Assiut University web-site: www.aun.edu.eg

BACTERIOLOGICAL ASSESSMENT OF RAW MILK AND YOGHURT FOR PRESENCE OF STAPHYLOCOCCUS AUREUS WITH SPECIAL REFERENCE TO ITS ENTEROTOXINS

M.F. HUSSIEN[1] and A.M. KOREIM[2]
[1] Food Hygiene Department, Animal Health Research Institute, Assiut Lab.
[2] Bacteriology Department, Animal Health Research Institute, Assiut Lab.

Received: 13 December 2015; **Accepted:** 10 January 2016

ABSTRACT

A total of 150 of raw milk and yoghurt samples (75 of each) were obtained from different small daries and supermarkets in Assiut city, Egypt and were examind for presence of *Staph aureus*. The isolated strains were examined for the production of Staphylococcal enterotoxins (SEs) using the enzyme - linked immunosorbant assay (ELISA) method. 39(52%) out of 75 examind raw milk samples were positive for *Staph aureus* while *the organism* failed to detect in yoghurt. Out of 39 *Staph aureus* isolates 12 (30.8%) were positive for at least 1SE, out of which 5(41.7%) were positive for SEA 3(25%) for SEC, 2(16.7%) for SED. Two isolates of *Staph aureus* are able to produce more than one type of enterotoxin as one strain produce SEA&C and another produce C&D, both in percentage of 8.3%. It could concluded that examind milk samples were contaminated with enterotoxigenic strains of *Staph. aureus* and the public health hazards of SEs were also discussed.

Key words: *Bacteriological Assessment, Raw Milk, Yoghurt, Staphylococcus Aureus*.

INTRODUCTION

Milk has long been referred to as the most perfect food for human consumption from birth to senility; it contains all the nutrients required for rapid growth and healthy development of the body. Milk and its products have been shown to be an ideal media for growth and multiplication of many microorganisms; including Staphylococcal food poisoning (Bone *et al*., 1989 and Wieneke *et al*., 1993). *Staphylococcus aureus* contamination can occur through its presence in raw milk itself or during its processing (Le Loir *et al*., 2003).

Yoghurt is the most popular type of fermented milk in Egypt, the nutritive value of yoghurt is attributed to the fat content, sugar and casein. Therefore, yoghurt is recommended for sick and convalescent people. It inhibits the bacterial flora of intestine which may lead to constipation, autointoxication and colitis as well as it helps in the absorbtion of calcium and phosphorous.

Staphylococcus aureus infection was estimated to be present in up to 90% of dairy farms and is responsible for 35% of the economic loss in the dairy industry (Bone *et al*., 1989). Moreover Staphylococcus aureus is facultative anaerobic Gram positive bacterium, the majority of *Staph. aureus* strains are catalse and coagulase positive which forms the basis of traditional identification methodology.

Corresponding author: Dr. M.F. HUSSIEN
E-mail address: Mahmoutfarghaly_1970@yahoo.com
Present address: Food Hygiene Department, Animal Health Research Institute, Assiut Lab.

Staphylococcus aureus is a leading cause of food poisoning resulting from the consumption of contaminated food with staphylococcal enterotoxins. Exactly, *Staph. aureus* is considered the world's third most important cause of food-born illnesses due to its ability to graw and produce enterotoxins (SEs) under a wide range of conditions.

The pathogenicity of *Staph. aureus* and its ability to cause diseases is attributed to a number of virulence factors such as the heat stable enterotoxins (Sande and Mckilli, 2004). Staph. aureus produces 15 enteotoxins (Atichou *et al*., 2004). The five classical enterotoxins (SEs type A, B, C, D and E) were known to be responsible for 95% of Staphylococcal food poisoning (SFP) cases, the rest of cases were due to the new types of SEs (SE G – SE O) (Wang *et al*., 2012). However, the role of newly identified enterotoxins in food poisoning is not fully clarified, and the development of methods for the detection of these novel SE genes is of critical importance for food poisoning investigations.

