

DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN A IN EXPERIMENTALLY INFECTED CHEESE

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SUMMARY: Three different types of cheese (processed or cooked, soft white and mish or salted cheese) were experimentally inoculated with enterotoxigenic strain of *Staphylococcus aureus* known to produce enterotoxin A. (1×10^7 organism per gram) to study their abilities to support the growth of the organism and the production of enterotoxin at different temperatures. Enterotoxin A was detected by direct extraction from the cheese and then subjected for the sensitive serological microslide assay technique.

In all inoculated samples staphylococcal count was reduced and no enterotoxin could be detected at 5 and 10°C after incubation for 72 hrs. Only processed cheese support staphylococcal growth when incubated at 20-23°C for 72 hrs., however no enterotoxin A was detected. Processed cheese showed a better staphylococcal growth than white and mish cheese when incubated at 30 and 37°C for a period of 24 to 72 hrs. respectively, in addition the enterotoxin A production in processed cheese was 2 and 4 folds more than that produced in white and mish cheese respectively. The highest enterotoxin A production was obtained in processed (0.65 ug/100 g.) and white cheese (0.45 ug/100 g.) incubated at 39°C for 72 hrs. In contrast, no enterotoxin A production was observed in mish cheese incubated at 39°C for 24 to 72 hrs.

INTRODUCTION

Cheese has been repeatedly incriminated in staphylococcal food poisoning outbreaks. Enterotoxigenic *Staphylococci* particularly those producing enterotoxin A, have been reported in cheese by various investigators (Hendricks et al., 1959; Allen and Stovall, 1960; Donnelly

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et al., 1967; Zehren and Zehren, 1968; Bergdoll, 1970; Reiser et al., 1974 and Niazi et al., 1986). The enumeration of enterotoxigenic Staphylococci in food is an unreliable goal for judgement of the safety of such food because the number of Staphylococci required for production of enterotoxin in cheese sufficient to be dangerous to the consumer is unknown (Zehren and Zehren , 1968). Donnelly et al. (1967) added that the presence of Staphylococci in finished product does not necessarily mean that they are originally present in numbers too small to produce significant amount of enterotoxin in handled milk or during cheesemaking. Reiser et al. (1974) recommended to use a rapid and sensitive method for the extraction of Staphylococcal enterotoxins directly from food materials as a valuable parameter for determining the safety of a given food.

This paper describes an attempt for direct detection of Staphylococcal enterotoxins in different types of cheese experimentally infected with enterotoxin A-producing strain and stored at different temperatures.

MATERIALS AND METHODS

Organism and preparation of inoculum:

Staphylococcus aureus strain S-76 isolated from white cheese in the Unit of Bacterial Toxins, Animal Health Institute, Dokki and known to produce Enterotoxin A and yield an amount of 4 µg per 1 ml of culture supernatant fluid at 37°C (Sac-culture method and optimum sensitivity plate technique) was used in these experiments. The enterotoxigenicity of this strain was rechecked by using a highly purified and lyophilized enterotoxin A and its specific antiserum (Robbins et al., 1974). The strain was grown at first on Brain-heart infusion agar slope, and the culture was harvested in sterile physiological saline and then washed 3 times with physiological saline to remove the residual enterotoxin. A suspension of washed cells was prepared after the last washing, and the number of cells was estimated by the optical density at a wavelength of 600 nm using a Spectronic-20 spectrophotometer.

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Preparation and inoculation of cheese samples:

Three different types of cheese were involved in this study: five samples of processed (cooked) cheese, five samples of Damiette white (soft) cheese and five samples of mish (salted) cheese. The cheese samples were subjected for bacteriological examination to ensure that they are free from coagulase positive Staphylococci. Each samples was cut off into slices of 2 weights (25-g each for Staphylococcal-counting and 100-g for separation of enterotoxin A) under aseptic circumstances. The slices were placed separately in sterile petri-dishes and after inoculation with the enterotoxin A-producing Staphylococcus strain, incubated aerobically at different temperatures at 5, 10, 20-23 (ambient Temp.), 30, 37 and 39°C each for 72 hours. The inoculum was 1 ml of a dilution of washed suspension of Staphylococcus aureus in sterile physiological saline (free from any residual toxins) and spread uniformly over the surface of each slice to give 1×10^7 organism per gram.

