Food Chemistry **33** (1989) 193–202

Effect of Staphylococcal Enterotoxin C_2 on the *in vitro* Rates of Protein and RNA Synthesis by Cow and Buffalo Liver

R. I. Mashaly, M. G. El-Ziny, M. H. Salem, S. A. El-Deeb & A. A. Ismail

Biotechnology Laboratory, University of Alexandria Research Center, (UNARC), Alexandria, Egypt

(Received 29 July 1988; revised version received and accepted 20 October 1988)

ABSTRACT

Out of 165 Staphylococcus species isolated from milk and cheese samples, 49 isolates (32 from milk and 17 from cheese) were identified as S. aureus *strains. Among the 49* S. aureus *strains examined, 35 (71·4%) produced one or more enterotoxin. Most strains (21 strains) produced only one type of enterotoxin of which type C was predominant.* In vitro *incubation of liver slices of cow and buffalo with enterotoxin C_2 resulted in a significant inhibition of protein and RNA synthesis in a dose-dependent manner. The maximum inhibition of protein and RNA synthesis was obtained at a dose of 5 μg enterotoxin.*

INTRODUCTION

Staphylococcal food poisoning is probably the main cause of food-borne disease in many countries all over the world. Dairy foods, especially cheese, are responsible for some of these outbreaks of food poisoning (Guitierrez *et al.*, 1982). About 10–20% of the human population are carrying enterotoxigenic *Staphylococcus aureus* strains (Casman, 1965) and they reach food, usually, through handling. At present five serologically different enterotoxins are recognized, enterotoxins A, B, C, D and E (Bergdoll *et al.*, 1971). Three types of enterotoxin C have been purified from separate strains

Food Chemistry 0308-8146/89/$03·50

of *S. aureus* and were classified as C_1 (Avena & Bergdoll, 1967), C_2 (Borja & Bergdoll, 1967) and C_3 (Reiser *et al.*, 1984). The staphylococci are among the most important etiological agents of mastitis, a disease of great sanitary and economic significance in Egypt and other Mediterranean and Balkan countries, where some breeds of sheep are kept mainly for milk production (Guitierrez *et al.*, 1982). Many surveys were carried out to detect the enterotoxigenic strains of *S. aureus* in different types of dairy food in Egypt (Ahmed, 1980; Fathalla, 1984) and enterotoxin C was the most predominant.

The present study was designed to obtain information about the presence and level of contamination of cow, buffalo and sheep individual milk samples and different types of cheese, with *S. aureus*. The ability of *S. aureus* isolates to produce enterotoxins A, B, C, D and E was also taken into consideration. This shows the dangers of *S. aureus* enterotoxins to which the human population in Egypt is exposed. The present investigation also aimed to study the *in vitro* effects of enterotoxin C_2 on the rate of protein and RNA synthesis in cow and buffalo liver. This underlines the problem with farm animals suffering from mastitis, which is widespread in Egyptian cows and buffaloes. The expected effect of enterotoxin C_2 on milk quality, regarding the amount of protein synthesized, may thus be evaluated.

MATERIALS AND METHODS

Incidence of *Staphylococcus aureus* and enterotoxins in dairy products

Eighty-eight milk and cheese samples were collected randomly from different areas in Alexandria city. The samples included 43 cheese samples (14 Domiati, 1 Pyramid, 16 Ras, 10 Edam and 2 processed) and 45 raw milk samples (28 cow's milk, 4 of which were from mastitic cases, 14 buffalo samples, apparently healthy, and 3 sheep milk samples from mastitic animals). The apparently healthy cows (24) and buffaloes (14) may have been suffering from early stages of mastitis. Samples of raw milk were collected in sterile bottles. The udders were washed thoroughly with warm water and soap and were dried carefully with paper towels, and teat orifices were swabbed with 70% alcohol. Cheese samples were collected in sterile widemouth stoppered bottles. In the laboratory 11 g of thoroughly mashed soft processed cheese were transferred to a sterile mortar; then 99 ml of sterile 2% sodium citrate solution were added to make a dilution. Eleven grams of hard cheese were also cut into small pieces, and were milled in a sterile mortar using sterile sand. A suspension was prepared by adding 99 ml of sterile 2% sodium citrate to make a dilution of 1:10.

