



Incidence of *Staphylococcus aureus* and its enterotoxins in yoghurt

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ABSTRACT

A total of 50 samples of balady and automatically packaged plain yoghurt (25 of each) were collected from different dairy shops, groceries and supermarkets at Kalubia Governorates. The collected samples were transferred immediately to the laboratory in an ice box for detection of *Staph.aureus*, its enterotoxins produced and detection of virulent genes responsible for enterotoxins production. The obtained results revealed that, *Staph. aureus* could be detected in three samples (12%) and two samples (8%) of examined balady and automatically packaged plain yoghurt, respectively. Isolated strains were tested for detection of enterotoxins producing genes by using PCR. The obtained results showed that one strain belonged to each of balady and automatically packaged plain yoghurt proved to have gene type (A) and (D), respectively. Also detection of enterotoxins by using ELISA revealed that one strain of both balady and automatically packaged plain yoghurt was enterotoxigenic, types (A) and (D) production at percentage of (33.33% and 50%), respectively. The public health importance and hygienic significance of the isolated *Staph. aureus* and their enterotoxins as well as the suggested measures for improving the quality and safety of the yoghurt was discussed.

Keywords: yoghurt, *Staph. aureus*, enterotoxins, PCR.

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1. INTRODUCTION

Yoghurt is one of the most unique and universal dairy products because of its beneficial and therapeutic properties. It is considered a main source of high quality fats, proteins, calcium, phosphorous and potassium along with significant quantities of several vitamins. The lactose content is easily absorbed even by lactose maldigestors, as it is converted to lactic acid by lactic acid bacteria (yoghurt starter culture). Yoghurt is valuable adjunct to any healthy diet (Ghadge et al., 2008). Yoghurt has a therapeutic value as it proved to prevent the intestinal putrefaction resulting from anaerobic decomposition, prevents the gastrointestinal disorder, prevents coronary heart disease, reduce the risk of colon cancer, exerts a hypocholesterolemic effect and produce antibiotics as acidophilin, lactocidin, nicin and lactoline that inhibit

the growth of many pathogens (Elson and Haas, 2005). In spite of advanced dairy manufacturing process, some dairy products as yoghurt may serve as a vehicle of food-borne pathogens various (Frazier and Westhoff, 1992). *Staph. aureus* is one of the most important pathogen in milk or its products (LeLoir et al., 2003). The presence of *Staph. aureus* in the yoghurt could also be because of unhygienic processing, handling and packaging (Prescott et al., 2004). *Staph. aureus* is ubiquitous microorganism, its major habitats including the skin and mucous membrane of nose (Schleifer, 1986), also, lactating animals and human handlers are main sources for this bacterium and frequently implicated in the transmission of this pathogen (Char et al., 1983). *Staph. aureus* food poisoning (SFP) is one of such organisms which can transmitted to human

through contaminated and untreated milk and milk products (Seifu et al., 2004). *Staph. aureus* food poisoning (SFP) is one of the most common food-borne disease resulted from the ingestion of *Staph. aureus* enterotoxins (SEs) already preformed in food which have superantigenic activity whereas half of them have been proved to be emetic, representing a potential health hazard for consumers (Hennekinne et al., 2012). Symptoms of *staph. aureus* food poisoning have a rapid onset (1-6 hr) and often include nausea, vomiting, diarrhea, subnormal temperature and sever abdominal pain (Jablonski and Bohach, 1997). Usually the condition is self-limiting and recovery is rapid therefore, minor outbreaks of SFP remain unreported. The enterotoxins are exoprotein, which are heat stable, resist boiling in presence of food material. Initially SEA, SEB, SEC, SED, and SEE were characterized (Bergdoll et al., 1973). The infective dose required to induce *Staph. aureus* food poisoning (SFP) in humans is estimated to be around 0.1 µg and it may vary with patient sensitivity (Evenson et al., 1988). According to the biological safety, *Staph. aureus* is classified into risk group 2 (Human Pathogens and Toxins, 2009), that require containment lab Level 2 facilities. Due to continuous demand for yoghurt and the increase of consumer's awareness of the product safety as well as to assure a safe supply, it is extremely necessary not only to increase the production of this important product but also to ensure the bacteriological safety of the product to safeguard consumers against health hazard. Therefore, the present study was planned out to cover the isolation of *Staph. aureus* and detection of enterotoxins produced by the isolated strains and detection of virulent genes responsible for toxins production, by using PCR in balady and automatically packaged plain yoghurt.

