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ENTEROTOXIGENIC STAPHYLOCOCCUS AUREUS IN RAW AND PASTEURIZED MILK AND SOME MILK PRODUCTS

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ABSTRACT

This study was carried out on 250 samples of milk and milk products (50 samples each of raw milk, pasteurized milk, soft white cheese, butter and ice cream). The samples were collected from different shops at Mansoura city, El Dakahlia Province, Egypt and bacteriologically analyzed to detect the prevalence of *Staph. aureus* and its enterotoxins using PCR and SET-RPLA kits. The results revealed that the incidence of *Staph. aureus* were 36, 4, 24, 8 and 4% with mean counts of 3.8±1.4, 1.78± 0.48, 3.4±1.25, 2.39±0.95 and 2.14±0.78 $\log_{10}$cfu/g or ml respectively. The examined positive *Staph. aureus* by PCR and SET-RPLA kits showed presence of the following enterotoxigenic genes in the examined raw market milk; white soft cheese and table butter samples (sea, seb and see); (sea, sed and see) and (sea and seb) respectively. Meanwhile, the enterotoxigenic genes could not be detected in the examined pasteurized milk and small scale ice cream samples thus, it is necessary to adopt a regime of good, safe and healthy production of such products with periodical cleaning and disinfection to ensure safe products for consumer.

Key words: Enterotoxiginic, *Stath. aureus*, milk, milk products.

INTRODUCTION

Milk and Milk products are highly nutritious products specially for young and old aged due to its contents of proteins, fats, sugars, minerals and vitamins hence, they may exposed to be contaminated with bacteria through animals or its contact environment or handling and distribution. *Staph. aureus* was one of the dominant bacteria associated with raw milk. This might be due to the fact that milk is a good nutritive medium for microorganisms growth especially in poor sanitary conditions and lack of cooling facilities. Sattar *et al.* (2001) and Mubarack *et al.* (2010) added that *Staph. aureus* introduced into the milk also by droplet infection or from udder surface and milker's hands.

Normanno *et al.* (2005); Bhatia and Zahoor (2007) and Rabello *et al.* (2007) mentioned that *Staph. aureus* commonly causes gastroenteritis resulting from consumption of contaminated food in which enterotoxigenic staphylococci have grown and produced toxins. As these toxins are excreted from the organism, they are referred to as exotoxins. Staphylococcal enterotoxins are considered a potential biological threat because of their stability at 100°C for 1 hour.

Zhang *et al.* (1998); Atanassova *et al.* (2001); Loir *et al.* (2003) and Alegro *et al.* (2007) assured that Staphylococcal enterotoxicosis has a very rapid onset and course characterized by vomiting, headache, abdominal pain, and diarrhea develop as early as one to six hours after consumption of contaminated food. The symptoms resolve spontaneously within 24–48 hours. Meanwhile, Lina *et al.* (2004) added that *Staph. aureus* enterotoxicosis are due to the classical enterotoxins (SEA, SEB, SEC, SED, SEE) and several new variants of SEs.

Bergdoll (1983) and Letertre *et al.* (2003) concluded that the first five (A to E) classical enterotoxins are known to cause 95% of the food poisoning globally and Argudin *et al.* (2010) isolate 22 types of SEs designated with letters A-V are currently known. While, Bennett, (2005) demonstrated that there is a strong association between the ability of *Staph. aureus* strains to produce one or more of the SEs and the occurrence of staphylococcal food poisoning. Weronika and Jacek (2014) found that 11.9% of the

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isolated strains were positive for one or more classical SE markers. The aim of this study was to detect enterotoxigenic *Staph. aureus* prevalence which is a potential source of food poisoning

MATERIALS AND METHODS

Two hundred and fifty samples of milk and milk products (50 samples each) of raw market milk, pasteurized milk, soft white cheese, table butter and small scale ice cream were collected from different shops at Mansoura city and sent to the laboratory in icebox for examination without delay.