Staphylococcus aureus (*S. aureus*) strains were isolated according to Bailey

& Scott (1974). Milk samples or 2 g of cheese were centrifuged at 3×10^3 rpm for 3 min, and sodium chloride broth tubes were prepared and inoculated with part of the obtained sediment. Inoculated tubes were incubated at 37°C for 24 h and loopfuls, from inoculated sodium broth as well as directly from the prepared samples, were streaked on mannitol salt agar plates (Bailey & Scott, 1974; Boothby *et al.*, 1979). Isolates were identified using the criteria of the *Bergey's Manual* (Breed *et al.*, 1957) and of the International Sub-Committee of the Taxonomy of Staphylococci and Micrococci cultures (1965).

Coagulase test: Coagulase activity was studied by the tube method using desiccated rabbit plasma (coagulase plasma ethylenediaminetetraacetic acid (EDTA), Difco). The test was carried out according to the method of Sperber & Tatini (1975). Only 4+, 3+ and 2+ reactions were considered as positive evidence of coagulase production.

Anaerobic fermentation of glucose and mannitol: Anaerobic use of glucose and mannitol as well as fermentation of these sugars were tested by the method recommended by the Sub-Committee of Taxonomy of Staphylococci and Micrococci (1965).

Thermonuclease test: Production of heat-labile nuclease by isolate produced heat-stable nuclease which was determined by the microslide and plate method (Barry *et al.*, 1973) using toluidine blue-deoxyribonuclease acid agar (TBD).

Acetoin test: Acetylmethylcarbinol production was studied by the method recommended by Baird-Parker (1979).

Hemolysin test: Production of β-hemolysin was investigated by the plate method of Bailey & Scott (1974).

Growth on 15% sodium chloride: The growth on 15% sodium chloride was studied by the method of Abd-El-Malek & Gibson (1948).

Hydrolysis of arginine: The method of Abd-El-Malek & Gibson (1948) was used.

Phosphate production: Phosphatase production was detected by the method of Baird-Parker (1965).

Egg yolk reaction: Egg yolk factor production investigated on Baird-Parker medium containing 5% egg yolk tellurite emulsion.

Pigmentation of colonies: Isolates were grown on Brain Heart Infusion agar. The appearance of yellow to orange pigmented colonies on Petri dishes was taken as preliminary indication of *S. aureus* (Guitierrez *et al.*, 1982).

Enterotoxin assay: All strains which were considered as *S. aureus* were assayed for enterotoxins A, B, C, D and E. The cellophane-over-agar technique was used for estimation of enterotoxin production. The enterotoxins were detected and identified by the optimal sensitivity plate

method (Robbins & Bergdoll, 1974). Enterotoxin antisera used were lyophilized sera for A, B, C, D and E enterotoxins, and were provided by Professor M. S. Bergdoll (Food Research Institute, University of Wisconsin, Madison, USA). Enterotoxin references were rehydrated lyophilized standards, also a gift from Professor M. S. Bergdoll. The final concentrations and dilutions used were as required for the OSP technique.

Effect of enterotoxin C_2 on protein synthesis: Cow and buffalo liver tissues were obtained from 1–2 year old animals within 10 min after slaughter, sliced and rinsed in buffer at 0–4°C. The incubation medium of 1 ml Krebs-Ringer bicarbonate (KRB) buffer, pH 7·4, containing 400 mg% glucose, 1 μCi of ^{14}C-lysine (342 mCi/mmole, New England Nuclear, Boston, Mass., USA) and the tested enterotoxin C_2 (gift from Professor M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison, USA). SEC_2 contained 18 μg N_2/ml, and 1 μg/ml solution gave one band by the gel diffusion technique of Oakley & Fulthorpe (1953), while no other antigen could be detected even when 1 mg/ml was used. Additionally the toxin showed one band on polyacrylamide gel electrophoresis plates.