2. MATERIAL AND METHODS

2.1. Collection of samples:

A total of 50 random samples of balady and automatically packaged plain yoghurt (25 of each) were collected from dairy shops, groceries and supermarkets. Yoghurt samples were collected in their plastic containers and transferred in an ice box to the laboratory directly without undue delay to be immediately examined, the samples examined on a biological safety cabinet (BSC) lab level 2, according to the recommendation of Public Health Agency of Canada (P.H.A.C., 2004). Preparations of collected samples were carried out according to APHA (1992).

2.2. Isolation and identification of *Staph. aureus* according to FDA (2001).

2.3. Detection of *Staph. aureus* enterotoxins by ELISA (Ewald, 1988):

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

2.4. Detection of *Staph. aureus* enterotoxins genes:

The genes responsible for enterotoxins production were carried out using PCR of isolated strains.

2.5. PCR Master Mix used for conventional PCR (cPCR):

oligonucleotide primers used in cPCR five pairs of primers were supplied from metabion (Germany) or Biobasic (Canada). They have specific sequence and amplify specific products.

DNA Molecular weight marker: Gel Pilot 100 bp ladder (cat. no. 239035) supplied from QIAGEN. Number of bands: 6 Size range: 100-600 bp. Gel Pilot 100 bp plus ladder (cat. no. 239045) supplied from QIAGEN (USA). Number of bands: 11 Size range: 100-1500 bp.

2.6. Material used for agarose gel electrophoresis

Agarose 1.5% (Sambrook et al., 1989). Ethidium bromide solution 10 mg / ml (Sambrook et al., 1989). Tris borate EDTA (TBE) electrophoresis buffer (1x) (WHO, 2002). Preparation of multiplex PCR Master Mix for Sea, Seb, Sec and See genes according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A. Agarose gel electrophoreses (Sambrook et al., 1989) with modification.

3. RESULTS

This study revealed that, the presence of putative pathogenic factor genes was

examined by employing a set of gene-specific primers in a PCR assay. The forward and reverse primers were selected from the published sequences as listed in Table (1).

The obtained results showed that one strain belonged to each of balady and automatically packaged plain yoghurt proved to have gene type (A) and (D), respectively. *Staph. aureus* could be detected in 12% and 8% of examined balady and automatically packaged plain yoghurt and one strain of each proved to be enterotoxigenic, types(A) and (D) at percentage of (33.33% and 50%), respectively, by using ELISA as recorded in Table (2).

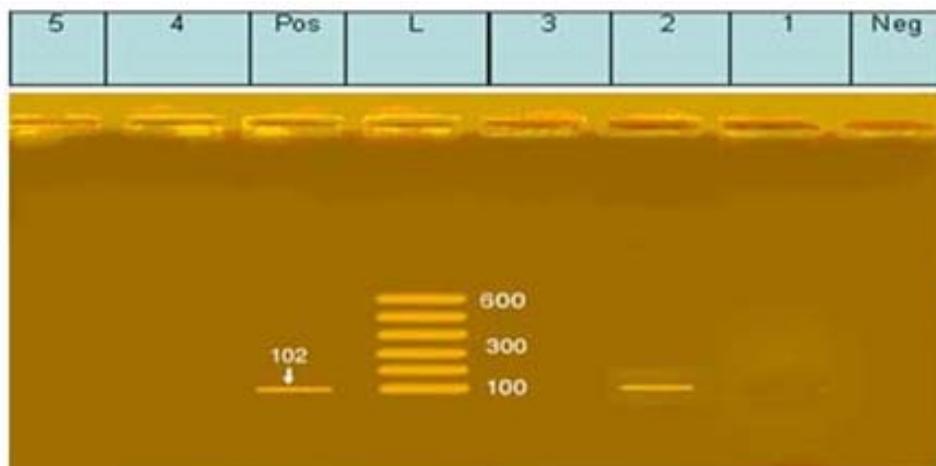
Table (1): Oligonucleotide primers sequences.

