قسم البكتريولوجيا
كلية الطب - جامعة أسيوط
رئيس القسم : أ٠د/ ماهر مختار زكي

معايرة السموم المعوية نوعي A ,C_3 المفرزة من ميكروب المكور العنقودي الذهبي في اللبن بطريقة التحليل الانزيمي الطيفي

شعبان هاشم ، أحمد عبدالحميد *، مصطفى خليل *، عماد نافع

للسموم المعوية المفرزة من ميكروب المكور العنقودي الذهبي في اللبن وما يتبعه من تواجده في مختلف منتجات الالبان خطورة كبيرة على الصحة العامة حيث أنه يسبب حالات كثيرة من التسمم الغذائي لذلك تم اجراء هذه الدراسة لمعرفة أسرع الطرق وأيسرها للكشف عن هذه السموم مباشرة في اللبن ٠

وقد تضمنت هذه الدراسة استخدام طريقة التحليل الانزيمي الطيفي لتقدير كمية السموم المعوية نوعي A ,C_3 المفرزة من ميكروب المكور العنقودي الذهبي في اللبن الخام وكذلك اللبن منزوع الدسم ٠

وقد لوحظ باستعمال هذه الطريقة أنه يمكن الكشف عن ٦٠ الى ٨٣٪ من مقدار هذه السموم في اللبن الذي لم يسبق تسخينه وذلك باستخدام طريقتين متتاليتين لاستخلاص هذه السموم ٠

وقد وجد أن الكشف المباشر بدون استخلاص هذه السموم من اللبن يعطي نتائج سلبيه كما لوحظ أن تسخين اللبن المحتوي على هذه السموم يؤدي الى تقليل مقدار هذه السموم التي يمكن الكشف عنها بمقدار لايزيد عن ٣٠٪ من النوع A ، ٢٠٪ بالنسبة للنوع C_3 ٠

وقد شملت الدراسة مناقشة خطورة تواجد هذه السموم في اللبن وكذلك أهمية استخدام هذه الطريقة الاقتصادية والسريعة للكشف الدوري عن هذه السموم المسببة للتسمم الغذائي٠

---

* قسم الرقابة الصحية على الأغذية - كلية الطب البيطري - جامعة أسيوط

Dept. of Bacteriology,
Faculty of Medicine, Assiut University,
Head of Dept. Prof. Dr. M.M. Zaki.

ENZYME-LINKED IMMUNONOSORBENT ASSAY (ELISA) FOR DETECTION OF STAPHYLOCOCCUS AUREUS ENTEROTOXINS A and C_3 IN MILK

(With 3 Tables)

By
S.H. AHMED; A.A-H. AHMED*; M.K. MOUSTAFA* and E.K. NAFIE

(Received at 6/3/1988)

SUMMARY

The enzyme-linked immunosorbent assay (ELISA) has proved to be a simple, reliable and sensitive test for the detection of Staphylococcal enterotoxins. It was employed in this investigation for the quantitative estimation of SEA and SEC_3 in raw and skim milk. It was observed that 60-83% of the toxin could be detected in unheated milk using the two successive extraction procedure of REISER, et al. (1974). Direct detection of SE in unheated milk gave false negative result. Heating SE containing milk samples caused immunological inactivation of the toxins, where the detectable amount of the toxin was reduced to 30% of SEA and 20% of SEC_3.

INTRODUCTION

Some strains of Staphylococcus aureus produce enterotoxins. The toxins are classified immunologically and designated from A to F. These enterotoxins are frequently the cause of food poisoning. The diagnosis of staphylococcal food poisoning is mostly done on clinical, epidemiological, and bacteriological evidence, but there is an urgent and widespread need for a simple and economical test (AHMED, 1980 & MEYER and PALMEIRI, 1980). Several immunological methods have been described: Single radial immunodiffusion (MEYER and PALMERIRI, 1980), reversed passive haemogglutination (SILOERMAN, et al. 1968), different solid-phase radio-immunoassays (BUKOVIC & JOHNNSON, 1981 and ROBERN, et al. 1975). However, all these methods have some drawbacks (e.g. time consuming extraction procedures, elaborate equipment, autoirradiation occurs and the reagents are consequently, short-lived, or there is a health hazard involved). Recently, more sensitive assay procedure, known as enzyme-linked immunosorbent assay (ELISA) introduced for the detection of staphylococcal enterotoxins BJORN, et al. 1981; NOTERMENS, et al. 1978; SAUNDERS & BARTLETT, 1977 and SIMON & TERPLAN, 1977). ELISA is, in its extreme sensitivity, comparable to radioimmunoassays, however, instead of a radioactive label an enzyme label is used, but it is simple to perform, and the reagents can be stored for more than a year.