The staphylococcal food poisning (SEP) is a mild intoxication occurring after the ingestion of food containing from 20 ng up to1 ug of Staphylococal enterotoxins (SEs) which is enough to induce symptoms in human beings (Nermano *et al*., 2007). The SFP symptoms appear 1-6 h after ingestion of contaminated food, depending on individual and toxic dose ingested. They include nauseas, abdominal cramps, diarrhea, general malaise, weakness and characteristic projectile vomiting. Clinical signs of SFP generally disappear within 24-48 h. Deaths occur

rarely and specifically in the very young or elderly (Jay *et al.*, 2005).

Enterotoxins are highly thermostable, normal cooking and pasteurization cannot totally inactivate them leading to food poisoning (Nagarajappa *et al*., 2012). Pasteurization kills *Staph. aureus* cells, and fermentation or ripening of cheese may prevent growth of *Staph. aureus* in raw milk cheese. However, once formed the thermostable SEs generally retain their biological activity (Le Loir *et al*., 2003 and Becker *et al*., 2007). Although SEs a generally heat resistant and a heat denatured enterotoxin, it can be renatured by prolonged storage or in the presence of urea. The SEs also are resist most proteolytic enzymes such as pepsin or trypsin thus keep their activity in the digestive tract after ingestion and are capable of causing food poisoning (Lee Loir *et al*., 2003).

Because of the importance of these toxins in the public health and food sectors, an efficient screening methods to detect the prevalence of Staphylococcal enterotoxins (SEs) in food are required. The present work was planned to investigate the prevalence of *Staph. aureus* in raw milk and yoghurt samples and identification of enterotoxigenic *Staph. aureus* isolates using ELISA technique.

MATERIALS AND METHODS

A total of 150 random raw milk and yoghurt samples (75 of each) were collected from small dairies and supermarkets in Assiut city, Egypt. The samples were collected in clean, sterile plastic bags as marketed to the consumers. The samples were transferred in an ice box as rapidly as possible to the laboratory were subjected to microbilogical examination:-

I- Isolation of *Staphylococcus aureus*: according to (**FDA, 2001**).

A- Isolation.

Samples were inoculated on Baird-Parker agar plates and incubated at 35 ^{0}C for 48h. The typical appearance of *Staph. aureus* colonies are circular, smooth, convex, moist, 2-3 mm in diameter, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone. Inoculated typical colonies on TSA slants were used for maintenance and further identification.

B- Identification.

1- Microscopical appearance.

2- Catalase test.

Growth from TSA slant was used for catalase test on glass slide or spot plate, and illuminated properly to observe production of gas bubbles.

3- Coagulase test.

Suspected *Staph. aureus* colonies were inoculated into small tubes containing 0.2-0.3 ml BHI broth and emulsified thoroughly and incubated at 35 ^{0}C for 18-24h then 0.5 ml of reconstituted coagulase plasma with EDTA was added to the BHI culture and mixed thoroughly. The tube was incubated at 35°C and examined periodically over 6 h period for clot formation.

4- Anaerobic utilization of glucose

Tube of carbohydrate fermentation medium containing glucose (0.5%) was inoculated with suspected organism. The tube surface covered with sterile paraffin oil at least 25 mm thick and incubated for 5 days at 37°C. Acid is produced anaerobically if indicator changes to yellow throughout tube, indicating presence of *S. aureus*.

5- Lysostaphin sensitivity.

Isolated colony transferred from agar plate with inoculating loop to 0.2 ml phosphate-saline buffer, and emulsified. Half of suspended cells was transferred to another tube (13 x 100 mm) and mixed with 0.1 ml phosphate saline buffer as control. Add 0.1 ml lysostaphin (dissolved in 0.02 M phosphate-saline buffer containing 1% NaCl) to original tube for concentration of 25 µg lysostaphin/ml. Both tubes were incubated at 35°C for not more than 2 h. If turbidity clears in test mixture, test is considered positive. If clearing has not occurred in 2 h, test is negative. *Staph. aureus* is generally positive.

II- Determination of *Staphylococcus aureus* enterotoxins: *Staph. aureus* isolates (39 strains) from raw milk samples were performed in Food Analysis Center, Faculty of Veterinary Medicine, Benha Univirsity, Egypt, by using ELISA technique according to (Ewald, 1988) as following:-

Accurately, RIDASCREEN set C (Art No.: R4101, R-Biopharm AG, Darmstadt, Germany) is an enzyme immunoassay for the determination of *Staph. aureus* enterotoxins by using their definite kits.

