dairy.

INTRODUCTION

Staphylococcus aureus is an important food-borne pathogen involved in a variety of invasive diseases. Of particular relevance is the ability of some *Staphylococcos aureus* strains to produce heat stable enterotoxins that cause staphylococcal food poisoning, which ranks as one of the most prevalent worldwide causes of gastroenteritis **(Boerema *et al*., 2006).**

Several studies have shown that 15% to 80% of the *Staphylococcus aureus* isolated from various sources (dairy products, ice cream, meat products) is able to produce enterotoxin **(Omoe *et al.,* 2005; Fueyo *et al.,* 2005 and Bania *et* al., 2006).**

Classically, enterotoxins from *Staphylococcus aureus* strains can be classified into 18 serological types: A-U (except S, F and T), most of these serotypes are heat stable **(Holtfreter *et al*., 2005).**

Staphylococcal enterotoxins (SE) A and B are two of the most important gastroenteritis causing agents. In some areas, more than 50% of food poisoning is caused by SEA. In USA and England *Staphylococcal* enterotoxins A & B are the most Food poisoning causing agents (> 60%) **(Kluytmans .,2005).**

Staphylococcus aureus nasal carriage is established constantly in 20%-40% of healthy human population and intermittently in 60% and only 10%-20% of people are non-carriers **(Ruimy *et al.,* 2010).**

If food providers don't abide by the rules of hygiene, they can transfer the contamination to food, A concentration of 10^5 bacteria/ gram in foods is sufficient for toxin production and induction of disease **(Paciorek *et al*., 2007).**

Staphylococcal enterotoxins are low molecular weight proteins (MW 26.900-29.600 KD). These are encoded by genes embedded in mobile genetic elements such as phages and pathogenicity islands **(Martin *et al*., 2004).**

There are several methods for detection of enterotoxigenic bacteria. The phenotypical methods (agglutination, SRID) are not reliable in specificity, because SE serotypes are antigenically similar **(Edwin *et al.,* 2011).**

On the other hand, commercial serologic kits can not detect all the serotypes and is limited to serotypes (A-E) **(Van Belkum *et* al., 2003)**.

Therefore, molecular techniques such as PCR and real-time- PCR are recommended for detection of *Staphylococcus aureus* enterotoxin genes **(Van Belkum *et al.,* 2003).**

In this study, genotypic method is utilized to detect *Staphylococcal* enterotoxins A and B genes. Furthermore, we used these methods to examine the contamination rate of traditional dairy products by *Staphylococcus aureus.*

MATERIALS AND METHODS

1-Sample collection.

One hundred samples of Milk and Milk products were collected randomly from different areas in Prot Said governorate as

II. Processing of samples : (Duncan *et al.,* 2004).

1 ml milk sample was added to 9 ml saline , or 10 ml gram of each dairy product sample was cutted by a steile knife and added to 90 ml saline, ,to be homogenized, the mixture was placed in
disposable, sterile polyethylene bag (Stomacher bag) to be inserted in the Stomacher machine.

III. Pacteriological identification:

Media used in cultivation:

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1- Cooked meat with 9% NaCl (Oxoid, UK)

Typical Formula*	gm/litre
Heart muscle	454.0
Peptone	10.0
'Lab-Lemco' powder	10.0
Sodium chloride	5.0
Glucose	2.0
pH 7.2 ± 0.2 at 25°C	

2- Baird Parker agar with Egg Yolk Emulsion (Oxoid, UK)
a- Baird-Parker agar

Typical Formula*	gm/litre
Tryptone	10.0
'Lab-Lemco' powder	5.0
Yeast extract	1.0
Sodium pyruvate	10.0
Glycine	12.0
Lithium chloride	5.0
Agar	20.0
pH 6.8±0.2at25°C	

b- Egg Yolk Tellurite Emulsion (Oxoid, UK)
Sterile egg-yolk 200 ml; NaCl 4.25 g; potassium tellurite 2.1 g; distilled water to give a final volume of 1000 ml.
3- Mannitol salt agar(OxoidUK)

Typical Formula*	gm/litre
'Lab-Lemco' powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0
pH 7.5 ± 0.2 at 25°C	

IV. Identification of the suspected colonies:
1- A smear from the suspected colonies was stained by Gram stain and examined under microscope.
2- Catalase reagent (Barry *et al.,* 2013)
3- Tube coagulase test
V . Mainteenance of the selected isolates
VI. Multiplex PCR for detection of sea & seb genes:
A) DNA extraction:
1- DNA extraction kit: i-genomic BYF DNA Extraction Mini Kit, (Cat. No. 17361) (**iNtRON Biotechnology, Korea).**
2- Additional required equipment and reagent:

B) DNA amplification:
Material for DNA amplification:
1- PCR Premix: 2x PCR Master mix Solution (i-Taq™) tubes (Cat. No. 25027 "1ml", 25028 "5 ml") **(iNtRON Biotechnology, Korea).**
C) DNA detection by gel electrophoresis;

Interpretation:
**The gel was examined and photographed under ultraviolet light as ethidium bromide intercalates between the bases of the DNA and fluoresces.
** Molecular size marker gave 11 bands ranging from 100-1500 base pairs (bp). VII.