Culture methods and counting of organism:

Samples of inoculated cheese were removed at regular time intervals of incubations (24, 48 and 27 hours) for counting the organism and separation of enterotoxin A in cheese samples.

Samples of 25-g each were blended in 225 ml of sterile distilled water using high speed blender. The number of Staphylococci present in the aqueous suspensions were determined by spreading 0.1 ml of ten-fold dilutions of suspension over the surfaces of Baird-Parker medium. The typical colonies were counted after 48 hours incubation at 37°C.

Separation and concentration of enterotoxin A:

Enterotoxin A was separated directly from cheese samples using the method described by Reiser et al. (1974). The procedure involved extraction of 100 grams cheese samples at pH 4.5, centrifugation (27,300 x g for 20 min. at 4°C), extraction of supernatant with CHCl_3 (pH 7.5) and extraction of enterotoxin from water layer with CG-50 ion exchange resin (pH 5.8). The enterotoxin was eluted from the resin, concentrated in 50% Carbowax 20-M overnight at 4°C. The concentrate was extracted with CHCl_3 and the water layer was dried. The dried material was dissolved in 1% crude trypsin in distilled water (to eliminate contaminating proteins) and placed for serological assay technique.

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All extracts were stored at -18°C for the microslide double gel diffusion technique (Casman and Bennett, 1965) used for enterotoxin quantification (sensitivity of 0.125 ug/ml toxin A).

RESULTS**Processed cheese:**

It is evident (Table 1) that incubation of processed cheese experimentally inoculated with 1×10^7 S.aureus cell per gram at 5 and 10°C for 72 hours progressively reduced the growth of S.aureus. At the same time enterotoxin A could not be detected in any of the inoculated portion of cheese. A relative good growth of S.aureus was obtained in cheese incubated at $20-23^{\circ}\text{C}$ for 72 hours, however no detectable amount of toxin A was determined in the inoculated cheese. Incubation of processed cheese at 30°C showed a good growth of the organisms allover the period of incubation (24-72 hours). In addition enterotoxin A was detected in all samples taken at different time intervals (Table 1). Maximum growth of S.aureus was observed in samples incubated at 37°C particularly after 48 and 72 hours incubation. The amount of enterotoxin A produced in processed cheese kept at 37°C increased by time and the amounts determined at the corresponding time intervals were relatively similar to those determined at 30°C .

The maximum enterotoxin A yield at 39°C (0.65 ug/100g.) corresponded to Staph. aureus count which was less than those determined in samples incubated at 37°C for 72 hours.

Damiette white cheese:

The average count of coagulase positive Staphylococci (organism/g.) and enterotoxin A production (ug/100g.) in white cheese inoculated with 1×10^7 S.aureus cell per gram at different temperature are shown in (Table 2). Marked reduction in the count of organisms was observed in cheese samples incubated at 5 and 10°C for 24-72 hours.

A relative good growth of Staph. aureus was seen in samples taken after 24 hour and incubated at $20-23^{\circ}\text{C}$, then the count progressively declined in samples taken after 48 and 27 hours.

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Table 1: Average count of *S. aureus* and assay of enterotoxin A produced in processed (cooked) cheese inoculated with 1×10^7 bacterial cell/g at different temperature.

Temp. (°C)	24		48		72	
	Count (per g)	Toxin (ug/100 g)	Count (per g)	Toxin (ug/100 g)	Count (per g)	Toxin (ug/100 g)
5	1×10^6	---	2.1×10^6	---	3×10^6	---
10	1.6×10^6	---	2.3×10^6	---	2.6×10^6	---
20-23*	1.1×10^7	---	1.7×10^7	---	2.7×10^7	---
30	3.4×10^7	0.40	5×10^7	0.45	1.2×10^8	0.50
37	1.7×10^9	0.45	2.1×10^{10}	0.50	2.4×10^{10}	0.55
39	7.7×10^9	0.55	4.5×10^8	0.60	2×10^8	0.65

(--) = Not detectable

* = Ambient temp. at the experimental time.