Enumeration and Isolation of Coagulase positive *Staph. aureus* according to (APHA 2001) 10 ml or g each of examined milk and milk product samples were taken aseptically and homogenized with 90 ml 0.1% peptone water in a stomacher for 3 minutes at 3000 rpm and filtered through a sterile cheese cloth filter, followed by six fold serial dilutions in 0.1% peptone water then 0.1 ml were taken from each dilution aseptically and inoculated onto Baird-Parker medium, the plates were incubated for 24-48 hours at 37°C. The plates containing 20-200 colonies were selected. Typical colonies of *Staph. aureus* were circular, smooth, convex, moist 2-3mm in diameter, grey to black (potassium tellurite reaction) with white margin and surrounded by outer clear zone (egg yolk reaction) the suspected colonies were streaked onto agar slant of nutrient agar medium and incubated at 37°C for 24 hours for further purification and identification by microscopical and biochemical examination by catalase, coagulase, thermostable nuclease and Voges-Proskauer tests.

Staph. aureus culture supernatant were collected by Sac cultural method (Donnelly *et al.*, 1967) and tested serologically by reversed passive latex agglutination technique using Oxoid SET-RPLA kits for the presence of SEA, SEB, SEC, SED and SEE.

Extraction of *Staph. aureus* enterotoxins from the examined samples were completed by blending of 10 ml of milk or milk product samples with 10 ml of sodium chloride solution (0.85%) and centrifuged. The supernatant was retained for toxin detection using Oxoid SET-RPLA kits Shingaki *et al.* (1981).

Detection of virulence genes in *Staph. aureus* using PCR (Reference Lab for Quality Control on Poultry Production, Animal Health Research Institute, Dokki -Egypt)

1-DNA extraction:
DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at $56^{O}C$ for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

2- Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in Table (1).

3- For multiplex PCR of enterotoxins, Primers were utilized in a 50-µl reaction containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 8 µl of water, and 7 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

4- Analysis of the PCR Products.
The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1xTBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 30 µl of the multiplex PCR products were loaded in each gel slot. Gelpilot 100 bp DNA ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions of Staphylococcus aureus enterotoxins.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Sea	GGTTATCAATGTG CGGGTGG	102	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Mehrotra *et al*. 2000**
	CGGCACTTTTTTC TCTTCGG							
Seb	GTATGGTGGTGT AACTGAGC	164						
	CCAAATAGTGAC GAGTTAGG							
Sec	AGATGAAGTAGT TGATGTGTATGG	451						
	CACACTTTTAGAA TCAACCG							
Sed	CCAATAATAGGA GAAAATAAAAG	278						
	ATTGGTATTTTTT TTCGTTC							
See	AGGTTTTTTCACA GGTCATCC	209						
	CTTTTTTTTCTTC GGTCAATC							

Statistical analysis:
The results are expressed as log mean ± standard error (SE). Data were statistically analyzed using statistical analysis systems.

RESULTS

Table 2: Mean counts of *Staph. aureus* in the examined samples expressed as log_{10}cfu/g or ml (n=50).

Examined products / Microbial count	Raw milk	Pasteurized milk	White soft cheese	Butter	Ice cream
Mean counts of Staph. aureus	3.8± 1.4	1.78±0.48	3.4±1.25	2.39±0.95	2.14±0.78

NB: n= number of the examined samples

Table 3: Incidence and Disribution of enterotoxins produced by *Staph. aureus* strains isolated from the examined samples by SET-RPLA kits and PCR (n=50).