According to the test kit manual, a loopful of the culture was mixed in sterile buffer saline and then shaken for 15 minutes. After centrifugation for 10 minutes at 3500 r.p.m, sterile filtration of the supernatant was applied. An aliquot (100 µl per kit well) of this solution was used in the test. Further, the last well was represented as positive control. They were mixed gently and incubated for one hour at room temperature (20-25°C) in the dark.

The liquid was dumped out of the wells into a sink to remove all of the remaining liquid from the wells. Therefore, the wells were then filled with 250 µl of washing buffer and the liquid was poured out again. The washing step was repeated 3 more times to remove the unbound conjugate.

Subsequently, 100 µl of enzyme conjugate were added to each well and incubated for one hour at room temperature in the dark after mixing gently. The liquid was dumped out of the wells into a sink and the wells were each filled with 250 µl of the washing buffer. The liquid was poured out again and the wells were emptied to remove all of the remaining liquid. The washing step was repeated 3 more times again.

Afterwards, 50 µl of substrate and 250 µl of chromogen solutions were added to each well. The solutions were mixed gently and incubated for 30 minutes at room temperature in the dark. Finally, 100 µl of the stop solution (1M H2 SO4) were added to each well with gentler mixing.

By using ELISA, the absorbance was measured at 450 nm in an ELISA plate reader (ELX800, BioTek Instruments, Bad Friedrichshall, Germany). The results were calculated from standard curve.

RESULTS

Table 1: Incidence of *Staphylococcus aureus* in the examined raw milk and yoghurt samples.

Type of samples	No. of samples	Positive		Negative	
		No.	%	No.	%
Raw milk	75	39	52	36	48
Yoghurt	75	0	0	75	100

Table 2: The distribution of *Staphylococcus aureus* enterotoxins recovered from raw milk samples.

No. of tested isolates	Positive isolates for SEs		Types and frequency of enterotoxins									
	No.	%	A	%	C	%	D	%	A & C	%	C & D	%
39	12	30.8	5	41.7	3	25	2	16.7	1	8.3	1	8.3

DISCUSSION

The results illusterated in Table (1) revealed that, 52% of the examined raw milk samples were contaminated with *Staphylococcus aureus*. Nearly similar results 52 and 56.66 were reported by Pourhassan and Taravat, (2011) and EL-Jakee *et al.* (2013), respectively. However, higher results were recorded by Al-Tarazi *et al.* (2003); Ekici *et al*. (2004) and Oliveira *et al.* (2011) in percentages of 80, 75 and 58%, respectively. While, lower results were estimated by Yagoub *et al.* (2005); Singh *et al.* (2011); Khuder *et al*. (2012); Meshref (2013); Thaker *et al*. (2013) and Vahedi *et al*. (2013).

Presence of *Staphylococus aureus* in raw milk cold be attributed to many causes such as subclinical mastitis, where *Staph. aureus* was the most isolated bacteria or due to contamination of raw milk by milkers and milk handlers. Also, inadequate refrigeration and long storage of milk before use help growth of *Staph. aureus* (Asperger, 1994). In addition, negligence of hygienic condition such as improper cleaning of bulk tank, dirty udder, milk equipments, milk handling techniques and improper storage will increase the proportion of Gram-positive and Gram-negative bacteria in the bulk tank milk (Bonfoh *et al.*, 2003).

Concerning examined yoghurt samples, *Staph. aureus* couldn't be detected in the present study. Similar results were obtained by Rodriguez *et al*. (1990); El-Bessery (2001) and Isam *et al.* (2011). The failure of *Staph. aureus* detection in this study may be due to pH of yoghurt, lactic acid and inhibitory substances produced by yoghurt culture which reduce or inhibit *Staph. aureus* growth in yoghurt (Pazakova *et al*., 1997). Moreover, such organisms was inhibited after few days during storage of fermented products (Estrada *et al*., 1999).

Table (2) illusterated the distribution of *Staph. aureus* enterotoxins recovered from raw milk samples by using ELISA technique, where the ability to

synthesize enterotoxins was found in 12 (30.8%) out of 39 isolates of *Staph. aureus*.

