DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN A, B, C, AND D BY LATEX BEAD TECHNOLOGY

By

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INTRODUCTION

Staphylococcal food intoxication, caused by ingestion of enterotoxins produced by certain strains of Staphylococcus aureus, is one of the major health hazards confronting food processors, food services, and food consumers. Staphylococci are among the hardiest of all non-sporeforming bacteria, as they will remain alive for months on the surface of sealed agar plates stored at 4 C (Davis et al., 1973). Since virulent strains are carried asymptomatically in the nasopharynx of 10-50% of normal adults, control of these organisms is quite difficult (Volk, 1982). Seven antigenetically distinct enterotoxins of Staphylococcus aureus, designated A, B, C_1 , C_2, D, E, and F have been described (Volk, 1982). When an enterotoxigenic strain of S aureus becomes established in a food product, environmental growth conditions may become optimum to allow for proliferation of the organism resulting in the production of enterotoxins. Ingestion of these enterotoxins usually results in the characteristic symptoms of staphylococcal food intoxication: diarrhea, vomiting, and enteritis in 2-6 hours. For regulatory and epidemiological purposes in investigating foodborne illnesses, it is important to be able to recognize the presence of staphylococcal enterotoxins in a suspect food product. To this end, various procedures have been developed: Single and double gel diffusion tube assay (Hall et al., 1965; Read et al., 1965), single and double gel diffusion plate or microslide assay (Casman and Bennett, 1965; Robbins et al., 1974; Meyer and Palmieri, 1980), passive and reversed passive hemagglutination assay (PHA and RPHA) (Johnson et al., 1967; Morse and Mah, 1967; Silverman et al., 1968), radioimmunoassay (Orth, 1977; Niyomvit et al., 1978), and enzyme-linked immunosorbent assay (Saunders and Bartlett, 1977; Kauffman, 1980). Of these, the RPHA test is the most simple and rapid procedure; however, the test occasionally shows a non-specific agglutination reaction with certain types of food

(Bergdoll, 1979). To replace the red blood cells, use of polystyrene latex particles was introduced by Salomon and Tew (1968). The system was improved by Oda et al. (1979) and Shingaki et al. (1981) and called passive latex agglutination test. The test kits are now commercially produced by Oxoid (Oxoid Limited, Wade Road Basingstok, Hampshire, RG-24 0PW England). Antiserum is taken from rabbits immunized with each of the specifically purified staphylococcal enterotoxins, A, B, C, or D. The corresponding immunoglobulin is purified by affinity chromatography. Polystyrene latex particles are sensitized with these purified immunoglobulin. These sensitized latex particles will agglutinate in the presence of the corresponding enterotoxin. The official procedure to detect the enterotoxins is the microslide diffusion method as recorded in Bacteriological Analytical Manual (FDA,1984). Recently U.S.D.A recommended the use of immunoassay for the detection and quantification of staphylococcal enterotoxins from meat products and/or broth culture fluids by use of the reverse passive latex agglutination technique as a presumptive test and the biotin-streptavidin enzyme linked immunosorbent assay for confirming the presence of the toxins and their quantification (Laboratory Communication No.52 March 19, 1986 issued by Ralph W Johnston).

The objectives of this study were to test the Oxoid staphylococcal enterotoxin system, to improve the procedure by introducing a capillary system, and to survey the occurrence of toxigenic strains of Staphylococcus aureus in meat using latex bead technology.

LITERATURE REVIEW

Incidence

Staphylococcal food intoxication remains to be one of the most prevalent form of food poisoning along with Clostridium perfringens and Salmonella spp. in the U.S.A. Ingestion of food containing enterotoxins results in : vomiting, nausea, retching, abdominal cramping, and prostration in 2-6 hours. Some individuals may not show all the symptoms associated with the illness (Bergdoll, 1979). Recovery generally takes two days, however, in severe cases it might take three days or even longer. Staphylococcal food intoxication is rarely fatal, although some cases have occurred among the elderly and infants.

Since recognition of staphylococci by Pasteur in 1880, cases of staphylococcal food poisoning have been reported, but the true incidence is unknown. This is because of poor response by victims to interviews conducted by health officials, misdiagnosis due to symptoms similar to those of other types of food poisoning, inadequate collection of samples for laboratory analysis, and improper laboratory examination (Bennett, 1986). According to the literature, Dack et al. (1930) were the first to show a case of food poisoning which was directly related to staphylococcus by inducing typical symptoms in human volunteers with a filtrate from a pure culture of the suspected organism. Between 1945 and 1947 staphylococcal food poisoning accounted for 82 % of all food poisoning out-breaks reported to the United States Public Health Service (Feig, 1950). More recent figures are not nearly so high. According to the Centers for Disease Control (CDC, 1977, 1979, 1983a, 1983b), total outbreaks attributed to staphylococcal food poisoning were 23.8 % in 1968, 25 % in 1972, 15.7 % in 1973, 23.6 % in 1975, 19.8% in 1976, 15.9% in 1977, 14.9 % in 1978, 19.8 % in 1979, 12.2 % in 1980, and 17.6 % in 1981. Many foods have been

involved in staphylococcal food poisoning. Foods most often implicated in staphylococcal food-borne disease in the United States are meats and meat products, poultry, baked foods, and salads including poultry, fish, egg, and potato salad (Bennett, 1986). Table 1 presents the foods mostly incriminated in staphylococcal food poisoning outbreaks in the United States from 1975 to 1981 (Bennett, 1986).

Properties of Staphylococcal Enterotoxins

Casman (1960 a) described two types of enterotoxins which were designated type F (food poisoning) and type E (enteritis). In 1963 Casman and Bennett (1963) established a permanent system of nomenclature to conform to the rules for naming other bacterial toxins, and to provide for naming of new enterotoxins in an organized manner. As a result type F became enterotoxin A and type E was known as enterotoxin B. Since then enterotoxin C (Bergdoll et al., 1965), D (Casman et al., 1967), E (Bergdoll et al., 1971) and F (Bennett et al., 1972) have been reported.

Staphylococcal enterotoxins are somewhat unique among bacterial toxins in being proteins that are heat resistant. The biological activity of enterotoxin B remained active after heating for 16 hours at 60 C at pH 7.3 (Schantz et al., 1965). Heating of one preparation of enterotoxin C for 30 minutes at 60 C resulted in no change in serological reaction (Borja and Bergdoll, 1967). Heating enterotoxin A at 80 C for 3 minutes or at 100 C for 1 minute caused it to lose its capacity to react serologically (Bergdoll, 1967).

All enterotoxins are simple proteins which upon hydrolysis yield 18 amino acids with aspartic acid, glutamic acid, lysine, and valine being the most abundant. The amino acid sequence for enterotoxin B has been determined (Huang and Bergdoll, 1970). The N-terminal amino acid is glutamic acid and the C-terminal amino acid is lysine. Enterotoxins A, B, C, and E are composed of 239 to 296 amino acid residues.

Table 1. Foods Incriminated in Staphylococcal Food Poisoning Outbreaks in the United States, 1975 to 1981.

FOOD	1975	1976	1977	1978	1979	1980	1981	TOTAL PRODUCT
Beef	2	2	1	0	2	0	3	10
Lamb	0	0	1	0	0	1	0	2
Ham	16	3	9	10	8	3	8	57
Pork	2	0	1	0	0	1	2	5
Sausage	0	1	1	0	0	1	1	4
Chicken	0	2	3	0	1	2	3	11
Turkey	1	1	1	0	3	3	4	14
Other Meat	2	2	0	0	1	2	1	8
Shellfish	0	0	1	0	0	0	1	3
Other Fish	2	0	0	0	0	0	1	3
Dairy Products	0	0	1	0	0	0	3	4
Baked Foods	1	2	1	0	0	3	7	14
Fruits and Vegetables	0	1	0	0	0	0	1	2
Potato Salad	1	1	1	1	2	3	0	9
Poultry, Fish, Egg Salad	6	0	1	1	2	1	2	13
Other Salads	1	3	0	5	1	0	2	12
Mexican Foods	1	0	0	0	0	0	0	1
Multiple Foods	8	3	1	4	5	5	1	27
Other Foods	1	5	1	0	1	0	0	8
Unknown	1	0	1	2	7	2	5	18
Total Out Breaks	45	26	25	23	33	27	44	223

FROM CENTERS FOR DISEASE CONTROL (1976; 1977; 1983a, 1983b).
FROM BENNETT (1986).

The disulfide bridge in enterotoxin B has been shown to be non essential for biological activity (Dalidowiez et. al., 1966). The effect of acetylation, succinylation, guanidination, and carbamylation on the biological activity of enterotoxin B obtained from strain S-6 was reported by Chu et al. (1969). These investigators found that guanidination of 90% of the lysine residues had no effect on emetic activity or on the combining power of antigen-antibody reactions of this toxin. Properties of enterotoxins A, B, C_1, C_2, and E are shown in Table 2.

In their active state, the enterotoxins have been reported to be resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin, and papain, but sensitive to pepsin at a pH about 2 (Bergdoll, 1967).

Enterotoxin B was first to be purified primarily because of the relatively large amount of enterotoxin produced by the B-type strains. The maximum amount of enterotoxin that can be produced in culture media under ideal conditions is about 5-6 microgram per milliliter, however, levels of 350 and 60 microgram per milliliter or more of enterotoxin B and enterotoxin C, respectively, can be produced (Reiser and Weiss, 1969). Chesbro et al. (1976) suggested that enterotoxin B is a heterogenous molecule. These investigators found that two electrophoretically distinct toxins can be identified in cultures, one produced in early to mid-log phase and the other in mid-late log phase of growth.

Ingestion of 25 micrograms of enterotoxin B results in vomiting and diarrhea in humans and monkeys. The emetic effect of enterotoxin is the result of central nervous system stimulation after the toxin acts on neural receptors in the gut. Infrequent human volunteer toxicity studies made with crude toxin, culture broths, or toxin-bearing foods have shown that man is one of the most susceptible species to the action of enterotoxin. This was confirmed in a small group of volunteers who were

Table 2. Comparison of Enterotoxins A, B, C, and E[a]

Property	Enterotoxins				
	A	B	C_1	C_2	E
Nitrogen content (%)	16.2	16.1	16.2	16.0	
Sedimentation coefficient	3.03	2.89	3.00	2.90	2.60
Diffusion coefficient x 10^7 cm^2 sec^{-1}	7.94	8.22	8.10	8.10	
Reduced viscosity (ml/g)	4.07	3.81	3.40	3.70	
Isoelectric point	7.3	8.6	8.6	7.0	7.0
Partial specific volume	0.726	0.726	0.732	0.742	
Maximum absorption (mM)	277	277	277	277	277
Extinction (E^1_{cm}[%])	14.6	14.4	12.1	12.1	12.5
Toxicity ED_{50} Mg/monkey	5	5	6	5-10	10-20
Molecular weight	27,800	28,366	26,000	34,100	29,600
C-terminal amino acid	serine	lysine	glycine	glycine	threonine
N-terminal amino acid	alanine	glutamate	glutamate	glutamate	serine

a Modified from Bergdoll et al. (1967_a, 1974) with the addition of C- and N-terminal amino acid information extracted from Bergdoll et al. (1965, 1967_a), Spero et al. (1965), and Huang et al. (1967). Enterotoxin E information from Borja et al. (1972).
From Miller (1975).

fed an aqueous solution of enterotoxin B preparation of 50 % purity (Raj and Bergdoll, 1969).