Statistical analysis of the results:

The results were calculated, tabulated and statistically analyzed. The collected data were entered, checked and analyzed using chi square (x^2) according to knapp **and Odds ratio** Using statistical computer program SPSS version II under windows 7 as follow:

Chi square x 2:

Used to compare between more than 2 percentages and determine whether there is a significant difference between the expected frequencies and the observed frequencies in one or more categories.

$$x^2 = \sum \frac{(O-E)^2}{E}$$

RESULTS

The present work was carried out on 100 samples of milk and milk products collected randomly from different areas in Prot Said Governorate.

Milk samples were 30; they were 20 samples of raw milk, 6 samples of pasteurized milk and 4 samples of powdered milk.

Cheese samples were 30; they were 10 samples of karish cheese, 5 samples of damitta cheese, 5 samples of rommy cheese, 5 samples of cheedar cheese and 5 samples of cooked cheese.

Yoghurt samples were 20; they were 10 samples of canned yoghurt and 10 samples of hand-made yoghurt.

Also 10 samples of cream and 10 samples of butter were examined.

Figure (10): showing agarose gel electrophoresis for PCR of 270. bp sea gene and 165 bp seb gene. (Lane M) is the (100 bp ladder marker), (Lane 2) is for sea, (Lane 3) is for seb, (Lane 4) is for both genes (sea & seb).

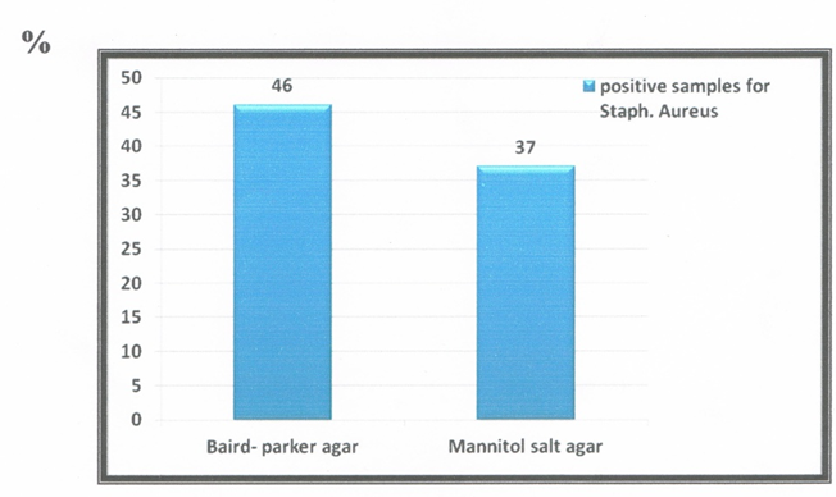


Figure (1): Comparison between media used in isolation of *Staphylococcus*

Table (2):-positive samples for *Staphylococcus aureus* in milk and milk products.

Sample type	No. of samples	Positive samples No. (%)	Negative samples No. (%)	Odds ratio 95%CI	X2	P. value
Milk	30	16 (53.3)	14 (46.6)	1.31 (0.42-4.09)	0.27	0.606
Cheese	30	15 (50.0)	15 (50.0)	1 (0.32-3.11)	0 . 0	1.0
Cream	10	4 (40.0)	6 (60.0)	0.44 (0.05-3.7)	0.8	0.371
Butter	10	4 (40.0)	6 (60.0)	0.44 (0.05-3.7)	0.8	0.371
Yogurt	20	7 (35.0)	13 (65.0)	0.29 (0.06-1.27)	3.6	0.06
Total	100	46 (46.0)	54 (54.0)			
X2		2.11				
P. value		0.716				

Sample type	No. of samples	Positive samples No. (%)	Negative samples No. (%)	Odds ratio 95%CI	X2	P. value
Raw milk	20	16 (80.0)	4 (20.0)	16 (2.78-108.85)	14.4	<0.001**
Pasteurized milk	6	0 (0.0)	6 (100.0)	0.0	1 2	<0.001**
Powdered milk	4	0 (0.0)	4 (100.0)	0.0	8	0.004*
Total	30	16 (53.3)	14 (46.7)			
X2		17.14				
P. value		<0.001**				