* Dept. of Food Hygiene, Faculty of Vet. Med., Assiut University.

This study was planned to evaluate the sensitivity of ELISA in detecting staphylococcal enterotoxins in milk.

MATERIAL and METHODS

Staphylococcal enterotoxins and antitoxins:

Staph aureus enterotoxins A and C_3 and their specific antitoxins, as well as antistaphylococcal enterotoxins A and C_3 IgG conjugated to peroxidase were kindly supplied by Dr. M.S. Bergdoll from the Food Research Institute, Madison, Wis., USA.

Milk Samples:

Buffaloe's milk (raw and skim) used in this study, was obtained from Dept. of Food Industries, Fac. of Agriculture, Assiut University, Assiut, Egypt.

Inoculation of milk with staphylococcal enterotoxins:

Staphylococcal enterotoxins were dissolved in 0.07 M phosphate buffer pH 7.2, containing 0.15 M Nacl. Two different concentrations of each toxin (10 and 100 ng/ml) were added to milk 24 hr before the assay. The inoculated milk samples were stored during this period at 4°C. The following types of experiments were made for raw and skim milk. (a) The pH of the inoculated milk was adjusted at 7.4, the assay was carried out directly on the inoculated milk before and after heat treatment. (b) Milk samples inoculated with SE were subjected to extraction procedure, the supernatant extracts were assayed for enterotoxin before and after heat treatment. (c) The supernatant extracts obtained from step (b) were 10 fold concentrated by dialysis against a solation of polyethylene glycol (PEG, 35% w/a), then the assays were done before and after heat treatment of the concentrated extracts.

Extraction procedure:

Different foods, naturally or artificially contaminated with enterotoxins, were extracted according to the procedure described by REISER, et al. (1974) 100 gm of the food was mixed with 1 to 2 volumes of distilled water and homogenized in a blender. The pH was adjusted to 4.5 with 2 N Hcl, and the homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was brought to pH 7.5 with 2 N NaOH, stirred with 10% chloroform for 5 min, and centrifuged again as described above. The supernatant was readjusted to pH 4.5, centrifuged and neutralized. Tween 20 was added to the extracts at a final concentration of 0.25%.

ELISA procedure:

The sandwich ELISA technique as described by ENGVALL and PERLMANN (1971) was employed with minor modifications of NOTERMANS, et al. (1978), wells of polystyrene trays were coated with anti SE-IgG from sheep (0.1 ml of anti SE-IgG diluted in 0.07 M, phosphate buffer, pH 7.2 containing 0.15 M Nacl (PBS) were added to each well; incubation was overnight at 20°C: After incubation, trags were washed by a continuous flow of tapwater containing 0.05% Tween 20 for 0.5 min). After coating, 0.1 ml of milk samples were added to each well. The trays were incubated at 20°C for 90 min, followed by washing as described above. The amount of absorbed SE was measured using anti SE IgG conjugated to peroxidase (0.1 ml of conjugate dilated in PBS containing 0.05 Tween 20 was added to each well. Incubation and washing were as described for sample incubation. The enzyme reaction was determined spectrophotometrically at 450 nm after addition of a solution of 5-amino salicylic acid and

H_2O_2 (0.15 ml of 0.07@ 5-aminosalicylic acid pH 6.0 containing 0.005% H_2O_2 was added to each well. After incubation of 30 min at 20°C, light absorption was measured).

RESULTS

The obtained results were recorded in Tables 1, 2 & 3.

DISCUSSION

According to the data summarized in Tables 1-3, assay of SE in milk using ELISA technique is markedly affected by the amount of fat and protein present. As seen in Table 1, ELISA failed to detect SE in raw milk with high concentration of fat and protein. In case of skim milk with lower concentration of fat little amount, not exceeding 30% of the inoculated toxin could be detected before heat treatment. Detection of SE after heating in raw and skim milk gave unsatisfactory results. The amount of SE that could be detected was reduced to not more than 4% in heated skim milk and to zero percent in raw milk.

The best method for quantitative estimation of SE in milk using ELISA is that of REISER, et al. (1974). By this method, the detection capacity of ELISA increased to 82% of the toxin inoculated as seen in skim inoculated with SEA and to 60% as in case of unheated raw milk injected with SEC_3 (Table 2).