Strains of Staphylococcus aureus producing staphylococcal enterotoxin A (SEA) are generally most prevalent in cases of food poisoning. Payne and Wood (1974) surveyed 200 strains of S. aureus by using a double diffusion immuno-precipitation technique for the production of enterotoxins A, B, C, D, and E. Of these, 83 strains (41.5 %) produced only a single enterotoxin. Fifty nine strains produced staphylococcal enterotoxin A (SEA), 3 produced staphylococcal enterotoxin B (SEB), 8 produced staphylococcal enterotoxin C (SEC), 11 produced staphylococcal enterotoxin D (SED) and only 2 produced staphylococcal enterotoxin E (SEE). Shiozawa et al. (1980) examined 586 strains of S. aureus isolated from diseased and healthy chickens and found that only 16 strains produced staphylococcal enterotoxin. Eight enterotoxigenic strains were isolated from diseased chickens and the other eight were from healthy chickens. Enterotoxin D producing strains were dominant in diseased chicken while enterotoxin C producing strains were dominant in healthy chickens. The incidence of enterotoxin production by S. aureus strains isolated from five Nigerian ready-to-eat products was investigated by Abiodun (1984). Overall, 97 (39.1 %) of the 248 strains tested elaborated one of the enterotoxins. Of these, 43 (17.3 %) strains from all products produced SED, 31 (12.5 %) strains produced SEA, and 29 (11.7 %) produced SEE. The frequency of isolation of enterotoxigenic S. aureus in dining hall workers of a Nigerian university was determined (Abiodun et al., 1986). Of the 186 workers sampled, 47 (25.3 %) were carriers of enterotoxigenic S. aureus in their anterior nares. Fifty-five (26.6 %) of the 207 strains of S. aureus tested produced enterotoxin. Eighteen (8.7 %) strains elaborated SEA, 14 (6.8 %) strains produced SEC, and 13 (6.3 %) produced SED.

Effect of Sodium Chloride on Enterotoxin Formation

Sodium chloride plays many roles when it is used as an additive in food products. It acts as a flavoring agent and it has intense effects on functional properties of various foods. In addition, NaCl acts as an antimicrobial agent by inhibiting growth and/or activity of spoilage and pathogenic microorganisms. Sofos (1983) suggested that inhibitory action of NaCl in foods was indirect because addition of salt lowered the water activity with concomitant decreases in the growth and biochemical activities of many spoilage and pathogenic bacteria. McLean et al. (1968) investigated the effect of meat-curing salt and temperature on staphylococcal enterotoxin B production by Staphylococcus aureus using the double-gel diffusion method and found that concentrations of NaCl up to 10 % had a relatively slight effect on total growth, but above 3 % caused a definite decrease in toxin production. In addition they showed that neither $NaNO_3$ in concentrations up to 1000 ppm nor $NaNO_2$ up to 200 ppm (maximum concentration permitted in cured meats) would affect either growth or enterotoxin production in broth culture. Markus and Silverman (1970) demonstrated that concentrations of NaCl of up to 10 % did not essentially alter the ratio of enterotoxin formation to growth. They also showed that as much as 200 micrograms of $NaNO_2$ per milliliter and 1000 micrograms $NaNO_3$ /ml did not affect either bacterial growth or toxin formation by strain S-100. Markus and Silverman (1970) found that enterotoxin A secretion was increased by the presence of Tween 40 or 60 whereas Tween 20 and 80 and Span 60 had no effect. Span 80, although having a minor inhibitory effect on growth, completely inhibited enterotoxin A secretion.

Growth and production of SEC by S. aureus strain 137 in 3 % protein hydrolysate powder N-Z Amine NAK broth with 0 to 12 % NaCl and initial pH of 4 to 9.83 were studied during an 8-day incubation period at 37 C by Genigeorgis et al. (1971). The

rate of growth of strain 137 decreased as the NaCl concentration was increased gradually to 12 %. Optimum pH for growth of this strain appeared to be between 5 and 6.5. Enterotoxin C was produced in broths inoculated with more than 10^8 cells/ml. The pH range supporting SEC production was 5.45 to 7.3 for the inoculum level of 10^8 cells/ml and 6.38 to 7.3 for the level of $3.5x10^6$ cells/ml. Tompkin et al.(1973) studied the combined effect of pH, NaCl, and sodium nitrite by using a dialysis sac technique and showed that enterotoxin level decreased as NaCl concentration was increased in Brain-Heart Infusion (BHI) broth at pH 7. They also demonstrated that the effect of nitrite on growth and enterotoxin production was pH dependent. Castellani and Niven (1955), Lechowich et al. (1956), and Buchanan and Solberg (1972) demonstrated that nitrite inhibition of S. aureus growth in broth media was dependent upon pH. Genigeorgis et al. (1969) studied the effect of pH, salt and nitrite on enterotoxin B production under anaerobic conditions in laboratory cured ham and found that undissociated HNO_2 affected enterotoxin production at 10 C. The probability of ham with an initial pH of 5.58 or higher becoming toxic decreased as the HNO_2 concentration increased. Staphylococcal enterotoxin B was not produced below pH 5.58 anaerobically at 10 C. Pereira et al. (1982) demonstrated that a direct relationship existed between the increase of NaCl concentration and decrease of enterotoxin formation and production of SEB was affected more than that of SEA. Additionally neither enterotoxin was detected at NaCl concentration above 10 %. Woods and Wood (1982) studied the mechanism of the inhibition of Clostridium sporogenes by NaCl and found that there was a progressive decrease in glucose utilization and loss of intracellular ATP with increased concentration of salt. Smith et al. (1987) investigated the effect of NaCl on uptake of substrate by S. aureus strain 196E and found that SEA production, induction and enzymatic activity of phospho-Beta-galactosidase, and glucose utilization were 3.5 to 4.1 times more

sensitive to salt than was growth. Troller and Stinson (1978) showed that growth of S. aureus 196E (SEA producer) or C243 (SEB producer) was reduced by approximately 20 % when the salt level was increased from 0 to 5.3 %. SEA level, however, was unchanged by the increase of the salt level but SEB production was decreased by more than 4 fold.

Effect of Water Activity on Enterotoxin Production

The importance of microbial moisture requirements in the spoilage of many types of foods has been recognized. Staphylococci are particularly tolerant of low moisture levels, a characteristic frequently exploited by the addition of relatively high concentrations of NaCl to selective media. Scott (1953) demonstrated that growth rates of S. aureus cultures in media were uniformly reduced as a_w was lowered by addition of solutes. It was further noted that the minimal a_w which would allow staphylococcal growth (0.86) was independent of the medium and of solutes employed to adjust the a_w of the medium as well as the minimal water content which allows growth. McLean et al. (1961) found that concentration of NaCl greater than 3 % (a_w<0.98) severely limited SEB production. They found that this inhibition was produced without affecting total growth of the Staphylococcus aureus. However, Genigeorgis and Sadler (1966) observed SEB production from S. aureus S-6 in broth containing 10 % NaCl. Troller (1971) studied the effect of a_w on growth and SEB production found that the toxin production was very sensitive to slight reduction in a_w levels without a commensurate effect on growth. The production of SEA was markedly inhibited by decreases in a_w, although its production is relatively less sensitive to a_w than that of SEB (Troller, 1971). In addition he demonstrated that the greater portion of enterotoxin synthesis occurs during late logarithmic phase of growth and therefore he concluded that generation time is a function of a_w.

Depending on the solute employed, reduction in a_w levels from 0.99 to 0.97 or 0.98 reduced enterotoxin B levels by 90 to 99 % (Troller, 1971).

Unlike staphylococcal strains producing SEA, SEB producing strains are rarely implicated in outbreaks of food poisoning. This would be caused by the sensitivity of SEB producing strains to slightly reduced a_w levels. Many of the foods which offer opportunities for the production of staphylococcal enterotoxins do not exceed a_w levels of 0.95 to 0.96 levels. This would easily prevent the production of SEB but not production of SEA (Troller, 1972).

Effect of pH on Enterotoxin Production

Genigeorgis et al. (1969) studied the effect of pH and NaCl on enterotoxin production in cured ham and found that under anaerobic condition enterotoxins were not formed below pH 5.3 at 30 C or below pH 5.58 at 10 C. Ham having a pH below 5.20 and incubated at 10 C showed a continuous decrease in the number of inoculated cells by 1-3 log during 16 weeks of incubation. Metzger et al. (1973) demonstrated that optimal conditions for SEB production in a medium containing 4 % N-Z Amine A, 0.2 % dextrose, and 1 % yeast extract were achieved with pH controlled at 7. Under an alkaline condition (pH 8), minimal amounts of toxin were produced whereas under acid condition (pH 6) a 50 % reduction was observed. With pH controlled at 7, deletion of 0.2 % dextrose from the medium resulted in 40 % reduction in the 8-hour yield. Markus and Silverman (1970) showed that optimal pH for the secretion of SEB was 8.0 to 8.5 in a nitrogen-free medium and 7.0 to 7.5 in the presence of protein hydrolysate. The optimal pH for SEA formation by strain 100, in either the presence or absence of protein hydrolysate was 6.5 to 7.0 (Silverman and Markus, 1970). Tompkin et al. (1973) demonstrated that a decrease in toxin production in the medium occurred as pH decreased from 7.0 to 4.5 .

Effect of Temperature on Enterotoxin Production

The optimum temperature for growth of S. aureus is 37 C (Iandolo et al., 1964). This temperature is also optimal for enterotoxin production (Baird-Parker, 1971). However, the temperature for maximum toxin yield by S. aureus S-6 and S-137 in liquid medium cultured under aerobic conditions was determined to be 40 C by Vandenbosch et al. (1973). Tatini et al. (1971) reported that production of SEA, SEB, SEC, and SED in BHI broth was stimulated by incubation at 40 and 45 C. Pereira (1982) demonstrated that optimal growth temperature for strain S-6 in the NAK medium at pH 7 was 39.4 C and maximal production of both SEA and SEB corresponded to the optimal growth temperature with no toxin produced at 50 C and very little at 20 C.