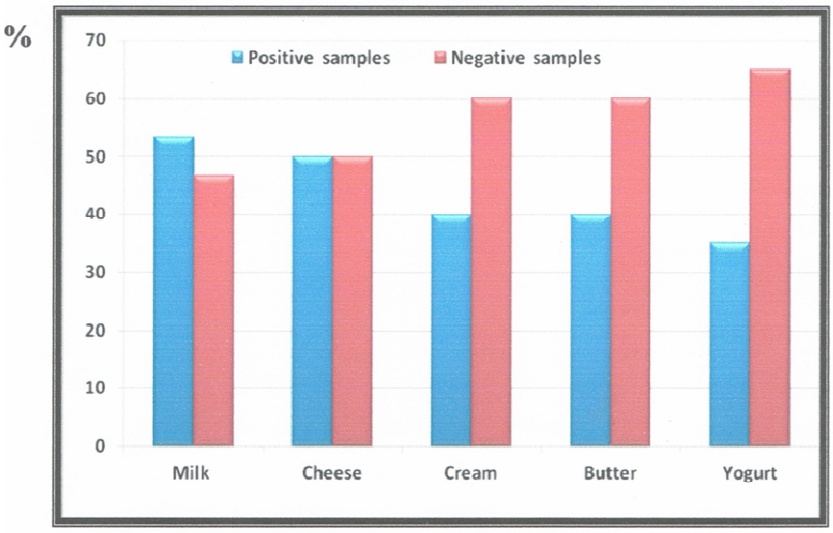
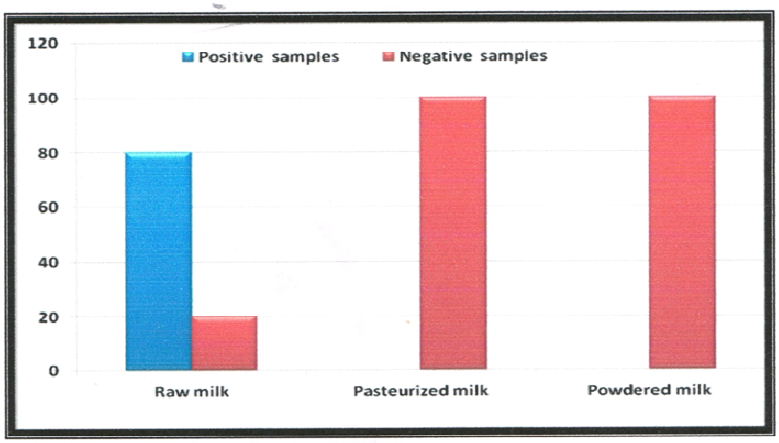
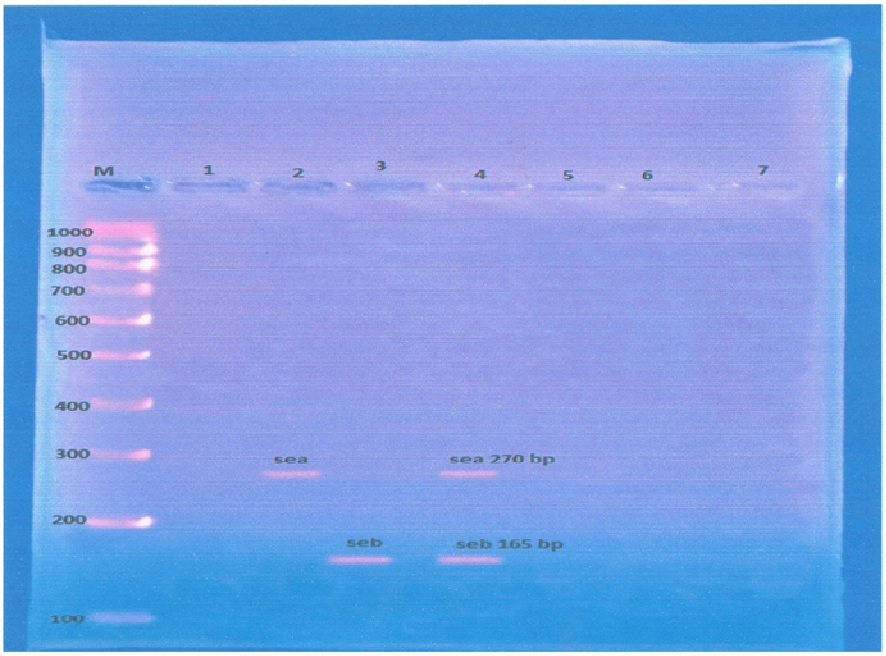


Figure (2): Frequency of *Staphylococcus aureus* in milk and milk products.

Figure (3): Frequency of *Staphylococcus aureus* in different types of milk.