Examined products	No and incidence % of the isolated strains		No of strains Producing enterotoxins		Types of produced enterotoxins				
	No/50	%	No	Frequency %	A	B	C	D	E
Raw market milk	18	36	3	16.66	SEA	SEB			SEE
Pasteurized milk	2	4	-	-	-	-	-	-	-
white soft cheese	12	24	3	25	SEA	-	-	SED	SEE
Butter	4	8	2	50	SEA	SEB	-	-	-
Ice cream	2	4	-	-	-	-	-	-	-

Results of Polymerase chain reaction:

Multiplex PCR for enterotoxiginic *Staph. aureus* genes:

Results of the isolated *Staph. aureus* from the examined raw milk by using multiplex PCR using sets of primers for enterotoxins (A,B,C,D and E) showed that

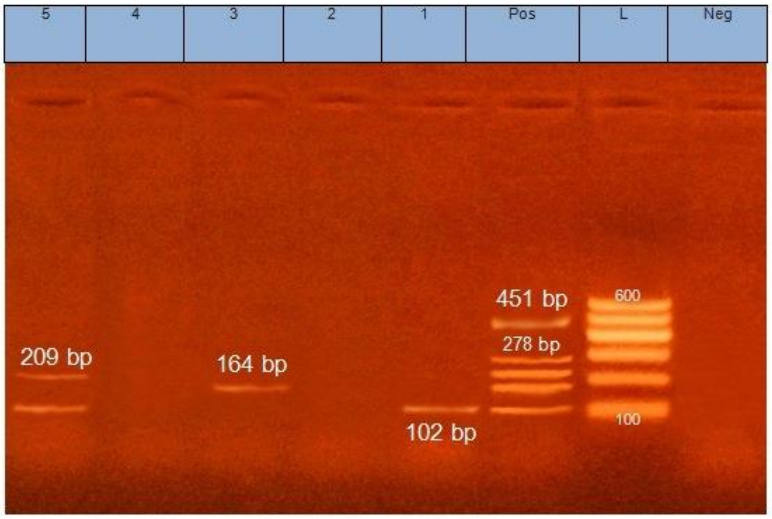

Fig (1): Agarose gel electrophoresis of *Staph. aureus* PCR products using enterotoxins *Staph. aureus* primer Pos=positive control, Neg=negative control, L=100 bp DNA ladder

Lane "1": positive amplification of 102 bp for enterotoxin A
Lane "2 "and Lane "4" were negative
Lane "3": positive amplification of 164 bp for enterotoxin B
Lane "5": positive amplification of 102 bp for enterotoxin A, 209 bp for enterotoxin E

Results of the isolated *Staph. aureus* from examined white soft cheese samples (Lane 1,2&3) and butter samples (Lane 4&5) by using multiplex PCR using sets of primers producing enterotoxins (A,B,C,D and E) showed that

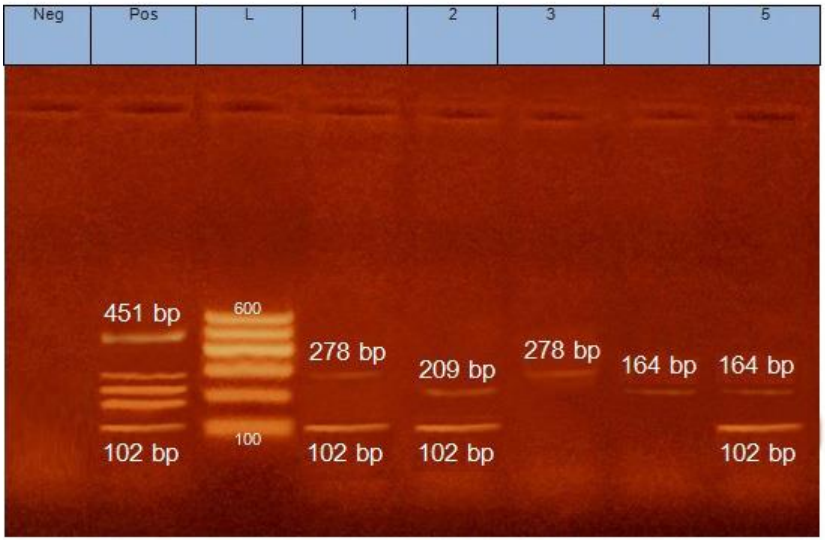

Fig (2): Agarose gel electrophoresis of *Staph. aureus* PCR products using enterotoxins *Staph. aureus* primer Pos=positive control, Neg=negative control, L=100 bp DNA ladder