Liver slices (50 mg) were incubated for 3 h in Dubnoff shaker at 60 cycles/min at 37°C under a 95% O_2–5% CO_2 atmosphere, then removed from the medium, rinsed twice in 0·9% NaCl and stored at −20°C. Frozen tissues were homogenized in 2 ml of 0·01M unlabelled lysine. Proteins were precipitated with trichloroacetic acid (TCA) and extracted from aliquots of liver homogenates according to the procedure of Seikevitz (1952). The dried TCA-precipitable proteins were dissolved in 1N NaOH. One aliquot was used for radioactive measurement, and duplicate aliquots were used for protein determination by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. The results were expressed as dpm of the radioactive lysine incorporated per mg protein.

Effect of enterotoxin C_2 on RNA synthesis: Preparation of samples and incubation conditions were similar to those described for protein synthesis, except that ^{14}C-lysine was replaced by 1 μCi of ^{14}C-uridine (56 mCi/mmole, New England Nuclear, Boston, Mass., USA) and that 100 mg (instead of 50 mg) tissue were used for incubation. Slices were then removed from the medium, rinsed twice in 0·9% NaCl and stored at −20°C. RNA was then isolated, solubilized and measured according to the method of Fleck & Munro (1962). An aliquot from the clear supernatant was used for radioactivity measurements. Results were expressed as dpm of ^{14}C-uridine incorporated per 100 mg tissues. Data were statistically analysed by analysis of variance and Duncan's Multiple Range Test (Snedecor & Cochran, 1967).

RESULTS

Staphylococcus aureus and its enterotoxins in raw milk and cheese

The results of biochemical tests showed that out of the picked-up colonies from mannitol salt agar plates, 165 isolates were identified as *Staphylococcus* spp. of which 49 isolates were *S. aureus* strains. The total number of strains isolated from milk were 32 (20 from cow's milk, 6 from buffalo's milk and 6 from sheep milk). The total number of strains isolated from cheese were 17 (9 from Domiati, 5 from Ras and 3 from Edam). Pyramid and processed cheese samples were free from any contamination. Among the 49 *S. autreus* strains examined, 35 (71·4%) produced one or more enterotoxin. Most strains (21 strains) produced only one type of enterotoxin, while others produced up to three enterotoxins simultaneously. From the 21 strains which produced a single enterotoxin, type C was predominant. Enterotoxin B-producing strains were not detected. Fourteen strains produced more than one enterotoxin, 10 of which produced enterotoxin C in combination with other enterotoxins (Table 1).

Effect of enterotoxin C_2 on protein and RNA synthesis

Table 2 indicates that enterotoxin C_2 significantly inhibited ($P < 0{\cdot}05$) protein and RNA synthesis by cow liver slices in a dose-dependent manner. The maximum inhibition of protein and RNA synthesis was obtained at a dose of 5 μg/ml enterotoxin.

TABLE 1

Enterotoxin Production by *Staphylococcus aureus* (*S. aureus*) Isolated from Milk and Cheese

Treatment	*Dose* (*μg/ml*)	*Protein synthesis* (*dpm/mg protein*)*	*RNA synthesis* (*dpm/100 mg tissue*)*
Cow			
Control	0	$2\,449 \pm 90^{a}$	$3\,452 \pm 589^{a}$
Enterotoxin C	1	$2\,171 \pm 211^{a,b}$	$2\,828 \pm 60^{a,b}$
	5	$1\,984 \pm 173^{b}$	$2\,692 \pm 210^{b}$
	10	$1\,904 \pm 225^{b}$	$2\,499 \pm 420^{b}$
Buffalo			
Control	0	$1\,555 \pm 117^{a}$	$1\,482 \pm 93^{a}$
Enterotoxin C	1	$1\,391 \pm 111^{a}$	$1\,300 \pm 63^{a,b}$
	5	$1\,149 \pm 82^{b}$	$1\,028 \pm 48^{c}$
	10	—	$1\,256 \pm 54^{b}$

* Mean values of 6 observations ± SE.