Notermans and Heuvelman (1983) investigated the combined effect of a_w, pH, and suboptimal temperature on growth and enterotoxin production of S. aureus strains producing SEA, SEB, SEC, and SEE and found that SEA was produced under nearly all conditions of a_w and temperatures which allowed growth of S. aureus. Although temperature clearly affected the amounts of SEA produced, production of SEA at a_w 0.99 and 0.96 occurred at all incubation temperature allowing growth of S. aureus. Production of SEC was strongly influenced both by temperature and a_w. When using NaCl as a humectant, SEC was produced only at a_w 0.99 and 0.96 in combination with a temperature of 24 C and 18 C. When sucrose was used as a humectant no production of SEC was observed at a_w 0.96. Staphylococcal enterotoxin F and SEC were produced under nearly similar conditions. Silverman et al. (1983) reported that growth of S. aureus S-100 was influenced both by a_w and temperature. Staphylococcus aureus grew faster at 37 C than at 20 C, although the maximum populations attained were of approximately equal magnitude. At 37 C very slight growth occurred at a a_w of 0.86 but appreciable growth occurred at a a_w of 0.87. At 20 C the limiting a_w for

growth was 0.90. McLean et al. (1968) showed that lowering the incubation temperature decreased the amount of toxin produced without affecting the total amount of growth. Hirooka et al. (1987) investigated the change in pH, growth, thermonuclease, and SEA production by S. aureus in artificially inoculated cream pies, and found that S. aureus count varied from $<10^2$ CFU/g at 20 C to $1.6x10^4$ CFU/g at 37 C after 12 hours of incubation and from $8x10^2$ CFU/g at 20 C to $5.4x10^6$ CFU/g at 37 C after 35 hours of incubation. Thermonuclease was detectable after incubation for 35 hours at 20 C (2.9 ng/g), 12 hours at 30 C (9.4 ng/g), and 12 hours at 37 C (72 ng/g). Staphylococcal enterotoxin A was detectable after incubation for 35 hours at 20 C (3.9 ng/g), 18 hours at 25 C (3.9 ng/g), and 14 hours at 37 C (4.8 ng/g).

Effect of Age of The Culture on Enterotoxin formation

McLean et al. (1968) showed that the maximal SEB production occurs at the beginning of the stationary phase of growth. Metzger et al. (1973) found that release of SEB into the culture medium was initiated during the mid log phase of growth. Markus and Silverman (1968) established that SEB was produced by cells entering the stationary phase of growth and that, for a given medium, the secretion of SEB could be varied by altering the cultural conditions. The secretion of SEA by strain S-100 was found to occur mainly during the exponential phase of growth and was directly related to cell numbers. Secretion of SEA was influenced to a much lesser extent by cultural conditions compared with cell numbers. The secretion of SEA in shaken flask cultures occurred mainly in the exponential phase of growth although approximately 20 % was secreted as the cells entered the early stationary phase of growth (Markus and Silverman, 1968, 1969). Unlike SEB, considered as a secondary metabolite, SEA resembled a primary metabolite by being secreted during the exponential phase of growth (Markus and Silverman, 1970). As with SEB, the production of detectable

levels of SEA appeared to occur mostly during late logarithmic and early maximum stationary phase of growth (Troller, 1972).

Effect of Lactic Acid Bacteria on Enterotoxin Formation

Hirsch and Wheater (1951) and Oxford (1944) indicated that antibiotic substances (diplococcin and nisin) produced by certain strains of Streptococcus cremoris and Streptococcus lactis are effective against staphylococci. Dahiya and Speck (1968) and Thompson and Johnson (1951) demonstrated that inhibition of S. aureus may occur as a result of production of hydrogen peroxide by lactic acid bacteria. Iandolo et al. (1965) reported that inhibition of S. aureus by Streptococcus diacetilactis may involve a depletion of nutrients in the growth medium, particularly nicotinamide.

Representative strains of 15 species of lactic acid bacteria were examined for their ability to influence growth of S. aureus and production of enterotoxin in associative culture by Haines and Harmon (1973). Among the organisms used as effectors, streptococci were the most inhibitory, followed by Pediococcus cerevisiae. Lactobacillus and Leuconostoc citrovorum were not inhibitory to growth and only slightly inhibitory to enterotoxin production. Enterotoxin was detected in all cultures in which the population of S. aureus reached $8x10^7$ CFU/ml. At a lower S. aureus population no enterotoxin was detected after incubation for 48 hours. Kao and Frazier (1966) tested cultures of lactic acid bacteria, mostly from food, for their effect on the growth of S. aureus in Trypticase Soy broth, and found that some of the effectors (Lactobacillus lactis and L. brevis) stimulated growth of S aureus during early hours of growth especially at higher temperatures of incubation, but most cultures were inhibitory and some (S. faecium and L. mesenteroides) were even killing by the time of attainment of the maximal phase of growth of S. aureus. The more effector bacteria there were in the inoculum, the greater was the overall inhibition or

stimulation of S. aureus. Inhibition was most effective at 10 or 15 C, less so at 20 or 25 C and least at 30 or 37 C.

Troller and Frazier (1963) reported that maximum inhibition of staphylococci by food bacteria occurred in the pH range of 7.4 to 6.2. They also indicated that maximum inhibition of growth of S. aureus in association with other organisms occurred at temperatures of 20 to 25 C. Peterson et al. (1964) reported similar findings regarding the inhibition of S. aureus by psychrophilic saprophytes.

Noleto et al. (1987) grew two S. aureus strains in BHI broth and a meat medium along with Bacillus cereus, Streptococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa and found that both S. aureus strains grew well and produced enterotoxin in the presence of S. faecalis in BHI broth; however, enterotoxin production was observable in the meat medium only when the S. aureus inoculum was greater than S. feacalis inoculum. Staphylococcus aureus strain FRI-100 grown with B. cereus produced enterotoxin in both media only when the S. aureus inoculum was much higher than the B. cereus inoculum (10 versus 10^4 colony forming units), whereas strain FRI-196E produced enterotoxin in both medium at all inoculum concentrations except in the meat medium, when the inocula of the two organisms were the same. Staphylococcus aureus grown with E. coli in BHI produced enterotoxin in all inoculum combinations except when E coli inoculum was greater than S. aureus inoculum. Staphylococcus aureus strain FRI-100 grown with P. aeruginosa in either medium produced enterotoxin only when the S. aureus inoculum was much greater than P. aeruginosa.

Effect of Nutrients on Enterotoxin Production

Many media have been proposed for the isolation and enumeration of coagulase-positive staphylococci from foods. These media have been designed with the purpose

of selecting the coagulase-positive staphylococci without having to conduct confirming coagulase tests, nevertheless, most of the available media require that coagulase tests be done on single colonies for final confirmation. Williams (1972) reported that Polymyxin Mannitol Phenolphthalein Diphosphate Agar allowed only coagulase positive staphylococci to produce recognizable acid from mannitol and phenolphthalein from phenolphthalein-diphosphate within 40 hours at 37 C.

An acid hydrolysate of casein supplemented with glucose, thiamine, and nicotinic acid was designed by Favorite and Hammon (1941). Instead of acid hydrolysate a pancreatic digest of casein called Amigen was used by Segalove (1947). The name Amigen was later changed to Protein Hydrolysate Powder (PHP; Mead Johnson International, Evansville, Inc.) and became an ingredient of choice for most investigators for staphylococcal enterotoxins producing strains (Surgalla et al., 1951; Kato et al., 1966). Casman et al. (1967) used N-Z Amine A for production of enterotoxin D. Kato et al. (1966) found that a medium containing 3 % PHP and 3 % N-Z Amine NAK supplemented with niacin and thiamine gave maximum yields of enterotoxin B. On the other hand Metzger et al. (1973) reported that 4 % N-Z Amine A plus dextrose and yeast extract supported maximum production of enterotoxin B. Wu and Bergdoll (1971) developed a defined medium which could detect SEB at 125 micrograms /ml. This medium contained, in addition to vitamins and inorganic salts, 18 amino acids, the quantity of which was based on the rate of utilization of each amino acid. In their studies arginine appeared to be essential for enterotoxin production. Miller and Fung (1973) used the chemically defined medium of Mah et al. (1967), and deleted non-essential amino acids in a step-wise manner and found that arginine, cystine, and phenylalanine were essential for growth and toxin production with monosodium glutamate as a source of carbon and energy. However, proline and valine also seemed to play an important role in enterotoxin formation when glucose

was used as the sole source of energy.

Morse et al. (1969) reported that glucose severely repressed enterotoxin formation in complex media containing 1 % PHP plus vitamins. Toshiko et al. (1979) compared 4 % N-Z Amine NAK made with distilled water, naturally hard water, and synthetic salt solutions to investigate the effect of magnesium and iron on SEA, SEB, and SEC production. They found that magnesium, and to a lesser extent iron, were limiting factors in the production of SEB and SEC but not SEA. Maximum enterotoxin production with NAK medium was achieved by the addition of 5 mg of Mg^{2+}/L and 0.5 mg of Fe^{2+}/L. Eventhough Keller et al. (1978) found that increasing Mg^{2+} levels from 9.6 to 39 mg/L in a synthetic medium containing 2.05 % amino acids with vitamins and inorganic salts resulted in 80 % increase in enterotoxin B production, Toshiko et al. (1979) did not find Mg^{2+} above 9 mg/L to increase SEB yields with NAK medium.

Thermal Inactivation of Staphylococcal Enterotocins

Satterlee and Kraft (1969) studied thermal inactivation of enterotoxin B in a phosphate-saline buffer, in the presence of two meat proteins, myosin and metmyoglobin, and in a ground beef slurry. They showed that when SEB was incubated at temperatures from 60 to 110 C, the initial thermal inactivation at 80 C was faster than at either 60 or 100 C. Thermal loss of enterotoxin in a ground beef slurry was rapid when compared to inactivation in a phosphate-saline buffer. They reported that unusual instability of the enterotoxin at 80 C may be the result of SEB complex formation at this temperature but unable to form at higher temperatures (100 or 110 C). A similar anomaly was observed by Smith and Gardner (1949) who demonstrated that lecithinase of Clostridium perfringens was more stable at 65 C than at 100 C.