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 Table 2: Average count of *S. aureus* and assay of enterotoxin A produced in Damiette white cheese (soft cheese) inoculated with 1×10^7 bacterial cell/g at different temprature.

Temp. (C°)	24		48		72	
	count (per g)	Toxin (ug/100 g)	Count (per g)	Toxin (ug/100 g)	Count (per g)	Toxin (ug/100 g)
5	1.6×10^6	---	5×10^5	---	1.6×10^6	---
10	1.7×10^6	---	2.7×10^6	---	1.8×10^6	---
20-23*	1×10^7	---	2×10^6	---	2.4×10^6	---
30	1.5×10^7	0.20	2×10^6	0.20	1.9×10^5	0.20
37	4×10^7	0.20	2.5×10^7	0.25	1×10^6	0.30
39	1.7×10^8	0.35	5×10^6	0.40	1.9×10^5	0.45

(---) = Not detectable.

* = Ambient temp. at the experimental time.

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Table 3: Average count of *S. aureus* and assay of enterotoxin A produced in Mish cheese (Salted cheese) inoculated with 1×10^7 bacterial cell/g at different temperature.

Temp. (C°)	Incubation time (h)					
	24		48		72	
	Count (per g)	Toxin (ug/100 g)	Count (per g)	Toxin (ug/100 g)	Count (per g)	Toxin (ug/100 g)
5	4.2×10^6	---	4.8×10^6	---	2.4×10^6	---
10	2.5×10^6	---	2.7×10^6	---	1.2×10^6	---
20-23*	1.4×10^6	---	3×10^5	---	9×10^4	---
30	4×10^7	0.125	3×10^5	0.125	2.3×10^5	0.125
37	1.4×10^7	0.125	5×10^6	0.125	3×10^5	0.125
39	1.2×10^6	---	3×10^5	---	1×10^5	---

(--) = Not detectable .

* = Ambient temp. at the experimental time.

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No enterotoxin A was detected in any white cheese samples incubated at 5, 10 and 20-23°C (ambient temperature) after 24 to 72 hours.

The organism showed a good growth after 24 hours then the count declined after 72 hours in samples incubated at 30, 37 and 39°C. In addition after 72 hours of incubation, the detectable amount of enterotoxin A that was obtained from samples incubated at 30, 37 and 39°C represented 50, 55 and 70% of that produced by the organisms experimentally inoculated in processed cheese with the same inoculum (1×10^7 cell/g.) at the same incubated temperature respectively (Tables 1 and 2).

Mish cheese:

Reduction in Staphylococcal growth and absence of enterotoxin production were recorded in mish cheese inoculated with *S.aureus* (1×10^7 cell/g.) at 5, 10 and 20-23°C after 24-72 hours incubation.

A relative good growth of the organism was remarked in samples at 30, 37°C incubation then the count progressively declined by time. Enterotoxin A produced in mish cheese was 0.125 ug/100 g. after 24 hours incubation at 30 and 37°C and the value was not altered in samples examined after 48 and 72 hours (Table 3).

In contrast, marked retardation in Staphylococcal growth as well as no enterotoxin A production were observed in mish cheese incubated at 39°C for 24-42 hours.

DISCUSSION

In our attempts to determine the enterotoxin A produced by coagulase positive *S.aureus* in different cheese by direct extraction and assay of the enterotoxin (Reiser et al., 1974) by means of the microslide serological technique (Casman and Bennett, 1965), it was clear that concentrations less than 1 ug/100 g of enterotoxin in cheese could be detected. This indicates the sensitivity of the method used and hence its reliability in judgement of the safety of a given food as small amounts of enterotoxin A (less than 1 ug/100 food) were also involved in food poisoning outbreaks (Reiser et al., 1974; Fraizer et al., 1978). In addition, the abilities of the most commonly used type of cheese in Egypt to support the growth of staphylococci and enterotoxin production

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under certain conditions is of particular significance with regard to the health hazard of the consumers. In this respect, Jeski et al. (1961), Fraizer et al. (1978) reported that staphylococci increase from thousands per milliliter milk to millions per gram cheese during cheese making.