A ten-fold concentration of the extract against PEG, resulted in an increase of the toxin concentration and consequently increased the sensitivity of ELISA in detecting the toxin. The increase of toxin concentration obtained was approximately 5 to 10 times (Table 3).

Enzyme linked immunosorbent assay (ELISA) have been improved to be very useful in detecting specific antigens in different heterogenous solutions (YOLKEN, et al. 1977; STIFFER-ROSENBERG, 1978; BERDAL, et al. 1979; DOSKELAND and BERDAL, 1980 and FREED, et al. 1982). Many cases of food poisoning caused by consumption of milk and milk products have been documented (HENDRICKS, et al. 1959 and AHMED, 1980). Milk may be contaminated with enterotoxigenic strains of staphylococci before and after heat treatment. Quantities of 0.5 to 5 ug of SE ingested with milk can produce symptoms of food poisoning. STIFFLER-ROSENBER and FEY (1978) could detect SE in supernatants and food extract down to 0.1 ng/ml levels by means of ELISA test.

Direct detection of SE in raw and skim milk before and after heat treatment at pH 7.4 gave unencourging results (Table 1). Similer findings were observed by LIND (1974) and NOTERMANS, et al. (1978). They reported that some of the major inconvenience with enterotoxin ELISA Sandwich procedure is the presence of protein and fat. Such interferring substances when present in high concentrations in supernatant and food extracts may lead to false-positive or false-negative results.

A very promising extraction procedure for detection of SE in milk using ELISA is that of REISER, et al. (1974).

Two successive extractions at pH 4.5 and pH 7.4 (Table 2). By this method 2 ng of SE per ml of milk could be detected. Our data obtained are similar to those obtained by BUNING-PFAUE, et al. (1980). They used ELISA for detectiono of SE in vanilla custard and

found that two successive extraction procedure at pH 4.5 and pH 7.4 followed by 20-fold concentration was sufficient to detect 0.1 ug SEB in 100 gm of custard. Also, a similar findings was recorded by NOTERMANS, et al. (1978). They used the same technique in extraction of staphylococcal enterotoxins A, B, C and E from minced meat. They succeeded to detect less than 0.5 ug of SE per 100 gm of meat with a recovery rate of 40-80% of the toxin.

Detection of SE in raw and skim milk after heating gave unsatisfactory result (Table 1). Heating SE in the extract of milk lead to rapid inactivation of the SE (Table 2, 3). SATTERLEE and KRAFT (1969) observed that heating SEB in the presence of either myosin or metmyoglobin resulted in a rapid loss of the immunological activity of the toxin.

STIFFLER-ROSENBERG and FEY (1978) found that, when ELISA used for detection of SE in food especially certain cheese products; some troubles are met with the non specific interferring substances present as gelatin, fat and protein. But this can be overcome when these substances or most of them are eliminated from the extracts.

In this study, it was possible to detect a very small amount of SE which is 10 to 100 times lower than that provoking clinical symptoms of food poisoning. Therefore, ELISA can be used economically for routine diagnosis of staphylococcal food poisoning after removing the non specific interferring substances present in food.

REFERENCES

Ahmed, S.H. (1980): Studies on acute gastrointestinal infections in Assiut Governorate. Ph.D. Thesis, Assiut University, Assiut, Egypt.

Akatov, A.K.; H. Mochmann; A.L. Khatenever; U. Richter; I.V. Kushko; W. Kavsch; S.D. Voropaeva and M.K. Mazurenko (1980): Occurrence of enterotoxigenic strains among Staphylococcus aureus isolated from different sources. Zh. Mikrob. Epidem. Immun. SSSR. 11, 56-60.

Berdal, B.P.; C.E. Farshy and J.C. Feeley (1979): Detection of Legionella pneumophila antigen in urine by enzyme linked immunospecific assay. J. Clin. Microbiol. 9: 575-578.

Bjorn, P.B.; O. Qrijan and T. Omland (1981): A Sandwich method for detection of Staphylococcus aureus enterotoxins. Acta. Pathol. Microbiol. Scand. Sect. B. 89: 411-415.

Bukovic, J.A. and H.W. Johnson (1975): Staphylococcal enterotoxinc: Solid-phase radio-immunoassay. Appl. Microbiol. 30: 700-701.

Büning-Pfaue, H.; P. Timmermans and S. Notermans (1980): Einfache Method für den Nachweis von Staphylokokken enterotoxin-B in Vanillepuding mittels ELISA. Test. Lebensm. Unters. Forsch. 173: 351-355.

Doskeland, S.O. and B.P. Berdal (1980): Bacterial antigen detection in body fluids: methods for rapid antigen concentrations and reduction of nonspecific reactions. J. Clin. Microbiol. 11: 380-384.