It is generally agreed that the heat stability of an enterotoxin varies with the type of toxin and the chemical and physical nature of the suspending medium. Denny et al. (1966) demonstrated that crude SEA is less resistant to thermal inactivation when compared to SEB and that the thermal inactivation of SEA was completed in 16.4 minutes at 250 F and 65 minutes at 212 F. Hilker et al. (1968) showed that the rate of thermal inactivation was in direct proportion to the initial SEA concentration in the sample. Denny et al. (1971) studied the heat inactivation of SEA and SEB in beef bouillon with three different concentrations of toxin and found that the inactivation of SEA was not directly proportional to concentration. Heat inactivation was more effective at the lowest initial concentration than at higher initial concentrations. Staphylococcal enterotoxin A was inactivated by less heat in a pH 7.2 phosphate buffer than in beef bouillon as detected by serology (Denny et al., 1971)

Jamlang et al. (1971) investigated the effect of pH, protein concentration, and ionic strength on heat inactivation of SEB and observed no activity after 20 minutes treatment at 70 C and 15 minutes at 80 C. However, it took 60 minutes at 100 C and 90 minutes at 90 C to achieve the same degree of inactivation. This again supported the findings of Satterlee and Kraft (1969) concerning low temperature protein aggregate phenomenon. A recovery of 35 to 40 % of the initial activity was achieved when the inactivated solutions were heated at 100 C for 6 minutes. Fung et al. (1973) investigated thermal inactivation profiles of SEB and SEC at 80, 100, and 121 C, and reported that SEC is more heat resistant than SEB. They also showed that after 24 hours of incubation at 25 C, some reactivation occurred in toxins that had been inactivated by heat. Reactivation was temperature dependent. Additionally stirring during heating prevented reactivation of enterotoxin. Humber et al. (1975) showed that SEA in beef bouillon was inactivated faster at pH 5.3 than at pH 6.2.

Production of Enterotoxins After Injury and Repair.

By using a method which permitted the selection of repaired cells from a population of heat injured and non-injured cells of S. aureus 196E, Smith et al. (1984) determined that the progeny of repaired cells retained the ability to produce SEA. There were large variations in the amount of SEA produced by the progeny of individual colony forming units before and after heating. In addition the average amount of SEA produced by the progeny of non-injured and repaired staphylococci were similar.

Thermal injury and death in S. aureus usually are accompanied by leakage of cellular constituents into the external medium. Compounds which protect bacterial cells against lethal or injurious effects of heat appear to decrease or prevent leakage of cellular constituents (Smith et al., 1985). Sucrose decreased heat lethality in S. aureus and heat injury in Salmonella typhimurium. In both organisms there was a decrease in leakage of 260-nm absorbing material (Allwood and Russel, 1967).

Injury and recovery characteristic of S. aureus after exposure to acetic, hydrochloric, and lactic acid were studied by Zayaitz and Ledford (1985). Cells of S. aureus were acid-injured at 37 C for 30 minutes in acidic isotonic saline solutions and were enumerated on Trypticase Soy Agar(TSA) and TSA with 7 % NaCl (TSAS). A difference of at least 25 % between counts on the non-selective (TSA) and selective (TSAS) medium was considered evidence of acid injury. The activities of coagulase and thermonuclease were reduced in injured cells. The absence of leakage of 260-280 nm absorbing material from acid-injured cells together with the absence of change in membrane fatty acids, indicated that membrane damage was not associated with acid injury.

Cells of S. aureus FRI-100 were exposed to a sublethal temperature of 50 C for 30 minutes in 0.1 M phosphate buffer using either microwave energy or a

conventional heating source. Following thermal stress, cells were allowed to recover. Injury was monitored as the difference between cell counts when an inoculum from the recovering cells was plated on TSA and TSAS. Total viable population following either heat treatment was 10^6 cells/ml as indicated by TSA counts. When the same suspensions were plated on TSAS, a viable count of $1.7x10^3$ cells/ml resulted from conventional heating compared with $5.6x10^2$ cells/ml following microwave irradiation. Greater membrane damage was sustained by the microwave-heated cells. In addition the microwave-heated cells regained their enterotoxin synthesis ability at a slower rate following recovery (Khalil and Villota, 1988).

Repair, growth, and enterotoxigenesis of Staphylococcus aureus S-6 injured by freeze-drying was investigated by Fung and Vandenbosch (1975). They demonstrated that mid-log growth phase S. aureus S-6 cells rapidly (0.5-1 h) repaired freeze-drying injury when rehydrated at mesophilic temperatures (20-50 C).

Methods for Detection of Staphylococcal Enterotoxin

To date several methods have been proposed for detection of staphylococcal enterotoxin from food. Biological tests which were available for the detection of enterotoxin were difficult to perform and of variable reliability. Of these, the monkey-feeding test was not sufficiently sensitive and the procedure of injecting cats or kittens was not specific (Casman and Bennett, 1965). Dolman and Wilson (1938) suggested that the problem might be solved by means of serological procedures.

Serological Methods: Since staphylococcal enterotoxins are simple proteins of reasonably good antigenicity, specific antibodies can be produced against them in various animals. A number of serological methods have been developed and used for the detection of staphylococcal enterotoxin by different investigators. Methods

proposed included hemagglutination, microslide gel diffusion, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA).

Hemagglutination Technique: Morse and Mah (1967), Johnson et al. (1967), and Silverman et al. (1968) proposed hemagglutination as a means of detecting enterotoxin. Of the several methods, reverse passive hemagglutination (Silverman et al., 1968) showed some promise with regard to sensitivity and relative ease of application. In this method, specific antibodies to staphylococcal enterotoxins are absorbed to sheep red blood cells treated with tannic acid. Bergdoll et al. (1976) substituted bisdiazotied benzidine for tannic acid in treating red blood cells. When such treated cells are reacted with specific enterotoxin, the cells agglutinated. Although this system seemed highly sensitive, Bennett et al. (1973) and Bergdoll (1979) experienced non specific reactions with this procedure. Bergdoll et al. (1976) and Bergdoll (1979) reported that antibodies in some sera failed to react with red blood cells. Reiser et al. (1974) reported that considerable treatment of food extract was required to eliminate non-specific reaction due to food proteins. Since there is no way to compare test results directly with a control, this method is not recommended as reliable for the detection of staphylococcal enterotoxin in foods (Bennett, 1986).

Gel Diffusion Tests: The principle of this method is based on linear migration of antibody and enterotoxin in gels. Some of the applications included Oudin single gel diffusion tube test, Oakley double gel diffusion tube test, and the capillary tube test (Fung and Wagner, 1971; Gandhi and Richardson, 1971; Fung, 1973). These techniques are mostly useful for the measuring of enterotoxin in culture fluids. These methods can be used in presumptive detection of enterotoxins in food extracts as long as good antibodies are available in the gel. For definitive identification the

microslide method must be used.

Microslide Method: The principle is based on the diffusion and migration of the antibody, the reference toxin, and the material being tested through the agar gel. The radial system was first described by Ouchterlony (1949) and was later miniaturized on a microscope slide by Crowle (1958). Casman and Bennett (1963) adapted a microslide method for the detection of staphylococcal enterotoxin. The main advantage of this system is that it facilitates direct comparison of the lines of precipitation formed by the interaction of a known antibody with known toxin, and the sample extract on the same slide. The sensitivity of this method is 0.1-0.01 microgram toxin /ml depending on the enterotoxin reference and antiserum concentrations. The microslide method has been described in detail by Bennett and McClure (1980), Bergdoll and Bennett (1984), and Bennett (1984). It is employed extensively for detection and identification of enterotoxins in culture fluids and in food extracts. AOAC (1984) recommended the microslide technique as the official method for detection of staphylococca enterotoxins.

Radioimmunoassay (RIA): Dickie (1970) suggested solid phase RIA as being potentially useful in meeting the requirements of a simple, rapid, and reasonably inexpensive approach in the quantitative determination of staphylococcal enterotoxins. Johnson et al. (1971) and Collins et al. (1972) reported the use of solid phase RIA for the detection of SEB. In the solid phase RIA suggested by Johnson et al. (1971), partially purified antibody was absorbed onto the internal a surface of polystyrene tube with protein adsorbing sites not covered by antibody blocked by addition of bovine serum albumin. The extract from the suspect test sample was added to the tubes. This provided an opportunity for adsorption of enterotoxin (if present) of a

food extract to react with the known antibody. After appropriate incubation, the labeled known enterotoxin was added and after washing the tube the radioactivity (I^{125}) was measured in a gamma counter. The sensitivity of this system is 0.0025-0.0015 microgram of enterotoxin/g of food and it requires 24-72 hour for completion (Johnson et al., 1973; Park et al., 1973). Collins et al. (1972) proposed a RIA system in which the antibody was coupled to bromacetylcellulose and particles were suspended in a small amount of borate bovine serum albumin solution. The sensitivity of their system was 0.01-0.001 microgram/ml for the detection of SEA and it required 3-4 hours for completion.

Although RIA system is highly sensitive, certain meat products such as salami have been reported by Johnson et al. (1973) to cause as much as 50 % or greater nonspecificity. Bennett et al. (1973) studied the sensitivity of the RIA method of Collins et al. (1972) and reported that the RIA method of Johnson et al. (1971) was ten times more sensitive than that of Collins et al. (1972). Park et al. (1973) using RIA with polystyrene tubes as a solid phase support, were able to detect as little as 25 ng SEA/g of cheddar cheese. Miller et al. (1978) developed a modification of RIA for the detection of SEA, SEB, SEC, SED, and SEE by using inactivated S. aureus cells containing protein A as immunoadsorbent. The sensitivity of this system is 1 ng or less toxin /g of food. Areson et al. (1980) demonstrated the practical usage of a single standard curve for the quantitation of toxin in foods as opposed to other RIA methods employing a standard curve for each sample under test. They claimed the sensitivity of their system to be 0.1 ng/ml for SEA and 0.5 ng /ml for SEB. Robern et al. (1975) developed the double antibody RIA method by using anti-rabbit gamma globulin from goats to separate the enterotoxin complex from the unreacted toxin. The method was highly sensitive and there was no need for purified antisera. As little as 0.33 ng SEC_2/ml of reconstituted dehydrated soup was detectable by this method.

Robern et al. (1978) detected SEA and SEB in fermented sausage at a level of 5 ng /ml of extract. Using the double antibody RIA, Lindroth and Niskanen (1977) were able to detect as little as 2-5 ng of SEA/g of minced meat and sausage extract. Niyomvit et al. (1978) employing Sepharose 4B as the solid phase support instead of plastic or bromacetylcellulose particles, were able to detect SEB at a level of 1.2 ng /ml in buffer, 2.2 ng/ml in non fat dry milk, and 6.3 ng/g in hamburger.