Five isolate (41.7%) produce SEA, three isolate (25%) produce SEC, two isolates (16.7%) produce SED, one isolate produce SE A&C and another isolate produce SE C&D, both in percentage of 8.3%.

Of the twelve Enterotoxigenic positive isolates of Staph. aureus, two isolates only are able to produce more than one enterotoxins (one produce A & C and another produce C & D), while, all the remaining 10 isolates were able to produce one enterotoxin only.

These findings were nearly similar to that detected by Nermano *et al*. (2007) and Rahimi *et al*. (2012) who detected similar findings of enterotoxin A. However, some of the present finding differ from those reported by other researchers in other countries, such studies have indicated that SEC was the most predominant type (Stephen *et al*., 2001; Jorgensen *et al*., 2001; Katsuda *et al*., 2005 and Loncarevic *et al.*, 2005).

Staphylococcal enterotoxins SEA and SEB are the most important gastroenteritis causing agents. In some areas, more than 50% of food poisoning is caused by SEA and SEB (> 60%) in USA and England (Kluytman and Werthein 2005). SEA is the most common enterotoxin recovered from food-poisoning outbreak in US (77.8%) followed by SED (37.5%) and SEB (10%) (Rall *et al*., 2008).

In conclusion *Staph. aureus* producing enterotoxins could be isolated from examind raw milk samples, so most official regulations should stricktly require the absence of *Staph. aureus* and its enterotoxins in milk and dairy products which could be highlighted Using ELISA technique**.**

REFERENCES

Al-Tarazi, Y.; Al-Zamil, A.; Shaltout, F. and Abdel-Samei, H. (2003): Sanitary status of raw cow milk marketed in northern Jordan. Assiut Vet. Med. J., 49 (96): 180-194.

Asperger, H. (1994): The significance of pathogenic microorganisms in raw milk. International Dairy Federation (IDF). Document No. 292. Belgium.

Atichou, M.; Henkens, R.; Sultana, A.; Ulrich, R. and Ibrahim, M. (2004): Detection of *Staphylococcus aureus* enterotoxin A and B genes with PCR-ELISA and hand-hand electrochemical sensor. Mo. Cell Probes, 18: 373-377.

Becker, H.; Bûrk, C. and Mârtlbaure (2007): Staphylokokhen-Enterotoxin: Bildung. Eigen Schaften und Nachweis. J. Verbr Lebensm, 2: 171-189.

Bone, F.J.; Bogie, D. and Morgan, S.C. (1989): Staphylococcal food poisoning from sheep mil cheese. Epidemiology Infect., 103: 449-458.

Bonfoh, B.; Wasem, A.; Traore, A.; Fane, A.; Spillmann, H.; Simbe, C.; Alfaroukh, I.; Nicolet, J.; Farah, Z. and Zinsstag, J. (2003): Microbiological quality of cow's milk taken at different intervals from the udder to the selling point in Bamako (Mali). Food Control, 14: 495-500.

Ekici, K.; Bozkurt, H. and Isleyici, O. (2004): Isolation of some pathogens from raw milk of different milch animals. Pakistan J. Nutrition, 3 (3): 161-162.

El-Bessery, M.M. (2001): Microbiological quality of market fermented milks in Assiut city. M.V.Sc. Thesis, Fac. Vet. Med., Assiut Univ., Egypt.

El–Jakee, j; Marouf, S.A.; Ata, Nagwa,; Eman H.; Abdel-Rahmam, Sherein, I.; Abd-El-Moez; Samy, A.A. and EL-Sayed, Walaa, E. (2013): Method for detecting of *Staphylococcus aureus* Enterotoxins in Food. Global Veterinaria, 11(3): 335-341.

Estrada, Z.A.; Mendoza, S.M.; La Garza, M.L. and Ferado, J. (1999): Behavior of enterotoxigenic strains of *Staphylococcus aureus* in milk fermented with yoghurt starter culture. Rev. Latinoam Microbiol., 41 (1): 5-10.

Ewald, S. (1988): Evaluation of enzyme-linked immunosorbant assay (ELISA) for detection of staphylococcal enterotoxins in foods. Int. J. Food. Microbiol., 6 (2): 141-153.