Lane "1": positive amplification of 102 bp for enterotoxin A and 278 bp for enterotoxin D
Lane "2" positive amplification of 102 bp for enterotoxin A and 209 bp for enterotoxin E
Lane "3" positive amplification of 278 bp for enterotoxin D
Lane "4": positive amplification of 164 bp for enterotoxin B
Lane "5": positive amplification of 102 bp for enterotoxin A and164 bp for enterotoxin B

DISCUSSION

Presence of lactic acid bacteria lowering the pH in raw milk that may prevent *Staph. aureus* growth and enterotoxin production (Alomar *et al.,* 2008 and Janstova *et al.,* 2012). Pinchuk *et al.* (2010) mentioned that bacterial counts of *Staph. aureus* need to reach 10^5-10^8 cfu/mL before sufficient amount of toxin to cause illness is produced while, Evenson *et al*. (1988) showed that growth of enterotoxigenic *Staph. aureus* up to 10^6 or more/g of food enables them to produce a sufficient amount of enterotoxins to cause intoxication. As little as 20ng of SE can induce nausea, violent vomiting, abdominal cramps, and diarrhea between 1 to 8 h after food consumption. The achieved results in Tables 2,3 and Fig. 1 declared that the highest contamination of *Staph. aureus* were found in raw market milk with mean counts of 3.8 ±1.4 $\log_{10}$cfu/ml with incidence percent of 36% mean while, 3 out of the examined 18 isolates by PCR and SET-RPLA kits were enterotoxigenic. The enterotoxigenic strains have sea, seb and see virulent genes, these results were nearly in accordance with Manfreda *et al.* (2005) who found 34.6% of milk samples were contaminated with *Staph. aureus*, 6.6% of which were enterotoxin producers. Enterotoxigenic strains were most frequently detected in milk with *Staph. aureus* count 4.47 $\log_{10}$cfu/ml; Bianchi *et al.* (2013) declared that 53% of raw milk were positive for one or more SE genes and *Staph. aureus* count were 3.5 ±2.38 $\log_{10}$cfu/ml with incidence percent of 32%. Also, Thabet *et al.* (2014) revealed that *Staph. aureus* was isolated with a percentage of 26.6% from raw milk; Hu Shou Kui *et al*. (2013) found *Staph. aureus* in 30.0% of examined raw milk and 43.7% of the isolated *Staph. aureus* produced enterotoxins. These results were lower than that reported by Weronika and Jacek (2014) and Gundogan and Avc (2014) who found the incidence percent in raw milk were 56% and higher than Rajeev and Amit (2010) who could isolate Staphylococcus from milk by 10%.

The obtained results of *Staph. aureus* count and its incidence in Pasteurized milk in Tables 2 and 3 declared that the mean counts were 1.78±0.48 $\log_{10}$cfu/ml with incidence percent of 4% mean while, the enterotoxigenic strains of *Staph. aureus* could not be detected by PCR and SET-RPLA kits. These results were in accordance with those obtained by Gad EL-Said *et al.* (2013) who reported that no enterotoxigenic *Staph. aureus* were detected in pasteurized milk and Asao *et al*. (2003) who added that pasteurizing raw milk would eliminate *Staph. aureus* from raw milk, however once the pathogens have produced enterotoxins the toxins will remain stable even after pasteurization. Also, Jicinska and Havlova (1995) concluded that because of its heat resistance, *Staph. aureus* can be detected even in pasteurized milk in addition to Anderson *et al.* (1996) shown that *Staph. aureus* enterotoxins are highly resistant to heat treatment, a good example is sea, which retained its biological activity even after exposure to 121°C for 28 minutes.

Jablonski and Bohach (2001) reported that 10^3 and 10^5cfu/g *Staph. aureus* is able to produce enterotoxin in amounts that can pose a health risk to the consumers.