Enzyme-Linked Immunosorbent Assay (ELISA): The principles of this system are similar to those of the RIA, the primary difference is the substitution of an enzyme conjugate for radioactivated antigen in the ELISA assay. The enzyme detection system can be divided into two categories, competitive and non-competitive. In competitive enzyme immuno assay (EIA), antigen in the test sample competes with enzyme labeled known antigen binding sites on a solid matrix (Swaminathan et al., 1985). Sanders and Bartlett (1977) employed double-antibody EIS to detect SEA in spiked foods. They detected 0.4 ng SEA /ml in Vienna-type sausage extract within 20 hour, 3.2 ng SEA /ml in milk within 1-3 hour, and 1.6 ng SEA /ml in mayonnaise extracts. The enzymes of choice are usually peroxidase or alkaline phosphatase. However, Morita and Woodburn (1978) used Beta-amylase coupled with SEB. They believed that Beta-amylase is not produced by S. aureus and thus eliminates a possible complication in assaying samples of culture media. Morita and Woodburn (1978) adapted the homogenous enzyme immune assay and were able to detect as low as 5 ng of SEB /ml in food extracts. Rubenstein et al. (1972) developed the enzyme multiple immunoassay technique which appeared to be simpler than some others because it did not require a solid surface or separation of unbound from bound reactants. Stiffler-Rosenberg and Fey (1978) described a competitive ELISA assay, using polystyrene balls coated individually with antibody against SEA, SEB, and SEC. The sensitivity of their

method was 0.1 ng or less of enterotoxin/ml of food extract. In addition they observed no cross reactions and/or non-specific interference. Kauffman (1980) proposed an enzyme immunoassay procedure, with sensitivity of 2 ng SEA /ml of food extract. Freed et al. (1982) employed the double-antibody sandwich ELISA to assay food extract containing SEA, SEB, SEC, SED, and SEE. They were able to detect enterotoxins at levels below 1 ng /g of food in 8 hour. Nortermans et al. (1983) using ELISA for detection of SEA, SEB, SEC, and SEE in extracts of minced meat (50 % beef 50 % pork), were able to detect less than 0.5 microgram of SEE per 100 gram of product and they observed no false-positive or false-negative reactions. Fey et al. (1984) compared 4 different ELISA systems for the detection of SEA, SEB, SEC, and SED and concluded that even though the sensitivity of both competitive and sandwich version of ELISA was the same, the sandwich method proved to be better. Meyer et al. (1984) developed a monoclonal antibody (McAb) capable of binding to particles shared in common by staphylococcal enterotoxins SEA, SEB, SEC, SED, and SEE. Edwin et al. (1984) developed clones for McAb products by using sensitized spleen cells from mice immunized with SEA and fused with mouse myeloma cells.

Latex Bead Technology: Most foods involved in staphylococcal food poisoning outbreaks contain low levels of enterotoxin, often less than 1 microgram per 100 g of food (Reiser et al., 1974). To detect such a low level without concentration of the enterotoxin extract only reverse passive hemagglutination (RPHA), radioimmuno assay (RIA), and enzyme linked immunosorbent assay (ELISA) tests are sufficiently sensitive (Park and Szabo, 1986). To eliminate the nonspecific agglutination reactions which occasionally occur in RPHA test, Salomon and Tew (1968) replaced red blood cells by polystyrene latex particles as an anti-enterotoxin carrier. This system was improved by Oda et al. (1979) and Shigake et al. (1981) by using highly purified anti-

enterotoxin prepared by affinity chromatography. The system is called Reverse Passive Latex Agglutination and the test kit is commercially available. Park and Szabo (1986) evaluated 4 sets of RPLA test kits for detection of SEA, SEB, SEC, and SED in food and found that the SET-RPLA test kits did not show any non-specific reaction in either phosphate-buffered saline or extracts from various foods, and they were able to detect as low as 0.75 ng enterotoxin/g of food. Berry et al. (1986) compared latex agglutination and ELISA for the detection of Clostridium perfringens type A enterotoxin and demonstrated that the latex agglutination test was as sensitive and as specific as the ELISA for detection of Cl. perfringens enterotoxin and was simpler and more rapid to perform.

MATERIALS AND METHODS

ORGANISMS

A total of 243 strains of Staphylococcus aureus were used in this study. Ninety six strains were isolated from eight different types of meat including, chicken wing (12), smoked ham (9), fish fillet (3), ground beef (22), gizzard (14), grill steak (19), cow liver (12), and pork loin chop (5). These meat isolates were used in our preliminary study. In the final study twenty-six strains of S. aureus were obtained from the food products and microbiology laboratory, Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas and 121 Staphylococcus aureus cultures were isolated from chicken wing (16), ground beef (24), chicken gizzard (18), pork loin chop (23), sirloin steak (8), cow liver (24), and fish fillet (8). Stock cultures of these strains were maintained on Brain-heart infusion Agar slant at 4 C.

Procedure for Isolation of S aureus from Meat

Five grams of meat samples were blended with 45 ml of sterile 10% NaCl tryptic soy broth (Difco). The meat samples were mixed in the Stomacher for 1 minute. The meat mixtures were then incubated at 37 C for 24 hour. One ml of the meat fluid was spread on plates of pre-set Baird-Parker agar (Difco) using bent glass rod. After 24 hour of incubation at 37 C, characteristic colonies of S. aureus (black colonies with halo around them) were picked and streaked on Brain-heart infusion slants for further studies.

Biochemical Tests

Individual colonies were tested for production of coagulase, production of

catalase, and fermentation of mannitol. Isolated colonies were streaked on Mannitol salt agar slants and incubated at 37 C for 24-48 hour. The change of color from red to yellow was considered as a positive reaction. To demonstrate the ability of Staphylococcus aureus cultures to produce catalase, the Capillary tube method of Fung and Petrisko (1975) was used. Formation of gas bubbles in a 3 % H_2O_2 liquid column was an indication of positive reaction. To demonstrate coagulase production 0.5 ml of S. aureus from an overnight broth culture was added to 0.5 ml of rehydrated coagulase plasma (Difco) and then incubated at 37 C for 4 hours. The formation of a clot in the plasma was interpreted as presence of coagulase.

Preparation of Cultures for Enterotoxin Test.

Staphylococcus aureus cultures were grown in Brain-heart infusion broth and incubated at 37 C for 24 hour. After 24 hour the liquid cultures were centrifuged at 15000 x g for 15 minutes at 4 C. The cell-free liquids were collected and diluted to 1:10 and 1:100 using buffered diluent. All the samples were kept at -20 C for further study.

Materials and Methods for Detection of Staphylococcal Enterotoxin

Toxin detection kit (SET-RPLA): A kit for detection of staphylococcal enterotoxin A, B, C, and D by reversed passive latex agglutination (Denka Seiken LTD, Japan, distributed in the U.S.A. by Oxoid company) was used. The test kit consists of following items:

DR901: (Latex sensitized with anti enterotoxin A), Latex suspension sensitized with specific antibodies (rabbit IgG) against Staphylococcal enterotoxin A.

DR902: (Latex sensitized with anti-enterotoxin B), Latex suspension sensitized

with specific antibodies (rabbit IgG) against Staphylococcal enterotoxin B.

DR903: (Latex sensitized with anti-enterotoxin C), Latex suspension sensitized with specific antibodies (rabbit IgG) against Staphylococcal enterotoxin C.

DR904: (Latex sensitized with anti enterotoxin D), Latex suspension sensitized with specific antibodies (rabbit IgG) against Staphylococcal enterotoxin D.

DR905: (Control Latex), Latex suspension coated with non-immune rabbit globulins.

DR906: Staphylococcal enterotoxin A.

DR907: Staphylococcal enterotoxin B.

DR908: Staphylococcal enterotoxin C.

DR909: Staphylococcal enterotoxin D.

DR910: Diluent, phosphate buffered saline containing 0.5% (w/v) bovine serum albumin.

Ninety-six well U shape Microtiter Plates and Sterile Plate Sealers (Dynatech Lab, Inc)

Microtiter test Reading Mirror(Dynatech Lab, Inc, Cook Microtiter System)

Mini Shaker, Micro Mixer (Cooke Microtiter, Japan)

Micropipet (25 and 10 microliter)(Micro/Pettor, SMI), and disposable pipet tip (Scientific Manufacturing Industries).

Five Inch Capillary tubes.

Performance of the SET-RPLA Test in Microtiter Plate

Twenty-five microliters of buffered diluent was placed in each well of column 1 in rows A, B, C, D and E of a Microtiter plate, using micropipet and a disposable tip.

Twenty-five microliters of reference enterotoxin A, B, C, and D was placed into the wells of column 2 rows A, B, C, and D, respectively.

Twenty-five microliters of one of the reference enterotoxin standards was placed in the well of column 2 in row E.

Twenty-five microliters of each test sample was placed in each well of a single respective column in rows A, B, C, D, and E, beginning with column 3.

Individual vials of latex Anti-A, Anti-B, Anti-C, Anti-D and Control latex suspension were mixed thoroughly but gently to produce uniform latex suspensions.

Twenty-five microliters of each latex Anti-A, Anti-B, Anti-C, Anti-D, and control latex was dispensed into each occupied wells of rows A, B, C, D, and E respectively.

The plates were covered with sterile plate sealers (Dynatech Lab Inc.) and mounted on the carrier of the mini-shaker and shaken carefully for 15 seconds.

The plates were incubated at 21 C and 37 C. The results were checked every 3-4 hour up to 24 hours.

To read the results, the Microtiter plates were mounted on the Microtiter test reading mirror. The pattern of settled red latex particles in each well was observed from the bottom of the plate. When the latex particles have settled into a distinct pile at the bottom of the particular well (button), the reaction was considered negative. Agglutination was determined by observing that all the latex particles in a given well were uniformly spread out over the entire surface of the well without any distinct pile or "button".

To ensure that the test was working properly, the following results were expected with regard to the controls employed. All wells of column 1 should be negative (no agglutination) as these were negative controls. All wells of column 2 of rows A, B, C, and D should be positive (agglutination), as these were positive controls. The single well in column 2 of row E should be negative. If any controls did not react properly, the test was considered invalid and the procedure was repeated

again.

The cell free liquid was used in 0, 1:10, and 1:100 dilutions.

Performance of the SET-RPLA Test in Capillary Tube

Negative control: Five heat sealed five-inch pasteur pipettes were labeled as A^-, B^-, C^-, D^-, and E^-. Ten microliters of buffered diluent was placed in the Capillary part of each pasteur pipet using a Micro/Pettor, SMI (Scientific Manufacturing Industries) and disposable tips. Ten microliters of each latex Anti-A, Anti-B, Anti-C, and Anti-D and control latex was placed into each of the pasteur pipette previously labeled as A^-, B^-, C^-, D^-, and E, respectively. The buffered diluent and the anti-toxins were allowed to mix by rapidly moving the pipette in three circular motions. The entire liquid column was then shaken into the narrow portion of the pipette by a quick jerk of the wrist similar to shaking a mercury column in a mercury thermometer. All the pipettes were covered at the top with aluminum foil and were incubated at 21 C and 37 C.