Gene	Primer	Primer sequence (5'-3')	Length of amplified product	Reference
<i>Sea</i>	GSEAF-1	GGTTATCAATGTGCGGGTGG	102 bp	Mehrotra et al., 2000
	GSEAR-2	CGGCACTTTTTTCTCTTCGG		
<i>Seb</i>	GSEBF-1	GTATGGTGGTGTAAGTGAAGC	164 bp	
	GSEBR-2	CCAAATAGTGACGAGTTAGG		
<i>Sec</i>	GSECF-1	AGATGAAGTAGTTGATGTGTATGG	451 bp	
	GSECR-2	CACACTTTTAGAATCAACCG		
<i>Sed</i>	GSEDF-1	CCAATAATAGGAGAAAATAAAAAG	278 bp	
	GSEDR-2	ATTGGTATTTTTTTTCGTTTC		
<i>See</i>	GSEEF-1	AGGTTTTTTCACAGGTCATCC	209 bp	
	GSEER-2	CTTTTTTTTCTTCGGTCAATC		

Table (2): Prevalence of *Staph. aureus* and its enterotoxins production in the examined yoghurt samples.

Types of samples	No. of examined samples	Positive <i>Staph. aureus</i> samples		Enterotoxins Types			
				A		D	
		No.	%	No.	%	No.	%
Balady	25	3	12	1	33.33	-	0.0
Automatically packed	25	2	8	-	0.0	1	50

Photograph (1) Agarose gel electrophoresis of cPCR amplified of enterotoxin SEA of *Staph. aureus*.



The genomic DNA of 5 *Staph. aureus* tested using specific primers for the SEA gene was amplified in 1 (33.3%) *Staph. aureus* strains for balady plain yoghurt isolates and giving product at 102 bp. Samples (1,2,3): balady Plain yoghurt Samples (4,5): automatically packaged plain yoghurt. Lane M: 100-600 bp DNA Ladder. Pos: positive control (at 102bp). Neg: Negative control. Lane 2 : *Staph. aureus* toxin positive Lane 1,3,4,5: *Staph. aureus* toxin negative

4. DISCUSSION

It is worth to mention that the presence of small numbers of *Staph. aureus* can not give complete assurance that a food is safe. Conditions inimical to *Staph. aureus* may result in diminished population or death of viable microbial cells , while sufficient toxins remain to elicit symptoms of (SFP).In contrast, the presence of large numbers of the organism in food is not,

however sufficient cause to incriminate a food as the vector of food poisoning (APHA,1992). Therefore, the potential for *Staph. aureus* food intoxication can not be ascertained without testing the enterotoxigenicity of isolated strains and/or demonstrating presence of *Staph. aureus* enterotoxins in food (Sherien, 2010). Although, the yoghurt starter cultures have inhibitory effect on *Staph. aureus* and can reduce the number of *Staph.*

Photograph (2): Agarose gel electrophoresis of cPCR amplified of enterotoxin *Staph. Aureus*



The genomic DNA of 5 *Staph. aureus* tested using specific primers for the SED gene was amplified in 1 (50%) *Staph. aureus* strains for automatically packed plain yoghurt isolates and giving product at 278bp. Samples (1, 2,3): balady Plain yoghurt Samples (4,5): automatically packaged plain yoghurt. Lane M: 100-600 bp DNA Ladder. Pos: positive control (at 278bp). Neg: Negative control. Lane 4: *Staph. aureus* toxin positive. Lane 1, 2, 3, 5: *Staph. aureus* toxin negative.

aureus added to milk by 1-2 log units during the cold storage (Pazakova et al., 1997), *Staph. aureus* could remain viable for 3 days at low inoculums (10^3 cfu/g) and for 10 days at high inoculum (10^5 cfu/g) at refrigerated storage (Halawa and Abozeid, 2000). Thus, at high contamination levels with *Staph. aureus* the antibacterial effect of yoghurt was insufficient to avoid the risk of food poisoning (Pazokova et al., 1997). The obtained results as recorded in Table (2) revealed that 12% and 8% of examined balady and automatically packaged plain yoghurt samples proved to contain *Staph. aureus*, respectively. Nearly similar findings of examined balady plain yoghurt samples were obtained by Hamaad (2004), Nashwa et al., (2010) and Sherien (2010). Higher incidences were reported by Hanna (1999), Manal (2000) and Eman (2007). The incidence of examined automatically packaged plain yoghurt samples in the current study were parallel to those obtained by Eman (2007). Lower incidences were obtained by Hassan (2003) and Hamaad (2004), meanwhile higher incidences were obtained by Gonui et al., (1996). The contamination of yoghurt with *Staph. aureus* could be due to lower sanitation and mishandling together with increased incidence of pathogenic staphylococcus carriers among producers (Grieger et al., 1990). The presence of putative pathogenic factor genes was examined by employing a set of gene-specific primers in a PCR assay. The forward and reverse primers were selected from the published sequences as listed in Table (1). In addition, table (2) revealed that three *Staph.aureus* strains were isolated from balady and two strains from automatically packaged plain yoghurt, were tested for the presence of genes responsible for enterotoxins production using cPCR. Out of which, one strain of balady contained gene type A and one strain of automatic contained type D enterotoxin producing gene. Detection of enterotoxins production showed that one strain of SEA (33.33%)