The achieved results of white soft cheese in Tables 2,3 and Fig 2 declared that the mean counts of *Staph. aureus* were 3.4±1.25 $\log_{10}$cfu/g with incidence percent 24% mean while, 3 out of the examined 12 isolates were enterotoxigenic detected in examined samples by PCR and SET-RPLA kits have the enterotoxigenic virulent genes sea, sed and see. These results were lower than Gundogan and Avc (2014) who found that 48% of white cheese were contaminated with *Staph. aureus*. While, Thabet *et al.* (2014) revealed that *Staph. aureus* was isolated with a percentage of 6.6% in Damietta cheese samples and Hu Shoukui *et al.* (2013) the positive rate of *Staph. aureus* in milk products including cheese were 7.5% and 43.7% of the isolated *Staph. aureus* produced enterotoxins. Gucukoglu *et al.* (2012) investigated that the enterotoxigenic *Staph. aureus* was detected in white cheese by 19%, two isolates from cheese samples 50% were found to be enterotoxigenic. Rahimi (2013) reported that 11.1% of examined cheese were found to be contaminated with *Staph. aureus* and the ability to synthesize classical staphylococcal enterotoxins (SEA-E) was determined in 7 of 20 (35%) isolates.

Bianchi *et al.* (2013) found that milk and dairy products account for 5% of all the incriminated foods poisoning.

Theresults of *Staph. aureus* incidence in Tables 2, 3 and Fig 2 of the examined table butter samples were 8% with mean count of 2.39±0.95$\log_{10}$cfu/g and the enterotoxigenic virulent genes of *Staph. aureus* was detected in 2out of 4 isolates from the examined table butter samples. The isolated enterotoxigenic strains of *Staph. aureus* by PCR and SET-RPLA kits have sea and sebvirulent genes. These results were nearly in accordance with Rahimi, (2013) who found 5.3% of butter samples contaminated with *Staph. aureus* and 35% of the isolated *Staph. aureus* were able to synthesize the classical staphylococcal enterotoxins (SEA-E). While, Gucukoglu *et al.* (2012) investigated that the enterotoxigenic *Staph. aureus* was detected in 30% of the examined butter samples and 25% of them showed enterotoxigenic character (SEB 100%).

The results in Tables 2 and 3 indicated that the incidence percent of *Staph. aureus* in ice cream samples were 4%, with mean counts 2.14±0.78 $\log_{10}$cfu/ml. The enterotoxigenic strains could not be detected in the examined samples either by PCR or by

SET-RPLA kits. Bostan and Akn (2002); Sagdc *et al.* (2003) and Hu ShouKui *et al.* (2013) could not found *Staph. aureus* in ice creams samples while, higher percentage were reported by Gunsen (2002) who found *Staph. aureus* in 5% of lemon ice cream samples; Rahimi, (2013) found 5.9% ice-cream contaminated with *Staph. aureus* and Gundogan and Avc (2014) found *Staph. aureus* in 36% of the examined ice cream samples. Nazem *et al.* (2010) isolate *Staph. aureus* from 5% of ice cream collected from supermarkets; lower percentage were reported by Rajeev and Amit (2010) who isolated Staphylococcus from Ice cream by 1%; Yucel and Ctak (2002) found *Staph. aureus* count $1.0x10^2$-$3.0x10^3$cfu/ml in ice cream samples. Guner *et al.* (2004) added that counts of *Staph. aureus* in ice cream were $1.2\text{-}1.7x10^3$cfu/g and El-Ansary (2015) found *Staph. aureus* count was $1.10x10^3 \pm 2.45x10^2$cfu/ml in Vanilla ice cream samples, which could be associated with potential food poisoning hazards. On the other side, Gucukoglu *et al.* (2012) investigated that the enterotoxigenic *Staph. aureus* was detected in 10% of ice cream samples.

Asao *et al.* (2003) mentioned that *Staph. aureus* were frequently contaminator for ice cream. Hence, improvement of the hygienic practice in processing, preparing and storage should be stressed and Schmitt *et al.* (1990) declared that the causes of staphylococcal enterotoxicosis are classical SEs. SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE and the production of SEs is unlikely at temperatures below 10^0C.