Positive Control: Five sealed pasteur pipettes were labeled as A^+, B^+, C^+, D^+, and E^+. Ten microliters of each reference enterotoxins A, B, C, and D was placed into the pipettes A^+, B^+, C^+, and D^+, respectively. Ten microliter of one of the reference enterotoxin standards (A, B, C, or D) was placed in the pipette E^+, then ten microliter of latex Anti-A, Anti-B, Anti-C, Anti-D, and control latex was placed into each of the pipettes A^+, B^+, C^+, D^+, and E^+, respectively. The reference toxins and anti-toxins were allowed to mix by rapidly moving the pipette in three circular motions. The entire liquid column was then shaken into the narrow portion of the pipette by a quick jerk of the wrist similar to shaking a mercury column in a mercury thermometer. The pipettes were covered with aluminum foil at the top and were incubated.

Test Samples: Heat sealed pasteur pipettes were labeled properly to represent the specific sample. For each sample five pipettes were used, labeled A, B, C, D, and E. Ten microliter of each sample was introduced into pipettes A, B, C, D, and E, then ten microliters of latex Anti-A, Anti-B, Anti-C, Anti-D, and control latex were added into pipettes A, B, C, D, and E, respectively. The test samples and the anti-enterotoxins were mixed as described previously for positive and negative controls.

All the tubes for positive and negative control and tubes for test samples were incubated at 21 C and 37 C. The results were checked every 3-4 hour. The reactions were observed by the aid of oblique light. When the tubes remained turbid without any agglutination or presence of white flocs the results were considered to be negative. Tubes which exhibited white particles or showed agglutination without any turbidity were positive.

For each sample zero, 1:10, and 1:100 dilution were used.

For confirmation of test samples with positive enterotoxin results, the official microslide method was used.

MICROSLIDE DOUBLE GEL DIFFUSION TEST.

Materials and Equipment for Microslide Test.

1-Microscope slide.

2-Electrical tape, 0.25mm thick, 19.1 mm wide, available from Scotch Brand, 3M Co, Electro-Products Division, St. Paul, MN 55011.

3-Plastic templates.

4-Silicone grease.

5-Pasteur pipet.

6-Staining jar.

7-Incubator, 35 +_ 1 C.

8-Water bath, 55-60 C

9-Desk lamp.

10-Synthetic sponge.

Media and Reagents.

1-Agar, bacteriological grade, 0.2% .

2-Gel diffusion agar, 1.2% .

3-Butterfield's phosphate-buffered dilution water.

4-Physiological saline solution, sterile (antisera dilution)

5-Thiazine red R stain.

6-Slide preserving solution.

7-Antisera and reference enterotoxins.

Preparation of Materials and Media.

1.2% Gel diffusion agar for gel diffusion slide. Fluid base for agar in distilled water was prepared as follows: NaCl 0.85%; Sodium barbital 0.8% ; Merthiolate 1:10,000 (Crystalline) the pH was adjusted to pH 7.4. Agar was prepared by adding 1.2% Nobel special Agar (Difco). Agar mixture was melted in a boiling water bath and filtered while hot, through two layers of filter paper. The agar was dispensed in small portions (15-25 ml) into screw cap test tubes.

Thiazine red R stain: 0.1 % solution of thiazin red R stain was prepared in 1 % acetic Acid.

Preparation of Slides

Double layer of electrician's plastic insulating tape was wrapped around both sides of glass slide, leaving 2.0 cm space in center. The tape was applied as follows: A piece of tape (about 9.5-10 cm long) was started 0.5 cm from the edge of the slide undersurface and was wrapped tightly around slide twice. The area between tapes was wiped with cheesecloth soaked with 95 % ethanol, and dried with dry cheesecloth.

The upper surface area between tapes was coated with 0.2 % agar in distilled water as follows: 0.2 % agar was poured over slide between two pieces of tape. The excess agar was drained into the beaker. The undersurface of slide was wiped. The slide was placed on a tray and was dried in an incubator.

Preparation of Slide Assembly.

Plastic templates were prepared as described by Casman et al. (1969). A thin film of silicone grease was spread on one side of a template with a smaller hole which would be placed next to agar. Melted and cooled (55-60 C) 1.2 % gel diffusion agar (0.4 ml) was placed between tapes. The silicone coated template was placed immediately on melted agar with an edge of bordering tapes. One edge of template was placed on one of the tapes and the opposite edge was brought to rest gently on other tape. Slide were placed in a petri-dish containing synthetic sponge with high humidity soon after the agar solidified and the slide was labeled with number, date, and other necessary information.

Slides were cleaned without removing the tape, rinsed with tap water, and brushed to remove agar gel. They were boiled in detergent solution for 15-20 minutes, and rinsed about 5 minutes in hot running water, and then boiled in distilled water. The templates were rinsed sequentially with tap water, distilled water, and 95 % ethanol. The templates were spread on towel a to dry.

The reagents were supplied as lyophilized preparations of enterotoxins and their antisera. The antisera was rehydrated in physiological saline. The reference enterotoxins were rehydrated in physiological saline containing 0.3 % proteose peptone, pH 7.0.

Slide Gel Diffusion Test.

In order to prepare the record sheet, the template hole pattern was drawn on a record sheet, the contents of each well indicated and each pattern was given a

number to correspond with a slide number.

Suitable dilution of anti-enterotoxin (antiserum) was placed in the central well and homologous reference enterotoxin was placed in an upper peripheral well. The test sample was placed in a well adjacent to the well containing reference enterotoxin. The control slide was prepared with only reference toxin and antitoxin. Wells were filled with reagents to convexity, using a Pasteur pipette. Bubbles were removed from all wells by probing with fine glass rod. The glass rod was broken into 2-1/2 inch lengths and the ends were smoothed in flame. The glass rods were inserted into all wells to remove trapped air bubbles that were not visible. Slides remained at room temperature in covered petri-dishes containing moist sponge strips for 48 and 72 hour examinations.

The template was removed by sliding it to one side. The slide was cleaned by dipping it momentarily in water and the bottom of the slide was wiped, then the slide was stained as described below. The slide was held over a source of light and against a dark back ground. The lines of precipitation were identified through their coalescence with a reference line of precipitation.

Lines of precipitation were enhanced by immersing slide 5-10 minutes in thiazine red R stain. If the slide was to be preserved, the staining procedure described by Crowle (1958) was used. The remaining reactant liquid was rinsed away by dipping the slide momentarily in water and immersing it for 10 minutes in each of the following baths: 0.1% thiazine red R stain in 1 % acetic acid, 1 % acetic acid, and 1 % acetic acid containing 1 % glycerol. The excess fluid was drained from the slide and the slide was dried in 35 C incubator.

The official method was used to confirm positive culture fluids of meat isolates obtained by the SET-RPLA method.

RESULTS AND DISCUSSION

In the preliminary study 96 strains of Staphylococcus aureus were isolated from eight different types of meat: chicken wing (12), smoked ham (9), fish fillet (3), ground beef (22), gizzard (14), grill steak (19), cow liver (12), and pork loin chop (5). All the meat isolates were tested for the production of catalase, coagulase, and production of acid from mannitol salt agar. Table 3 shows the biochemical characteristics of meat isolates. All ninety six strains were resistant to 10 % salt and were able to produce catalase (catalase positive); however, 10 strains were not able to produce coagulase (coagulase -) and ferment mannitol (mannitol -). These strains were isolated from pork chop (5) and smoked ham (5). There were also three strain from fish fillet which were coagulase and catalase positive but they were not able to ferment mannitol. (mannitol -)

All strains were examined with the Oxoid SET-RPLA for their toxin production regardless of their coagulase production. The Oxoid procedure was followed exactly except that the culture fluid was not diluted to 1:100.

Of the ninety-six strains of S. aureus, 37 strains showed positive reaction for SEA and +/- reaction for SEC and SED. The positive isolates were from ground beef (13), cow liver (11), and steak (13). Sixteen strain produced SEA including ground beef (9), chicken wing (6), and steak (1). Seven strains showed + reaction for SEC (gizzard), 3 strains showed + reaction for SEA and SEC (gizzard), 3 strains showed +/- reactions for SEA, two strains showed + reaction for SEC and +/- reaction for both SEB and SEC, two strains showed + reaction for both SEA and SEB and +/- reaction for SEC, one strain showed + reaction for SEA and +/- reaction for SED, and one culture showed + reaction for SEA and SEB and +/- reaction for SEC and SED. Table 4 summarized the toxin profile of meat isolates using Oxoid SET-RPLA.

Table 3 : Biochemical Characteristics of Meat Isolates in the Preliminary Study.

Sample	#	CATALASE	COAGULASE	MANNITOL	10% SALT
Cow Liver	(12)	+	+	+	+
Grill Steak	(19)	+	+	+	+
Ground Beef	(22)	+	+	+	+
Chicken Wing	(12)	+	+	+	+
Fish Fillet	(03)	+	+	-	+
Chicken Gizzard	(14)	+	+	+	+
Pork Chop	(05)	+	-	-	+
Smoked Ham	(05)	+	-	-	+
Smoked Ham	(04)	+	+	+	+

Table 4: Positive Reactions for Enterotoxin Meat Isolates in the Preliminary Study.

TOXIN PROFILES	FOOD	#	TOTAL
A, c, d	GROUND BEEF COW LIVER GRILL STEAK	13 11 13	37
A	GROUND BEEF CHICKEN WING GRILL STEAK	9 6 1	16
C	CHICKEN GIZZARD	7	7
A, C	CHICKEN GIZZARD	3	3
a	CHICKEN WING GRILL STEAK	2 1	3
a, c	CHICKEN GIZZARD	2	2
A, b, c	CHICKEN GIZZARD GRILL STEAK	1 1	2
A, B, c	CHICKEN GIZZARD GRILL STEAK	1 1	2
A, B, C	GRILL STEAK	1	1
A, d	GRILL STEAK	1	1
A, B, c, d	COW LIVER	1	1

A, B, C are the 3^+ reactions.
a, b, c, and d are +/- reactions.

In, total 75 % of the strains were toxigenic with strains producing SEA, SEC, and SED (49.33%) being the most prevalent followed by strain SEA producing (21.33 %). If +/- reactions were considered to be positive. If +/- reactions were considered as negative reactions, then 56 strains (74.66 %) were SEA producers, 7 (9.33 %) were SEC producers, 3 (4 %) were SEA and SEB producers, 3 (4 %) were SEA and SEC producers, and 1 strain (1.33 %) was an SEA, SEB, and SEC producer. These results were achieved after 24 hr of incubation at 21 C.

According to Oxoid SET-RPLA's procedure, dilution of culture fluid to 1:100 would be the ideal concentration for the reaction in order to eliminate the chances of false positive reactions. The large number of positive enterotoxin reactions obtained in preliminary study may have been the result of false positive reactions since the culture fluid was not diluted to 1:100. In the main body of the study, the effects of dilution (0, 1:10, and 1:100), incubation temperature (21 C, 37 C, and 45 C), and assay methods (Microtiter and a proposed Capillary tube method) of laboratory cultures and meat isolates were determined.