Engvall, E. and P. Perlmann (1971): Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8: 871-874.

Freed, R.C.; M.L. Evenson; R.F. Reiser and M.S. Bergdoll (1982): Enzyme-linked immunosorbent assay (ELISA) for detectionof Staphylococcal enterotoxin in food. Appl. Environ. Microbiol. 44: 1349-1355.

Hendricks, S.L.; R.A. Belknap and W.J. Hausler, Jr. (1959): Staphylococcal in toxication due to cheddar cheese. I. Epidemiology. J. Milk food Technol. 22: 313-317.

Lind, I. (1974): Protein A production in different strains of Staphylococcus aureas under varied growth conditions Acta. Path. Microbiol. Scand. Sect. B. 82: 821-828.

Meyer, R.F. and M.J. Palmieri (1980): Single radial immunodiffusion method for screening Staphylococcal isolates for enterotoxin. Appl. Environ. Microbiol. 40: 1080-1085.

Notermans, S.; H.I. Verjans, J. Bol, and M. Van Schotheorst (1978): Enzyme-linked immunosorbent assay (ELISI) for determinationof Staphylococcus aureus enterotoxin type B. Health Lab. Sci. 15: 28-31.

Reiser, R.; D. Conaway and M.S. Bergdoll (1974): Detection of Staphylococcal enterotoxin in foods. Appl. Microbiol. 27: 83-85.

Robern, H.; M. Dighton; Y. Yano and N. Dickie (1975): Doubble antibiody radioimmunoassay for staphylococcal enterotoxin C_2. Appl. Microbiol. 30: 525-529.

Satterlee, L.D. and A.A. Kraft (1969): Effect of meat and isolated meat proteins on the thermal inactivation of staphylococcal enterotoxin B. Appl. Microbiol. 17: 906-909.

Saunders, G.C. and M.L. Bartlett (1977): Double-antibody Solid phase. enzyme immunoassay for the detection of staphylococcal enterotoxin A. Appl. Environ. Microbiol. 34: 518-522.

Siloerman, S.J.; A.R. Knott and M. Howard (1968): Rapid, Sensitive assay for staphylococcal enterotoxin and a comparison of serological methods. Appl. Microbiol. 16: 1019-1023.

Simon, E. and G. Terplan (1977): Nachweis von staphylokokken Enterotoxin B mittels ELISA-Test. Zentralbl. Veterinaer. Med. Reihe B. 24: 842-844.

Stiffer-Rosenberg, G. and H. Fey (1978): Simple assay for Staphylococcal enterotoxin A, B and C: modification for enzyme-linked immunoassay. J. Clin. Microbiol. 8: 473-479.

Yolken, R.H.; H.B. Greenberg; M.H. Merson; R.B. Sack and A.Z. Kapikian (1977): Enzyme-linked immunosorbent assay for detectionof Escherichia coli heat labile enterotoxin. J. Clin. Immunol. 6: 439-444.

Table (1)
Detection of staphylococcal enterotoxins (SE) in raw and skim milk before and after heat treatment

Amount and type of SE added to milk (ng/ml)		Detectable amount of SE (ng/ml)			
		in raw milk		in skim milk	
		unheated	after heating*	unheated	after heating
SE	10	ND	ND	3	ND
	100	ND	ND	18	2
SEC_3	10	ND	ND	2	ND
	100	ND	ND	15	4

ND = non detectable

* = heating at 100°C for 20 min.

Table (2)
Detection of staphylococcal enterotoxins (SE) in extracts from raw and skim milk before and after heat treatment

Amount and type of SE added to milk (ng/ml)		Detectable amount of SE (ng/ml)			
		in raw milk		in skim milk	
		unheated	after heating*	unheated	after heating
SEA	10	7	ND	8	ND
	100	74	5	82	3
SEC_3	10	6	ND	7	2
	100	66	10	68	9

ND = non detectable
* = heating at 100°C for 20 min.

Table (3)
Detection of staphylococcal enterotoxins (SE) in concentrated extracts of raw and skim milk before and after heat treatment

Amount and type of SE added to milk (ng/ml)		Detectable amount of SE (ng/ml)			
		in raw milk		in skim milk	
		unheated	after heating*	unheated	after heating
SEA	10	76	ND	82	ND
	100	79U	9	850	7
SEC_3	10	35	ND	40	4
	100	430	16	460	18

ND = non detectable
* = heating at 100°C for 20 min.
U = 10-fold concentration.