and SED (50%) from balady and automatically packaged plain yoghurt, respectively. Photograph (1) showed that agarose gel electrophoresis of cPCR amplified of enterotoxin SEA of *Staph. aureus* at (102 bp) from balady plain yoghurt. Photograph (2) showed that agarose gel electrophoresis of cPCR amplified of enterotoxin SED of *Staph. aureus* at (278bp) from automatically packaged plain yoghurt. PCR technique is rapid pathogen testing and vital to the food industry and facilitates the public health protection. PCR is highly sensitive specific and rapid method and substitute biochemical and serological characterization of the pathogen (Shiral et al., 1991). PCR detects strains possessing toxin genes independent of their expression. The possession of genes for super-antigens seems to be a frequent and habitual trait of *Staph. aureus* (Taj et al., 2014). These results suggest that the PCR assay is a rapid and extremely sensitive procedure, which is a very good tool for the detection of enterotoxins genes in clinical isolates of *Staph. aureus* (Tkacikova et al., 2003 and Anvari et al., 2008). The isolated strains of *Staph. aureus* from small and large scale plain yoghurt by Hanna (1999) proved to be non enterotoxigenic strains. Although the selected five isolates were strongly producing Coagulase and DNase, only two were enterotoxigenic which confirm what was stated by APHA (1992) and Marth and Steel (2001) that attempts to associate enterotoxin production by *Staph. aureus* with specific biochemical properties were generally failed. Consequently, confirmation of the toxin by serological or other means provide the only proof that, the particular strain is enterotoxigenic. Some isolates of *Staph. aureus* may produce enterotoxins (SEs) that cause food poisoning if sufficient amount of SEs is ingested. The enterotoxigenic strain needs to grow to levels $>10^5$ cfu/g before the toxin is produced at detectable levels. In addition, SE formation is influenced by parameters

such as temperature, pH, water activity, redox potential and bacterial antagonisms (e.g. starter cultures used in the production of fermented milk products can prevent *Staph. aureus* growth and SEs production). The SEs share common structural and biological proportion and belong to a family of Staphylococcal and Streptococcal pyrogenic toxin super-antigens. They contribute to bacterial virulence, cause emesis and may induce toxic shock (Dinges et al., 2000). SEs production is optimal in a neutral pH but decreases in acidic pH, its production is usually inhibited in pH below 5 (Novick, 2000). The enterotoxin A is of human origin, it may contaminate milk and dairy products during different stages of production and processing or even at consumer outlet (El-Baradie, 1993). In addition, the presence of enterotoxin D can be attributed to the increased incidence of staphylococcal mastitis where strains of *Staph.aureus* were isolated from bovine mastitis and were designated as animal strains (Masud et al., 1993). Enterotoxins are highly stable, resist most proteolytic enzymes, such as pepsin, so they keep their activity in the digestive tract after ingestion, SEs are highly heat resistant, but can be inactivated by heat treatments used in the sterilization of canned foods when they are present at low concentration (Le Loir et al., 2003). The results in this study failed to comply with EOSQ (2005) which stipulated that, yoghurt should be free from pathogenic microorganisms and their enterotoxins. Although, *Staph.aureus* is a robust bacterium and can survive for long periods at low temperatures below those, which permit growth. Yet, refrigeration at 4°C may be considered the only viable method for control of growth and toxin production (ICMSF, 1996 and Marth & steel, 2000).

5. CONCLUSION

To improve the quality of the yoghurt and to safeguard consumers from being infected by *Staph. aureus*, the following suggesting

are to be considered including; select high quality raw milk; sanitary precautions with handlers, utensils and the surroundings during milking and processing; adequate and proper cooling of yoghurt. Consumers should understand the importance of correct refrigerated storage of yoghurt and avoid consuming of yoghurt labeled with unknown trade name or not carry expiring date. Generally, application and implementation of (HACCP) system as a hazards control system should be done in dairy processing group to decide on whether good manufacturing practice (GMP) is being done and to ensure a maximum safety to consumers.