Effect of Dilution and Incubation Temperature on Detection of Enterotoxin by SET-RPLA.

In the first part of this study, 26 laboratory Staphylococcus aureus cultures were examined at 0, 1:10, and 1:100 dilutions and incubated at 21 C, 37 C, and 45 C for the detection of their toxin profiles, using the Microtiter plates system and the commercial Oxiod kit. Results of 45 C incubation were discarded due to evaporation of reagents during incubation. All 26 strains were coagulase, catalase, and mannitol positive (Table 5). The data of Microtiter plate system at 0, 1:10, and 1:100 dilution at 21 C and 37 C for 24 hour is shown in Table 6. At zero dilution of 26 Strains of

Table 5: Biochemical Properties of Laboratory S. aureus Cultures.

Sample	#	Coagulase	Catalase	Mannitol
S.aureus	26	+	+	+

TABLE 6 : Detection of staphylococcal enterotoxin A, B, C, and D by Oxoid ; Microtiter Plate System at 0, 1:10, and 1:100 dilultion of Laboratory culture fluid at 21 and 37 C for 24 Hour.

	Staphylococcal Enterotoxin											
	A			B			C			D		
Sample	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100
S-14	+	+	+	+(-)	-	-	-	-	-	-	-	-
S-15	+	+	+	+	-	-	-	-	-	-	-	-
S-16	+(-)	-	-	-	-	-	-	-	-	-	-	-
S-17	+	+	-	-	-	-	-	-	-	-	-	-
S-18	-	-	-	-	-	-	-	-	-	+	+	+
S-19	+	+	+_(+)	+	+	-(+_)	-(+)	-	-	-	-	-
S-20	-	-	-	+	+	+	-	-	-	-	-	-
S-21	-	-	-	+	+	-	-	-	-	-	-	-
S-22	-	-	-	+	+	+	-	-	-	-	-	-
S-23	-	-	-	+	+	+	-	-	-	-	-	-
S-24	-	-	-	+	+	+	-	-	-	-	-	-
S-25	-	-	-	+	+	+	-	-	-	-	-	-
S-27	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 6 : CONTINUED

	Staphylococcal Enterotoxin											
	A			B			C			D		
Sample	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100
S-28	+	-	-	+	-	-	+	-	-	+(-)	-	-
S-29	+(-)	-	-	+	+	+	+(-)	-	-	-	-	-
S-30	-	-	-	+	+	+	-(+)	-	-	-	-	-
S-31	-	-	-	+	+	+	-	-	-	-	-	-
S-32	-	-	-	-	-	-	-	-	-	-	-	-
S-33	+	+	+/-(+)	+	+	+	-	-	-	-	-	-
S-35	+	-	-	+	-	-	+	-	-	+	-	-
S-36	+	+	+	+	-	-	+	-	-	-	-	-
S-37	-	-	-	-	-	-	-	-	-	-	-	-
S-38	+	-	-	+	-	-	+	-	-	+	-	-
S-40	+	-	-	+	-	-	+	-	-	+	-	-
S-41	+	-	-	-	-	-	-	-	-	-	-	-
S-42	+	+	+	+	+	+	-	-	-	-	-	-

+, -, and +/- indicates positive, negative, and minimal positive reactions at 21 C. Data from 37 C are identical except data presented in parenthesis ( ).

S. aureus, three cultures produced SEA, eight cultures produced SEB, none produced SEC, one culture produced SED, and three cultures produced SEA and SEB. In addition, three cultures showed agglutination for SEA, SEB, and SEC , and four cultures showed agglutination for SEA, SEB, SEC, and SED. The manufacturer of the Oxoid kit speculated that when more than three toxins are detected from one sample, the results should be considered as "suspects". Further dilutions are recommended to check for false positive. At 1:10 dilution, three cultures produced SEA, nine produced SEB, none produced SEC, two produced SED, three produced SEA and SEB, and one produced SEA and SED. At 1:100 dilution, two cultures produced SEA, eight cultures produced SEB, none produced SEC, two produced SEA and SEB, and two produced SEA and SED. The results obtained from 37 C were very similar to those obtained at 21 C.

Table 7 shows the results of the proposed Capillary system at 0, 1:10, and 1:100 dilution at 37 C for 4 hours and 21 C after 16 hours. At zero dilution, three cultures produced SEA, eight SEB, none produced SEC, three produced SEA and SEB, and two produced SEA and SED. At 1:10 dilution, four cultures produced SEA, seven produced SEB, none produced SEC, one produced SED, two produced SEA and SEB, and one produced toxins SEA and SED. At 1:100 dilution, one culture produced SEA, and four produced SEB. In most cases the reactions at 21 C are identical to the reactions achieved at 37 C, except that at 1:100 dilution the number of positive reactions are less at 37 C.

By comparing the data of Microtiter system at 0, 1:10, and 1:100 dilution and 21 C and 37 C we made the following concluisons:

1) At zero dilution many false positive results occurred. Of 26 cultures 7 cultures produced false positive results (26 %).

2) At 1:10 dilution the chance of false positive results was lower than at 1:100

TABLE 7 : Detection of staphylococcal enterotoxin A, B, C, and D by Oxoid; improved Capillary method at 0, 1:10, and 1:100 dilution of Laboratory culture fluid at 21 and 37 C for 4 hour.

	Staphylococcal Enterotoxin											
	A			B			C			D		
Sample	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100
S-14	+	+_	-	-	-	-	-	-	-	-	-	-
S-15	+	+	+	-	-	-	-	-	-	-	-	-
S-16	+	-	-	-	-	-	-	-	-	+_(+)	-	-
S-17	-(+)	-	-	-	-	-	-	-	-	-	-	-
S-18	-	-	-	-	-	-	-	-	-	+	+_(+)	-
S-19	+_(+)	+_(+)	-	-(+)	-	-	-	-	-	-	-	-
S-20	-	-	-	+	+	+	-	-	-	-	-	-
S-21	-	-	-	-	-	-	-	-	-	-	-	-
S-22	-	-	-	+	+	+	-	-	-	-	-	-
S-23	-	-	-	+	-	-	-	-	-	-	-	-
S-24	-	-	-	+	+	+	-	-	-	-	-	-
S-25	-	-	-	+	+	+	-	-	-	-	-	-
S-27	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 7: CONTINUED

	Staphylococcal Enterotoxin											
	A			B			C			D		
Sample	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100	0	1:10	1:100
S-28	-	-	-	-	-	-	-	-	-	-	-	-
S-29	-	-	-	+	+	-	-	-	-	-	-	-
S-30	-	-	-	+	+	+_	-	-	-	-	-	-
S-31	-	-	-	+	+	+_	-	-	-	-	-	-
S-32	-	-	-	-	-	-	-	-	-	-	-	-
S-33	+	+	+(-)	+	+	+(-)	-	-	-	-	-	-
S-35	-	-	-	-	-	-	-	-	-	-	-	-
S-36	+	+	-	-	-	-	-	-	-	+_(+)	-	-
S-37	-	-	-	-	-	-	-	-	-	-	-	-
S-38	-	-	-	-	-	-	-	-	-	-	-	-
S-40	-	-	-	-	-	-	-	-	-	-	-	-
S-41	+	+	-	-	-	-	-	-	-	-	-	-
S-42	+	+	+	+	+	+	-	-	-	-	-	-

+, - and +_ indicates positive, negative, and minimal positive reaction respectively at 37 C.
Data from 21 C are identical except data presented in pranthesis ( ).

TABLE 8: Detection of Staphylococcal Enterotoxin A, B, C, and D by Oxoid; Microtiter Plate System, at 1:100 Dilution of Laboratory Culture Fluid at 21 C after 24 hour and Capillary System, 0 Dilution at 37 C.

	ENTEROTOXIN									ENTEROTOXIN							
	A		B		C		D			A		B		C		D	
Sample	MS	CS	MS	CS	MS	CS	MS	CS	Sample	MS	CS	MS	CS	MS	CS	MS	CS
S-14	+	+	-	-	-	-	-	-	S-28	-	-	-	-	-	-	-	-
S-15	+	+	-	-	-	-	-	-	S-29	-	-	+	+	-	-	-	-
S-16	-	-	-	-	-	-	-	-	S-30	-	-	+	+	-	-	-	-
S-17	-	-	-	-	-	-	-	-	S-31	-	-	+	+	-	-	-	-
S-18	-	-	-	-	-	-	-	+	S-32	-	-	-	-	-	-	-	-
S-19	+_	+_	-	-	-	-	+_	-	S-33	+_	+	+	+	-	-	-	-
S-20	-	-	+	+	-	-	-	-	S-35	-	-	-	-	-	-	-	-
S-21	-	-	-	-	-	-	-	-	S-36	+	+	-	-	-	-	+¯	+_
S-22	-	-	+	+	-	-	-	-	S-37	-	-	-	-	-	-	-	-
S-23	-	-	+_	+	-	-	-	-	S-38	-	-	-	-	-	-	-	-
S-24	-	-	+	+	-	-	-	-	S-40	-	-	-	-	-	-	-	-
S-25	-	-	+	+	-	-	-	-	S-41	-	+	-	-	-	-	-	-
S-27	-	-	-	-	-	-	-	-	S-42	+	+	+	+	-	-	-	-

MS: Microtiter Plate System, 1:100 dilution and 21 C for 24 hour.
CP: Capillary System, 0 dilution and 37 C for 4 hour.

dilution but there were still two cases of 26 cultures with false positive results.

3) At 1:100 dilution false positive reactions were minimized, but, there is a possibility of missing some positive reactions. For example, Staphylococcus aureus S-41, a known weak SEA producing strain, did not show positive reaction in Microtiter system at 1:100 dilution. However, it showed positive reaction at 0 dilution.

4) Only a slight difference was observed between the reactions at 37 C and 21 C.

5) In all cases, clear cut results were achieved after 16-24 hour of incubation.

By comparing the data of the Capillary system at 0, 1:10, and 1:100 dilution and 21 C and 37 C, we concluded that:

1) At zero dilution of culture fluid, no false positive reaction were observed, and since the culture fluid was not diluted, the chance of missing positive reaction was zero .

2) 1:10 and 1:100 dilutions were not suitable for the Capillary system since there have been many toxin producing cultures which did not show positive reactions at 1:10, and 1:100 dilution. At 1:100 dilution of 26 cultures only 7 cultures showed positive reaction, and at 1:10 dilution 11 cultures showed positive reaction as compared to 13 positive reactions at 0 dilution.

3) There was a slight difference between the results at 21 C and 37 C.