[a,b,c] Within each column of each treatment group, means with different superscript letters differ significantly ($P < 0{\cdot}05$).

TABLE 2
Effect of Enterotoxin C on the *in vitro* Rates of Protein and RNA Synthesis by Cow and Buffalo Liver

Source of S. aureus *strains*	*Number of isolated* S. aureus *strains*	*Number of toxigenic strains*	*Types of toxin produced*										
			Number of strains producing single enterotoxin					*Number of strains producing more than one enterotoxin*					
			A	*B*	*C*	*D*	*E*	*AC*	*AD*	*AB*	*CD*	*ACE*	*ABD*
Raw milk (45)	32	22	3	—	8	—	2	5	1	1	1	—	1
Cheese (43)	17	13	2	—	2	1	3	1	1	—	—	3	—
Total	49	35	5	—	10	1	5	6	2	1	1	3	1
%		71·4	14·4		28·6	2·8	14·4	17·1	5·7	2·8	2·8	8·6	2·8

Table 2 also shows that enterotoxin C_2 significantly inhibited ($P < 0.05$) protein synthesis by buffalo liver at a dose of 5 μg/ml. RNA synthesis was also inhibited ($P < 0.05$) by enterotoxin C_2 at a dose of 5 and 10 μg/ml. The maximum inhibition of protein and RNA synthesis was obtained at a dose of 5 μg/ml enterotoxin.

DISCUSSION

Staphylococcus aureus and its enterotoxins in raw milk and cheese

The laboratory-controlled investigation of samples of raw milk and cheese revealed greater percentages (71·1%) for the isolation of *S. aureus* from raw milk samples than those from cheese (39·5%). Milk can be contaminated by *S. aureus* that are present on the skin of the udder of the animal during milking (Davidson, 1961) from animals that have Staphylococcal mastitis, and through milkers, water supply and flies (Harrey & Hill, 1967). Similar findings were reported by Ahmed (1980).

S. aureus isolated from cheese (39·5%) can be attributed to excessive manipulation during processing and storage, since most Egyptian cheeses are processed in small factories lacking automation. Similar percentages were reported by Abou-Donia *et al.* (1985) and Ahmed (1980). The nature and composition of the cheeses may affect their liability to Staphylococcal infection; hence the presence of *S. aureus* was greatest in Domiati cheese followed by Ras cheese then by Edam cheese. In addition, *S. aureus* seem to take advantage of the high salt content over other organisms and this may explain the highest percentage of Staphylococci in Domiati cheese. Hegazi (1984) found that Staphylococci were dominant in milk containing up to 9 or 12% NaCl, and that cheese poisoning by enterotoxin-producing Staphylococci is a real threat when the white pickled cheese is left for ripening in non-refrigerated whey. Additionally, the high water content in this type of cheese is a proper condition for the growth of microbes. Samples of Pyramid cheese (made from goat's milk and ripened by fungi) were free from *S. aureus* probably as a consequence of the high acidity in this type of cheese. The two samples of processed cheese were also free from *S. aureus*; one of the two samples had heavy growth of the fungi and the second was obtained freshly after packing directly. On the other hand, Ahmed (1980) found that 9% of the examined processed cheese samples contained *S. aureus*. In the present study, the failure to detect *S. aureus* in the Pyramid or processed cheese samples may be due to the small number of isolates examined.

The enterotoxigenicity of *S. aureus* isolates reached 71·4%. The published data showed large differences in enterotoxigenicity of *S. aureus* isolates from

milk and cheese. Among the enterotoxigenic isolates from raw milk, enterotoxin C producers were dominant. This agrees with previous studies in Egypt (Abou-Donia *et al.*, 1985) and in other countries (Olson *et al.*, 1970; Pandurangaroa, 1987). At the second position, isolates producing SEA and SEC were dominant followed by isolates producing SEA. In cheese, however, isolates producing SEA, SEC and SEE or SEE alone were dominant followed by SEA or SEC. On the other hand Dorsey *et al.* (1976) suggested that SEE would be less frequently associated with food poisoning outbreaks than other serotypes.