6. REFERENCES

- Anvari, S.H., Sattari, M., Foroz andeh Moghadam, M., Najar Peerayeh, S.H., Imanee Fouladi, A.A. 2008. Detection of *Staph. aureus* enterotoxins A to D from clinical samples by PCR. Research J. of Bio. Sci. 3(8):826-829.
- American Public Health Association, A.P.H.A. 1992. Compendium of methods for the microbial examination of foods. 3rd ed., American Public Health Association. Washington, DC., USA.
- Bergdoll, M. S., Robbins, R.N., Weiss, K., Borja, C.R., Huang, Y., Chu, F.S. 1973. The Staphylococcal enterotoxins similarities control. Microbiol. Immunol. 1:390-396.
- Char, N.L., Pao, P., Rao, P.R. 1983. Studies on mastitis in buffaloes livestock. Adviser, 8(12):19-22, Dairy Sci. Abst., 46:47.
- Dinges, M.M., Orwin, P.M., Schlievert, P.M. 2000. Exotoxin of *Staphylococcus aureus*. Clin. Microbiol. Rev., 13(1):16-34.
- Egyptian Organization for Standardization and Quality Control, E.O.S.Q. 2005. Yoghurt ES:1000/2005, yoghurt should be free from pathogenic

- microorganisms and their enterotoxins.
- EL-Baradie, G.A.1993. An avidine-biotin ELISA to determine the Staphylococcal enterotoxin A in soft cheese. *Alex. J. Agric. Res.*, 38:365-378.
- Elson, M.,Hass, M.D. 2005.Dairy products online. WWW.health.Ne/scr/ article.
- Eman, F.M. 2007. Microbiological evaluation of yoghurt produced commercially in Cairo and Giza markets. M.V.Sc. Thesis Fac. Vet. Med., Cairo Univ.
- Evenson, M.L., Hinds, M.W., Bernstein, R.S., Bergdoll, M.S. 1988. Estimation of human dose of Staphylococcal enterotoxin A from a large outbreak of Staphylococcal food poisoning involving chocolate milk. *Int. J. Food Microbiol.* 7:311-316.
- Ewalid, S. 1988. Evaluation of enzyme-linked immunosorbant assay (ELISA) for detection of staphylococcal enterotoxins in foods. *Inter. J. Food Microbiology*, 6 (2): 141-153.
- Food and Drug Administration (FDA). 2001. Bacteriological analytical manual, chapter 12 *Staphylococcus aureus*
- Frazeir, W.C., Westhoff, D.C. 1992. Food microbiology. McGrew Hill, New York. pp.295
- Ghadge, P.N., Prasad, K., Kadam, P.S. 2008. Effect of fortification on the physico-chemical and sensory properties of buffalo milk yoghurt. *Electronic. J. Environ, Agri. and Food chem.*, 7(5):2890-2899.
- Grieger, C., Badidova, D., Bednarcikova, E., Burdova, O., Haber, M. 1990. Detection of Staphylococcal enterotoxins in milk and milk products. *Veterinari Medicina*, 23(3):171-178., *Dairy Sci. Abst.*, 53:417.
- Gonui, S.A., Karapinar M., Kargozlu, N. 1996. Microbiological quality control of delicatessen food products. *Turkish J. of Biology*, 20 (3):263 – 271.
- Halawa, M.A., Abouzeid, A.M. 2000. Behaviour of Enterohaemorrhagic Escherichia coli and Enterotoxaemia *Staphylococcus aureus* in yoghurt and acidified milk. *Vet. Med. J.*, Giza 48(2):319-326.
- Hamaad, A.M. 2004. Micrbiological studies on raw milk and some dairy products. M.V.Sc. Fac. Vet. Med., Sadat city, Menufiya Univ.
- Hanna, A.A. 1999. Microbiological studies on milk and some milk products. Ph.D. Thesis, Fac. Vet. Med., Assuit Univ.
- Hassan, M. G. 2003. Quality assessment of some dairy products at consumer level. PhD. Thesis, Fac. Vet. Med. Cairo Univ. Bani Suef.
- Hennekinne, J.A., DeBuyser, M.L., Dragacci, S. 2012. *Staphylococcus aureus* and its food poisoning toxins, characterization and outbreak investigation. *F.E.Ms. Microbiol Rev.* 36(4): 815-36.
- Human Pathogens and Toxins 2009. Government of Canada, Second Session, Fortieth Parliament, 57-58 Elizabeth II Act. S.C. C.24. .
- International Commission on Microbiological Specification for foods (ICMSF) 1996. Microorganisms in foods,- their Significance and Methods of Enumeration 2nd Ed ., Univ .of Toronto Buffalo , London.
- Jablonski, L.M., Bohach, G.A. 1997. Food Microbiology Fundamental and Frontiers. Dayle, M.P., Rbeuchat, L. and Montivelle, T.J. (eds), American Society for Microbiology, Washington DC.
- Le Loir, Y., Baron, F., Guatier, M. 2003. *Staphylococcus aureus* and food poisoning. *Genet Mol Res.* 2:63-76.
- Manal, M.A. 2000. Studies on enterotoxigenic Staphylococci in milk and dairy products