4) Clear cut results were observed after 3-4 hour of incubation at 37 C.

Table 8 compared the Capillary system at 0 dilution and 37 C for 3-4 hr (proposed combination) with the Microtiter system at 1:100 dilution and 21 C for 24 hr (recommended combination by kit manufacturer). We concluded that the results of Capillary system at zero dilution and 37 C for 3-4 hour are the same as the Microtiter system at 1:100 dilution and 21 C after 24 hr incubation. Therefore the Capillary system not only reduces the reaction time from 24 hr to 3-4 hr but also

prevents false positive reactions and without the need of diluting the culture fluid. In addition it prevents the possibility of missing the positive reactions by the process of dilution.

Toxin Profile of Staphylococcus aureus Strains Isolated from Meat.

Of 121 Staphylococcus aureus cultures isolated from eight different types of meat, 20 cultures fermented mannitol and produced catalase, but were coagulase negative. These strains were isolated from fish fillet(8), chicken gizzard (11), and cow liver (1). The rest of the S. aureus isolates were mannitol, catalase, and coagulase positive (Table 9). All the coagulase positive isolates were tested for the toxin production by SET-RPLA using Microtiter plates at 1:100 dilution of culture fluid, 21 C for 24 hr, and by use of the proposed Capillary tube at zero dilution, 37 C for 3-4 hour. None of the isolates produced SEA, SEB, and SED, (Table 10) but nine strains of S. aureus isolated from chicken produced SEC as detected by the Microtiter plate system and the Capillary tube system. Seven strains of S. aureus isolated from sirloin steak showed a +/- reaction in Capillary tube and a negative reaction in the Microtiter plate. In order to prove the accuracy of the SET-RPLA, eight strains of S. aureus isolated from chicken (seven positive for SEC and one negative for SEC.), and four strains of S. aureus isolated from sirloin steak (+/- for SEC in Capillary tube and negative in Microtiter plate.) were tested by the official micro slide gel double diffusion test. All seven strains of S. aureus which produced SEC as detected by SET-RPLA in the Microtiter plate and the Capillary tube systems also produced SEC by the microslide technique. Four strains of S. aureus which showed +/- reaction for SEC by SET-RPLA in Capillary tubes and showed negative reaction in Microtiter plates, did not produced any SEC by the microslide technique. Therefore, we concluded that the SET-RPLA (Microtiter system and Capillary tube

system) is as accurate as microslide the technique. Also, we concluded that the Capillary system is a reliable system that can replace the Microtiter system in the SET-RPLA method. The advantages are elimination of the dilution procedure, reduction of reaction time for rapid detection of staphylococcal enterotoxins, and high sensitivity.

The general observation from this study indicates that Staphylococcus aureus occurs in many meat products ranging from beef, poultry, pork, and fish in the market place. In the preliminary study of the 96, cultures most of the isolates were catalase and coagulase positive with some variations of mannitol fermentation. In the second study 101 cultures were catalase, mannitol, and coagulase positive and 20 isolates were coagulase negative.

The toxin profiles of the isolates in the preliminary study were not reliable since the culture fluid was not diluted during the testing. The toxin profiles of the isolates in the secondary study were highly reliable and showed low numbers of toxigenic strains of S. aureus isolated from the products. This is not completely surprising since other workers also found low number of toxigenic strains of S.aureus in their studies. For example Shiozawa et al. (1981) examined 586 strains of S.aureus from healthy and diseased chicken and found only 16 toxigenic strains.

Our study also concentrated on testing and improving the new RPLA enterotoxin test. The Microtiter system and the Capillary system are both reliable compared with the reference microslide method. However, the Capillary tube method has the advantages of reduction of reaction time and avoidance of diluting the sample. The exact reason for the accelerated reaction time is not known. It can be speculated that the latex beads coated with anti-toxins move about much more actively in the Capillary tube than in the Microtiter plate wells due to electrostatic forces generated by the glass. In moving about, the chances of interacting with toxin and other beads

with anti-toxin are greatly enhanced and thus form visible clumps in 3-4 hrs. In the Microtiter system the beads react slower and will not be visible until the formation of a sheet of beads at the bottom of the well.

Table 9 : Biochemical Properties of S. aureus Cultrues Isolated from Meat

Sample	#	Catalase	Coagulase	Mannitol
Fish fillet	8	+	-	+
Gizzard	11	+	-	+
	6	+	+	+
Cow liver	24	+	+	+
	1	+	-	+
Ground Beef	24	+	+	+
Chicken Wing	16	+	+	+
Sirloin Steak	8	+	+	+
Pork Loin Chop	23	+	+	+
Total	121	121^{+}	101^{+}	121^{+}

Table 10: Toxin Ppofile of Meat Isolates, Microtiter 1:100 and Capillary System 0 Dilution.

		MICROTITER PLATE				CAPILLARY TUBE			
Sample	#	A	B	C	D	A	B	C	D
GIZZARD	6	-	-	-	-	-	-	-	-
COW LIVER	24	-	-	-	-	-	-	-	-
GROUND BEEF	24	-	-	-	-	-	-	-	-
CHICKEN WING	9	-	-	+	-	-	-	+	-
	6	-	-	-	-	-	-	-	-
PORK LOIN CHOP	23	-	-	-	-	-	-	-	-
SIRLOIN STEAK	7	-	-	-	-	-	-	+/-	-

Table 11: Comparison of SET-RPLA (Microtiter and Capillary System) with Microslide Gel Double Diffusion.

		STAPHYLOCOCCAL ENTEROTOXIN		
SAMPLE	#	MICROTITER	CAPILLARY	MICROSLIDE
CHICKEN WING	7	+	+	+
	1	-	-	-
SIRLOIN STEAK	4	-	+/-	-

CONCLUSION

General

1. Meat products in the market place harbor Staphylococcus aureus, most are coagulase positive

2. The number of toxigenic strains varies according to the study involved. In the preliminary study the high percentage of positive toxigenic strains identified was probably due to false positive results of the Microtiter system since diluted (1:100) fluid samples were not used. In the second study using the improved Capillary method only 9 % of the isolates were toxigenic strains (toxin C producer).

3. In general the SET-RPLA system (both Microtiter plate system and Capillary tube system) is a sensitive, reliable, and easy method for the detection of staphylococcal enterotoxins. It can replace the more tedious official microslide method.

Microtiter Plate System

1 . The most suitable concentration for reaction in this system was a 1:100 dilution of culture fluid, However at this dilution there was a possibility of missing true positive reactions due to the dilution process.

2. Incubation of reactants in the Microtiter plate at 21 C for 16-24 hours was required in order to achieve clear cut results.

3. Incubation of reactants in the Microtiter plate at 45 C was not recommended due to evaporation of reagents.

4. The system was highly sensitive, reliable, promising, and easy to use.

Capillary Tube System

1. Zero dilution of culture fluid was the most suitable and promising concentration for reaction. No false positive reaction was observed at this concentration and since the culture fluid was not diluted the chance of missing positive reactions was minimized.

2. Dilution of culture fluid to 1:100 was not recommended since the chance of missing a positive reaction was very high.

3. Clear cut results were achieved after 3-4 hours of incubation at 37 C.

4. The system was highly sensitive, reliable, rapid, and easy to use and can replace the Microtiter plate system because it reduced the reaction time and eliminated the process of dilution and therefore prevented the possibility of missing positive reactions.

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DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN A, B, C, AND D BY LATEX BEAD TECHNOLOGY

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ABSTRACT

Staphylococcal intoxication remains a major food-borne disease internationally. Many methods have been developed to detect the enterotoxins. The newest technique is the Latex Bead Reverse Passive Agglutination Test .

The commercial system by Oxoid involves placing a liquid sample (0.025 ml) with or without toxin into a well of a Microtiter plate and then reacting with a liquid (0.025 ml) containing antitoxin coated latex beads and allowing the reaction to proceed for 24 hrs at 21 C before reading reactions. All four enterotoxins are detected by antitoxin A, B, C, and D beads. Positive reactions will result in the formation of a sheet of beads at the bottom of the wells due to antibody-antigen reactions whereas negative reactions will result in a "button" formed at the bottom of the wells. Sensitivity of the system is 2 ng/ml.

To test the accuracy of SET-RPLA and to examine toxin profiles of Staphylococcus aureu, 243 strains of S. aureus were employed in this study. In the preliminary study, 96 strains of S.aureus isolated from different types of meat were used. All 96 strains were catalase, coagulase, and mannitol positive except 10 strains from pork chop and smoked ham which were coagulase and mannitol negative and 3 strains from fish fillet which were mannitol negative. The data from SET-RPLA using Microtiter plate system showed that 75 % of the isolates were toxigenic; however, these results considered to be non reliable since the culture fluids were not diluted to 1:100. The large number of positive enterotoxin reactions may have been due to false positive reactions.

To study the effect of dilution (0, 1:10, and 1:100), incubation temperature (21C, 37C, and 45C), and assay method (Microtiter plate method and Capillary tube method) 26 laboratory S. aureus cultures were used. All strains were catalase, coagulase, and mannitol positive. Results of 45C incubation were discarded due to evaporation of

reagents during incubation.

Data obtained from Microtiter plate system showed that:

1. Dilution of culture fluid to 1:100 is necessary in order to minimize false positive reactions. However at this dilution there was possibility of missing some positive reactions.

2. Clear cut results were achieved after 16-24 hours of incubation.

Data from Capillary tube system indicated that:

1. At 0 dilution of culture fluid no false positive reaction was observed.

2. 1:10 and 1:100 dilutions were not suitable in the Capillary system since there was high possibility of missing positive reactions.

3. Clear cut results were achieved after 3-4 hrs of incubation at 37C.

By comparing results of Microtiter system and Capillary tube method we concluded that results of the Capillary tube method at 0 dilution and 37 C after 3-4 hrs of incubation were the same as Microtiter plate method at 1:100 dilution and 21 C after 24 of incubation. Therefore, Capillary tube method not only reduced the reaction time from 24 hrs to 3-4 hrs but also eliminated the dilution process and therefore prevented the chance of missing positive reactions due to the dilution process.

One hundred and twenty one strains of S. aureus isolated from different types of meat have been employed in final part of this study using Microtiter plate at 1:100 dilution and 21C and the Capillary tube method at 0 dilution at 37C. Of 121 strains tested 20 strains were catalase and mannitol positive but coagulase negative and 101 strains were catalase, coagulase, and mannitol positive. Of 101 coagulase positive strains only 9 strains from chicken wing showed positive reactions for enterotoxin C both by the Microtiter plate system and Capillary tube method. Toxigenic strains (7 cultures) and non-toxigenic strains (5 cultures) were tested with the official microslide method. The results from official microslide method were the same as SET-RPLA indicating that SET-RPLA is as accurate as official method.