Effect of enterotoxin C_2 on protein and RNA synthesis

These results show that *in vitro* protein and RNA synthesis by liver tissues of cow and buffalo were significantly inhibited by graded doses of enterotoxin C_2. The inhibition of liver protein and RNA synthesis by enterotoxins may explain why mastitic milk has a low total protein content. Most cases of mastitis are caused by *S. aureus* infection. The microorganism secretes the enterotoxin C_2 during its growth inside the udder causing mastitis and the passage of toxin to the infected milk (Niskanen *et al.*, 1978). Some changes in milk composition then occur (particularly in milk proteins). Mastitic milk showed a 33% decrease in milk total proteins and a 38% decrease in milk casein (Ingr *et al.*, 1974). Mastitic milk thus showed longer coagulation time (Ingr *et al.*, 1973); its curd formation and drainage were slower, the curd particles took longer to harden and had a coarse texture and bitter taste. On the other hand, whey proteins (specially immunoglobulins), blood serum albumin and protease-peptones are increased in the mastitic milk (Singh & Ganguli, 1970).

In conclusion, these results indicate that protein metabolism is one of the main targets of enterotoxins. Further studies to evaluate the *in vivo* effects of enterotoxins on the rate of protein and RNA synthesis in lactating cows and buffaloes are urgently needed.

REFERENCES

Abd-El-Malek, Y. & Gibson, T. (1948). Studies in the bacteriology of milk. II. The Staphylococci and micrococci of milk. *J. Dairy Res.*, **15**, 249–60.

Abou-Donia, S., Fathalla, M., Rakha, A., El-Shazly & Youssef, N. (1985). Enterotoxins and thermonuclease production by *S. aureus* isolated from Domiati, Karish and Ras cheese. *J. Dairy Sci.*, **68**.

Ahmed, A. (1980). Studies on the enterotoxigenicity of *Staphylococcus aureus* isolated from milk and milk products. PhD thesis in milk hygiene, Faculty of Vet. Med., Assiut University, Egypt.

Avena, R. M. & Bergdoll, M. S. (1967). Purification and some physiological properties of enterotoxin C, *S. aureus* 361. *Biochem.*, **6**, 1474–80.

Bailey, W. & Scott, E. (1974). *Diagnostic Microbiology*. (4th edn), The C. V. Mosby Co., St. Louis, MO, p. 111.

Baird-Parker, A. C. (1965). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. Gen. Microbiol.* **38**, 363–87.

Baird-Parker, A. C. (1979). Methods for identifying staphylococci and micrococci. In *Identification Methods for Microbiologists*. (2nd edn), ed. F. A. Skinner & D. W. Lovelock. Academic Press, London, pp. 201–10.

Barry. A. L., Lachica, R. V. & Atchinson, F. W. (1973). Identification of *S. aureus* by simultaneous use of tube coagulase and thermonuclease test. *Appl. Microbiol.*, **25**, 496–7.

Bergdoll, M. S., Borja, C. R., Robbins, R. N. & Weiss, K. F. (1971). Identification of enterotoxin E. *Infect. Immun.*, **4**, 593–5.

Boothby, J., Genigeorgis, C. & Fanelli, M. J. (1979). Tandem coagulase/thermonuclease agar method for the detection of *S. aureus*. *Appl. Environ. Microbiol.*, **37**, 298–302.

Borja, C. R. & Bergdoll, M. S. (1967). Purification and partial characterization of enterotoxin C produced by *S. aureus* 137. *Biochem.*, **6**, 1467–73.

Breed. R., Murry, E. & Smith, N. (1957). *Bergey's Manual of Determinative Bacteriology*. (7th edn), Williams and Wilkins Co., Baltimore, MD, p. 1094.