- PhD. Thesis, Fac. Vet. Med., Cairo University.
- Marth, E.H., Steel J.J. 2001. Applied dairy microbiology 2nd Ed. Dekker, Am, Inc., USA.
- Masud, T., Ali, A.M., Shah, M.A. 1993. Enterotoxigenicity of *Staph. aureus* isolated from dairy products. Australian Journal of Dairy Technol., 48(1):30-36.
- Mehrotra, M., Wang, G., Johnson, W.M. 2000. Multiplex for detection of genes for *S. aureus* enterotoxins. exfoliative toxins. Toxic shock syndrome toxin 1. And methicillin resistance. J. Clin. Microbiol. 38:1032-1035.
- Nashwa, M.A.A., Hanaa, M.S., Sohair, R.B. 2010. Prevalence of enterotoxigenic strains of *Staphylococcus aureus* in some milk products. J. Egypt Vet. Med. Ause 70(1):57-65.
- Novick, R.P. 2000. Pathogenicity factors and their regulation .In: Gram Positive Pathogens (Fischetti, V.A. Novick, R.P., Feretti, J.J., Portnoy, D.A. and Rood, J.I., eds.). ASM Press, Washington, D.C., USA, pp. 392-407.
- Pazakova, J., Turek, P., Laciakova, A. 1997. The survival of *Staphylococcus aureus* during the fermentation and storage of milk. J. Appl. Microbiol., 82(5):659-662.
- Public Health Agency of Canada, P.H.A.C., 2004. In Best M., Graham M. L., Leitner R., Ouellette M. and Ugwu K. (Eds.), Laboratory Biosafety Guidelines (3rd ed.). Canada: Public Health Agency of Canada.
- Prescott, M.L., Harley, S.P., Klein, A.D. 2004. Microbiology. W.M.C. Brown publisher, Dubuque, pp. 951-952.
- Sambrook, J., Fritscgh, E.F., Mentiates 1989. Molecular cloning. A laboratory manual. Cold spring Harbor Laboratory press, New York.
- Schleifer, K.H. 1986. Gram-positive cocci. In Bergys's Manual of Systematic Bacteriology. Ed. Holts, J.C., pp. 99-1103, Baltimore-Williams and Wilkins.
- Seifu, E., Buys, E.M., Donkin, E.F., Petzer, I.M. 2004. Antibacterial activity of the lacto-peroxidase system against food-borne pathogens in Saanen and South African Indigenous goat milk, Food Control 15:447-452.
- Sherien, EL.B.N. 2010. Studies on some types of yoghurt at local markets. Thesis, M.V.Sc. Fac. Vet. Med. Sadat branch, Minufiya Univ. Food Dep.
- Shiral, H., Nish, I., Ramourthy, K.T., Bhattacharya, S.K., Pal, S.A. Takeda, Y. 1991. Polymerase chain reaction (PCR) for detection of the cholera enterotoxins operon of yibriocholera, J. Clin. Microbiol. 29.
- Taj, Y., Fatima, I., Ali, SW., Kazmi, SU. 2014. Detection of genes for superantigen toxins in methicillin-resistant *Staphylococcus aureus* clinical isolates in Karachi. J. Coll Physicians Surg Pak. 24(2):101-5.
- Tkacikova, L., Tesfaye, A., Mikula, I. 2003. Detection of the Genes for *Staphylococcus aureus* enterotoxins by PCR. Acta. Vet. BRONO 72:627-630.
- World Health Organization (WHO), 2002. Department of communicable diseases surveillance and response.