F.D.A. (2001): Food and Drug Administration (Bacteriological Analytical Manual). Staphylococcus aureus. Chapter: 12, January 2001.http://www.fda.gov/Food/ScienceResearch/ LaboratoryMethods/ Bacteriological AnalyticalManualBAM/UCM071429.

Isam, A.M.; Eshraga, A.E.; Abu-Elgasim, A.Y and Efadil, E.B. (2011): Physicochemical, microbiological and sensory characteristics of yoghurt produced from camel milk during storage. Electronic J. Envi. Agricult. Food Chemistry, 10 (6): 2305-2313.

Jay, M.J.; Loessner, J.M; and Golden, A.D. (2005): Staphylococcal gastroenteritis. In: Modern Food Microbiology. 7th edition. Springer

Jorgensen, H.J.; Mark, T.; Hogasen, H.R. and Rovik, L.M. (2001): Implication of milk and milk products in food-borne diseases in France and in different industrized countries. Int. J. Food Microbiol., 67: 1-17.

Katsuda, K.; Hatab, E.; Kobayshi, H.; Kohmoto, M.; Kawshima, K.; Tsunemitsu, H. and Eguchi, M. (2005): Moleculer typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxigenes and coagulase genes polymorphisms. Vet. Microbiol., 105: 301-305.

Khuder, M.H.; Abbas, B.A. and Idbeis, H.I. (2012): Detection of enterotoxin genes of

Staphylococcus aureus isolates from raw milk. Basrah J. Vet. Res., 11 (1):254-264.

Kluytman, J.A.J.W. and Werthein, H.F.L. (2005): Nasal carriage of *Staphylococus aureus* and prevention of nosocomial infections. Infection, 33: 3-7.

Le Loir, Y.; Baron, F. and Gautier, M. (2003): Staphylococcus aureus and food poisoning. Genet. Mol. Res., 2: 63-76.

Loncarevic, S.; Jorgensen, H.; Lovseth, A.; Mathiser, T. and Rorvik, L.M. (2005): Diversity of *Staphylococcus aureus* enterotoxin types within single samples of raw milk and raw milk products. J. Appl. Microbiol., 98: 344-350.

Meshref, A.M.S. (2013): Bacteriological quality and safety of raw cow's milk and fresh cream. Slov. Vet. Res., 50 (1): 21-30.

Microbiological quality of cow's milk taken at different intervals from the udder to the selling point in Bamako (Mali). Food Control, 14: 495-500.

Nagarajappa, S.; Thakur, M.S. and Manonmani, H.K. (2012): Detection of enterotoxigenic Staphylococci by Loop-mediated isothermal amplification method. J. Food Safety, 32: 59-65.

Nermano, T.G.; La Salandra, G.; Dambrosia, A.; Quaglia, N.C.; Corrent, M.; Paris, A.; Santagada, G.; Firina, A.; Crisetti, E. and Celano, G.V. (2007): Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* strains isolated from meat and dairy products. Int. J. Food. Microbiol., 115: 290-296.

Oliveira, Lílian, P.; Barros, Ludmilla, S.; Silva, V.C. and Cirqueira, Marina, C. (2011): Study of *Staphylococcus aureus* in raw and pasteurized milk consumed in the Reconcavo area of the State of Bahia, Brazil. J Food Process Technol., 2 (6): 1-6.

Pazakova, J.; Turek, P. and Laciakova, A. (1997): The survival of *Staphylococcus aureus* during the fermentation and storage of yoghurt. J. Appl. Microbiol., 82: 659-662.

Pourhassan, M. and Taravat, N. (2011): The Spatial Distribution of Bacteria Pathogens in Raw Milk Consumption on Malayer City, Iran. Shiraz E-Medical J., 12 (1): 1-9. Available at http://semj.sums.ac.ir/vol12/jan2011/88047.htm.

Rahimi, E.; Mommtaz, H.; Shakerian, A. and Kavyani, H. (2012): The detection of classical enterotoxins of *Staphylococcus aureus* in raw cow milk using the ELISA method. Turk. J. Vet. Anim. Sci., 36 (3): 319-322.