DISCUSSION

Staphylococcus aureus is one of the most important pathogen in food poisoning, due to its wide spread and ability of many strains to synthesize one or more enterotoxin. It causes gastroenteritis symptoms-like nausea, vomiting, abdominal cramps and diarrhea **(Scherrer *et al.,* 2004 & Normanno *et al.,* 2007).**

Among food stuffs implicated in food poisoning; milk, dairy products, meat, poultry and eggs, specially handled food, play an important role since enterotoxigenic strains have been frequently isolated from food handelars **(De Buyser *et al.,* 2001 & Normanno *et al.,* 2005).**

Staphylococcus aureus strains were isolated by cultivation on two selective media, Baird-Parker agar and Mannitol Salt agar .

Better results were obtained by Baird-Parker agar. 46%.of samples were positive by Baird-Parker agar versus 37% by Mannitol: Salt agar, This, was attributed to the enrichment of Baird-Parker medium by selective agents; glycine, lithium chloride and potassium tellurite. These results agreed with Niskanen and Aalto (1978) who .detected Staphylococcus growth on Baird-Parker agar in 83 % of samples versus 59.4% by Mannitol Salt agar, on the other hand **Alkhafaji (2013)** detected equal growth of *S. aureus* on both media.

The percentage of samples of milk and milk products contaminated with *S. aurues* in the current study were 46% which is higher than that reported by **Normanno *et al.,* (2007) and Imanifooladi *et al.,* (2010)** who detected *S. aureus* in 17 % and 32 % respectively of the analyzed samples of milk and milk products. On the other hand **Alkhafaji (2013)** reported higher percentage (48%) of samples contaminated by *S. aureus.*

In our study the main bulk of samples were from raw milk and raw milk products like karish cheese and hand-made yoghurt which also could be a cause of our high result.

Regarding contamination of different types of milk, the/current study revealed the organism in 80% of examined samples of raw. Milk while pasteurized and powdered milk show no growth. The result was statistically significant ($P < 0.001$). This result is greatly higher than mat- obtained by **El-Jakee *et al.,* (2010)** who detected *S. aureus* in 106 out of 554 collected milk samples in percentage of 19.13%. Also lower results were reported by **Badia (2004), El-Gabry (2006**) **and Thaker *et al.,* (2013)** who detected *S. aureus* in raw milk at rate of 27.21%, 21.2% and 6% respectively. This low percentage is attributed to that the researchers followed strict hygienic conditions during milking, handling and storage of milk samples while ours were collected without any precautions to.test as they are already sold. Rommy and damitta cheese also show a high rate to contamination (60%). The least percentage was in cheedar and cooked cheese (20%) each. This may be due to unhygienic storage and handling of rommy and damaitta cheese while cooked and cheedar cheese were sold sealed.

The present study detected *S. aureus* in 35% of all examined samples. Canned yoghurt was *S. aureus* free after cultivation on Baird-Parker agar whileseven out of ten (70%) of the examined hand-made samples were contaminated.

The present study detected *S.aureus* in 40% of the examined samples of both cream and butter, which came

after milk (53.5%) and cheese (50%). This result differs from that reported by **Imanifooladi *et al.,* (2010),** who found also the highest contamination rate in cream (18%) and attributed that to excessive manipulation of cream .

Detection of SE gene does not indicate presence of biologically active molecules or production of enough amount of toxin to cause. disease. But it allows the determination of potentially enterotoxigenic strains of *S. aureus* **(Ercolini *et al.,* 2004).**

SEA is most commonly reported in food stuffs. It .is also considered as the main cause of Staphylococcal food poisoning, probably due to its extraordinary high resistance to proteolytic enzymes **(Balaban and Rasoolly, 2009 and Argudin *et al.,* 2010).**

SEB is also incriminated in food poisoning besides it has'been, studied for potential use as an inhaled bioweapon causing respiratory distress **(Ler *et al.,* 2006).**

Regarding genotypic findings by PCR, sea gene was isolated with a higher frequency than seb gene as it was detected in 52.6% of all isolated enterotoxin genes, followed by seb (31.6%) and lastly (sea+seb) (15.8%).

Our results were in agreement with **Imanifooladi *et al.,* (2010)** as they detected higher sea gene (50%) than seb gene (30%) in all isolated enterotoxigenic S. aureus strains in milk and milk products.

Also **Normanno *et al.,* (2007)** found sea gene (18.4%) was higher than seb gene (6.4%). Similarly El-Jakee *et al.* (2010) found that sea gene was higher than seb gene in a ratio of (17.9%) to (14.2%).

While *S. aureus* organism is heat labile, its produced enterotoxin is heat stable. Hence the importance of Multiplex PCR technique in detecting genes encoding enterotoxigenic strains especially in food poisoning outbreak.

CONCLUSION

In conclusion Sporadic presence of potentially staphylococcal enterotoxin producing strains in raw and raw milk products represents a health risk to onsumers. Bacteriological detection of S. aureus is better by Baird-Parker agar medium. Raw milk and raw milk products were found to be highly contaminated. Strains produce enterotoxin (A) gene was found in large extent than those produce enterotoxin (B) gene.

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