Casman, E. (1965). Staphylococcal enterotoxin. *Ann. N.Y. Acad. Sci.*, **128**, 124–31.

Davidson, I. (1961). Observation on the pathogenic staphylococci in a dairy herd during a period of six years. *Res. Vet. Sci.*, **2**, 22–40.

Dorsey, A., Bennett, R. & Amos, W. (1976). Incidence of enterotoxin type E producing Staphylococcal of food, human and animal origin. *Abstracts of the Annual Meeting of the American Society for Microbiology*, **76**, 189.

Fathalla, M. (1984). Study of the Relationship Between the Presence of *Staphylococcus aureus* and its Products as Thermonuclease and Enterotoxin in Milk and Some of its Derivatives. PhD Thesis, Public Health Inst., Alexandria University.

Fleck, A. & Munro, H. (1962). The precision of ultraviolet absorption measurements in the Schmidt-Thannhanser procedure for nucleic acid estimation. *Biochem. Biophys. Acta.*, **55**, 571–83.

Guitierrez, L., Menes, I., Garcia, M., Moreno, B. & Bergdoll, M. (1982). Characterization and enterotoxigenicity of staphylococcal isolates from mastitic ovine milk in Spain. *J. Food Protection*, **45**, 1282–6.

Harrey, W. & Hill, H., (1967). *Milk Production and Control*. (4th edn), Lewis and Co., London.

Hegazi, F. (1984). The microbial flora of salted raw milk. *Syst. Appl. Microbiol.*, **5**, 527–33.

Ingr, I., Pleva, J., Rysanek, D. & Renda, V. (1973). Effect of subclinical mastitis on certain nutritive, technological and hygienic properties of milk. *Nahrung*, **77**, 225–32.

Ingr, I., Pleva, J., Rysanek, D. & Renda, V. (1974). Reduction of milk yield and its major constituents in cows suffering from mastitis. *Veterinarni Madicina* **19**, 527–32.

International Subcommittee on Staphylococci and Micrococci (1965). Recommendations of Subcommittee. *Int. Bull. Bact. Nomencl. Taxon.*, **15**, 109–10.

Lowry, O., Rosebrough, N., Farr, A., & Rondall, R. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

Niskanen, A., Koiranen, L. & Roine, K. (1978). Staphylococcal enterotoxin and thermonuclease production during induced bovine mastitis and the clinical reaction of enterotoxin in udders. *Infection and Immunity* **19**, 493–8.

Oakley, C. & Fulthorpe, A. (1953). Antigenic analysis by diffusion. *J. Pathol. Bacteriol.*, **65**, 49–60.

Olson, J., Corman, E., Bear, E. & Stone, J. (1970). Enterotoxigenicity of *Staphylococcus aureus* culture isolated from acute cases of bovine mastitis. *Appl. Microbiol.*, **20**, 605.

Pandurangaroa, C. C. (1977). Enterotoxigenic Staphylococci in milk. *Indian J. Microbiol.*, **17**, 105.

Reiser, R. F., Robbins, R. N., Noleto, A. L., Khoc, G. P. & Bergdoll, M. S. (1984). Identification, purification and some physiological properties of Staphylococcal enterotoxin C_3. *Infect. Immun.*, **45**, 625–30.

Robbins, R. & Bergdoll, M. (1974). Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Appl. Microbiol.* **28**, 946–50.

Seikevitz, P. (1952). Uptake of radioactive alanine *in vitro* into the proteins of rat liver fractions. *J. Biol. Chem.*, **195**, 549–65.

Singh, L. & Ganguli, N. (1970). Alterations in the properties of milk proteins during mastitis, *XVIII Int. Dairy Congr.*, **1E**, 638.

Snedecor, G. & Cochran, W. (1967). *Statistical Methods* (6th edn), Iowa State University Press, Iowa, USA.

Sperber, W. H. & Tatini, S. R. (1975). Interpretation of the tube coagulase test for identification of *S. aureus*. *Appl. Microbiol.*, **29**, 502–5.