Bergdoll (1989) concluded that a very small amount of *Staph. aureus* enterotoxins ranging from 20 ng to < 1 µg is needed to cause a typical symptoms of staphylococcal food poisoning. An outbreak in Japan caused by low-fat milk contaminated with SEA showed that the total intake of SEA per individual was estimated to be 20–100 ng, More recently, Ostyn *et al.* (2010) in France found an outbreak caused by contaminated cheese, doses of SEE ingested by symptomatic persons were estimated to be about 90 ng, based on the mean weight of the cheese portion (about 200 g) and the total amount of SEE in food samples were 0.45ng/g.

CONCLUSION

The presence of enterotoxigenic *Staph. aureus* in raw milk and milk products poses a potential health hazard to the consumers. However, not only identification of such strains but also appropriate conditions for *Staph. aureus* enterotoxin genes during production and storage of milk and milk products should be taken into account in hazard risk analysis.

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الميكروب العنقودى الذهبى المفرز للسموم فى اللبن الخام والمبستر وبعض منتجات الالبان

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تعد الالبان ومنتجاتها من الأغذية الضرورية للإنسان فى جميع بلدان العالم لما تحتويه من عناصر غذائيه ضروريه لبناء الجسم ولكنها تعتبر من أكثر المصادر المسببه للتسمم الغذائى إذا ما تم معاملتها بطرق خاطئه من الناحية الصحية أثناء إنتاجها وتصنيعها لذا أجريت هذه الدراسة بغرض معرفة مدى تواجد ميكروب المكور العنقودى الذهبى فى اللبن الخام وبعض منتجاته فى مدينة االمنصورة - محافظة الدقهليه حيث تم جمع عدد ٢٥٠ عينه بواقع ٥٠عينة من كل من اللبن الخام واللبن المبستر والجبن الابيض الطرى والزبد والايس كريم. حيث تم تحديد المحتوى البكتيري لتواجد ميكروب المكور العنقودى الذهبى وكذا معرفة مدى تواجد السموم المعوية المفرزة منه حيث كانت نسب العزل لميكروب المكور العنقودى الذهبى كالتالي ٣٦، ٤، ٢٤,٨، ٤% على الترتيب. وباعداد ٣,٨±١,٤، ١,٧٨±٠,٤٨، ٣,٤±١,٢٥، ٢,٣٩±٠,٩٥و٢,١٤±٠,٧٨لوج.١/جم اومللى على الترتيب كما تبين وجود بعض العترات التى تنتج السموم المعوية باعداد ٣، ٣و٢ عينات لكل من عينات اللبن الخام والجبن الابيض الطرى والزبد المختبرة على الترتيب بنسبة ١٦,٦٦و٢٥و٥٠% على الترتيب بينما العينات المختبرة من اللبن المبستر والايس كريم كانت سلبية للسموم المعوية للميكروب الموجب لتجلط البلازما والتي تقوم بإثارة مراكز القئ في المخ وتشكل أحد الأسباب الرئيسية للتسمم الغذائي، والذي يحدث عادة بعد تناول الأطعمة الملوثة، لاسيما منتجات الالبان الملوثة بالميكروب عن طريق سوء التعامل والتخزين في درجات حرارة مرتفعة لذلك تم فحص جينات الضرواه لكل منها واجراء اختبار تفاعل البلمرة المتسلسل لتحديد وجود جينات الضراوة sea, seb, sed and see والتي تؤثر على قدرة الميكروب فى احداث حالات مرضية عند تناول الالبان الملوثة بهذا الميكروبحيث ثبت تواجدها فى بعض الميكروبات المعزولة من العينات. وقد نوقشت الأهمية الصحية للمعزولات وكذلك كيفية الإقلال من تواجدها باتباع نظم سلامة الغذاء أثناء الاعداد والتداول.