The maximal staphylococcal count obtained in either milk (Thatcher and Ross, 1960) or cheese (Jezeski et al., 1961) is proportional to the inoculum level, so that staphylococci in low numbers would have less chance of reaching a cell density that would produce significant level of enterotoxin than would staphylococci in high numbers (Donnelly et al., 1967).

The progressive reduction in staphylococcal count by time, and the absence of enterotoxin A production in any cheese inoculated with 1×10^7 organism/g and incubated at refrigerator temp. (5°C) and 10°C for 72 h. respectively, were anticipated. These results were also remarked in white cheese and salted cheese samples incubated at 20-23°C (ambient temp.) for 72 hours but not in processed cheese which showed a slight growth by time, however, no enterotoxin A was detected. In this respect, Vandenbosch et al. (1973) and Pereira et al. (1982) demonstrated that at 20°C a rather long incubation period would be required for the staphylococci to produce enough enterotoxin to results in food poisoning. They added that lowering of temperature would result in slower growth and decrease or stop of the enterotoxin production by the organism. Furthermore, Noleto and Bergdoll (1982) stated that there is a level of growth at which enterotoxin is first detectable (10^6 to 10^7 CFU/ml) at 1 ng/ml level. Pereira et al. (1982) have observed trace amounts of enterotoxin A production at 20°C in staphylococcal culture media.

The obtained results demonstrate that processed cheese showed a better staphylococcal growth than white and mish cheese inoculated with (10^7 organism/g) and incubated at 30 and 37°C for a period of 24-72 hours respectively. In addition, the enterotoxin A production by *S. aureus* grown in processed cheese was 2 and 4 folds more than that produced by white and mish cheese respectively. These findings are closely related to results obtained by other authors (Markus and Silverman, 1970; Czop and Bergdoll, 1974) who found that enterotoxin A is directly related to the growth of *S. aureus*. Donnelly et al. (1967) added

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that enterotoxin production is more likely during cheese making when sequence of temperature may be from approximately 31 to 38°C to 25°C (room temperature) for as long as 24 hours.

According to Vandebosch et al. (1973) and Pereira et al. (1982), maximum enterotoxin A production by *S.aureus* in liquid cultured media under aerobic condition is 39-40°C as optimum temperature for the growth of *S.aureus* organism. These communications support our findings for processed cheese and white cheese but not for mish cheese.

The inability of white cheese to obtain good growth and production of enterotoxin A than was remarked with processed cheese could be attributed to the presence of various other microorganisms in the raw milk and raw cheese (Graves and Frazier, 1963) which are lactic acid producers, particularly *E.coli*, *Proteus* spp., *Lactobacillus* spp. and are capable to decrease the pH (Digiacinto and Frazier, 1966) and consequently affect the growth and enterotoxin production (Haires and Harmon, 1973 ; Pereira et al., 1982) as it is known that *S.aureus* is a bad competitor. Furthermore, Frazier, 1978 reported that the growth of *S. aureus* in cooked food, and/or sterilized food recontaminated with *S. aureus* after their manufacture is better than that in the raw foods. Although *S.aureus* is a halotolerant organism, a high salt content as that found in mish cheese (3.5-7.5%, El-Essawy et al., 1985) represented an inhibitory factor for growth of such organism. This result substantiates the findings of Busta and Jeszeski (1963). Pereira et al. (1982) added that there is a direct relationship between the increase of NaCl concentration and the decrease in enterotoxin A production.

The data presented in this study suggest that enterotoxin A production, - the most common one associated with food poisoning outbreaks from cheese contaminated with *S.aureus* - could develop in cheese at storage when the storage temperature is above 10°C. The enterotoxin A was produced at 30°C in different types of cheese. This is particularly significant since this approaches the room temperature especially at summer time. Since processed cheese as well as white one are more subjected to mishandling in terms of poor control of temperatures. If the temperature of a display case or storage chamber containing processed or cooked cheese was allowed to rise significantly or if cooked cheese were stored in home in unrefrigerated place, growth of *S.aureus* and subsequent enterotoxins production could result.

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