Rall, V.L.; Vieira, F.P.; Rall, R.; Vieitis, R.L.; Fernandes, A.J.R.; Candeias, J.M.; Cardoso, K.F. and Araujo, J.P.J. (2008): PCR detection of Staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. Vet. Microbiol., 10: 408-413.

Rodriguez, A.C.; Pintor, D.M.; Gonzalez, P.A.; Alvarez, M.R. and Hardisson, D.A. (1990): Microorganisms of Food hygiene interest in commercial yoghurts in the Canary islands. Alimentaria, 212: 55-58.

Sande, M. and Mckilli, P. (2004): Virulence and recovery of *Staphylococcus aureus* relevant to food using improvement traditional approaches. Food Control, 15: 5-10.

Singh, V.; Kaushal, S.; Tyagi, A. and Sharma, P. (2011): Screening of bacteria responsible for the spoilage of milk. J. Chem. Pharm. Res., 3(4): 348-350.

Stephen, R.; Annemuuler, C.; Hassan, A.A. and Lammler, C.H. (2001): Characterization of enterotoxogenic *Staphylococcus aureus* stains isolated from bovine mastitis in north-east Switzerland. Vet. Microbiol., 78: 373-382.

Thaker, H.C.; Brahmbhatt, M.N. and Nayak, J.B. (2013): Isolation and identification of *Staphylococcus aureus* from milk and milk products and their drug resistance patterns in Anand, Gujarat. Vet. World, 6 (1):10-13.

Vahedi, M.; Nasrolahei, M.; Sharif, M. and Mirabi, A. (2013): Bacteriological study of raw and unexpired pasteurized cow's milk collected at the dairy farms and super markets in Sari City in 2011. J. Prev. Med. Hyg., 54: 120-123.

Wang, X.; Meng, J.; Zhang, T.; Zhang, Y.; Zhang, B. and Xia, X. (2012): Characterization of *Staphylococcus aureus* isolated from powdered infant formula milk and infant rice cereal in China. Int. J. Food Microbiol., 153 (1-2): 142-147.

Wieneke, A.A.; Roberts, D. and Gilbert, R.J. (1993): Staphylococal food poisoning in United Kingdom. 1969 – 90. Epidemilogy Infect., 110: 519-531.

Yagoub Sanaa, O.; Awadalla Nazik, E. and El Zubeir Ibtisam, E.M. (2005): Incidence of some potential pathogens in raw milk in Khartoum North (Sudan) and their susceptibility to antimicrobial agents. J. Animal Veterinary Advances, 4 (3): 341-344.

التقييم البكتريولوجي للبن الخام والزبادي لوجود ستافيلوكوكاس اوريوس مع إشارة خاصة للسموم المعوية

محمود فرغلي حسين ، ايمن منير إبراهيم كُريّم

E-mail: Mahmoutfarghaly_1970@yahoo.com Assiut University web-site: www.aun.edu.eg

تم فى هذه الدراسه تجميع عدد ١٥٠ عينه من اللبن الخام والزبادى (٧٥ عينه لكلا نوع) من محلات الالبان والسوبر ماركت بمدينه أسيوط لعزل ميكروب المكور العنقودى الذهبى ، وتم عزله بنسبه ٥٢٪ من اللبن الخام ، ولم يتم عزله تماما من عينات الزبادى. هذا وقد تم أجراء أختبار الاليزا على ميكروبات االمكور العنقودى الذهبى المعزوله من عينات اللبن الخام. وتم تحديد ١٢ عترة (٣٠.٨) من ٣٩ عترة معزولة لها القدرة على أفراز السموم، ووجد أن عترتين فقط أفرزتا نوعين مختلفين من السموم المعويه، واحده (SEA&C) والثانيه (SEC&D) بنسبه ٨.٣٪ لكلا منهما ، بينما باقى العترات (١٠ عترات) أفرزت نوع واحد من السموم المعويه وفى مقدمتهم SEA بنسبه ٤١.٧٪ (٥عترات) ، SEC بنسبه ٢٥٪ (٣عترات) ثم SED بنسبه ١٦.٧٪ (عترتين)، هذا وقد تم مناقشة الاهميه الصحية لهذا الميكروب ومدى خطورة السموم المعويه المفرزه على صحة المستهلك.