RAPID METHODS FOR DETERMINING STAPHYLOCOCCAL TOXINS AND SALMONELLAE ASSOCIATED WITH POULTRY PRODUCTS

by

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TABLE OF CONTENTS

I. INTRODUCTION

Food has long been recognized as an unintentional vehicle for the distribution of pathogenic organisms and their toxins to susceptible human consumers. Physical disorders ranging from mild cases of gastroenteritis to serious impairment, and in extreme cases, deaths have resulted. Although viral, fungal, protozoan, and helminthic agents may cause diseases associated with food consumption, the most important food poisoning agents belong to the bacterial group. Three major groups of bacteria are currently recognized as causing food-borne disease. The first group, causing food infection, includes organisms such as Salmonellae (Dewberry, 1959; Steele, 1963; Edwards and Galton, 1967; Taylor and McCoy, 1969), Shigellae (Keller and Robbins, 1956; Drachman et al., 1960; Bryan, 1969) and Escherichia coli (Neter et al., 1951; Taylor and Charter, 1952; Taylor, 1955; Bryan, 1969), which cause diseases through growth and multiplication in the intestine of susceptible persons after the organisms are ingested in contaminated food. The second group, causing food intoxication, includes organisms such as Staphylococcus (Hobbs, 1953; Dack, 1956, 1963b; Bergdoll et al. 1959a, 1967a; Thatcher and Robinson, 1962; Casman and Bennett, 1965; Lamanna and Carr, 1967; Bryan, 1968; Angelotti, 1969) and Clostridium botulinum (Pedersen, 1955; Ingram and Roberts, 1966; Lamanna and Carr, 1967; Riemann, 1969a; Sakaguchi, 1969) which produce toxins that can

cause disease when ingested. The third group includes organisms such as Clostridium perfringens (McClung, 1945; Dische and Elek, 1957; Despaul, 1966; Hauschild and Thatcher, 1967, 1968; Hobbs, 1969), Bacillus cereus (Hauge, 1955; Hobbs, 1969) and enterococci (Buchbinder et al., 1948; Hartman et al., 1965; Bryan, 1969) which cause diseases for reasons not yet well known, although toxins are generally implied.

The benefits of large scale food processing, storage, and supply due to advancement of food technology are sometimes diminished by the proportionally large scale outbreaks of food-borne disease caused by the above organisms when proper sanitary procedures are not observed.

In order to retard or eliminate the increasing hazard from food poisoning, it is essential to control the route of contamination, proliferation, and survival of the causative microorganisms in food. This can be achieved only after the occurrence and prevalence of those organisms in food are well understood, thus the need for rapid and reliable detection of pathogens and their toxins in foods.

Many procedures have been developed to enumerate, isolate and characterize pathogenic organisms and their toxins, in both culture media and natural environments, especially in foods. This subject has been adequately reviewed and studied by many individuals (Dack, 1956; Ayres et al., 1962; Slanetz et al., 1963; Molin and Erichsen, 1964; Graham et al., 1968;

Thatcher and Clark, 1968; Riemann, 1969b). These reviews also emphasize that rapid methods are needed for determining the agents, epidemiology, and nature of the pathogens involved in food poisoning cases.

The purposes of this study were to develop rapid, simple, and accurate methods for quantitative and qualitative detection of staphylococci and their toxins, and Salmonellae in poultry products. Also, related miniaturized methods for isolation and detection of organisms in food were devised and applied to general bacteriological work.

II. LITERATURE REVIEW

A. Rapid and Miniaturized Methodology

The concepts and applications of many miniaturized microbiological techniques were reviewed by Hartman (1968). Rapid and miniaturized methods were developed to increase the efficiency of routine microbiological work in terms of savings of: (1) time in operation and collection of data; (2) space for operation and incubation; (3) labor in performing the experiments; and (4) material for experimentation.

Several rapid techniques pertinent to this study are discussed.

Various types of multiple inoculating devices, such as needles and pins of several kinds (Garrett, 1946; Holliday, 1956; Zamenhof, 1961; Haque and Baldwin, 1964; Cooke, 1965; Smirnoff and Perron, 1965; Watt et al., 1966), Velveteen for replica plating (Lederberg and Lederberg, 1952; Shifrine et al., 1954; Wiseman and Sarles, 1956; Shiflett et al., 1966; Lee and Wolfe, 1967), Pasteur pipettes (Massey and Mattoni, 1965; Seman, 1967), and capillary tubes (Hartman and Pattee, 1968; Valland, 1969) were developed for microbiological studies.

The "Velveteen" method has the problem of resultant smearing of colonies during operations (Hartman, 1968). Clogging by agar may result when Pasteur pipettes are used as

inoculators on solid media (Hartman and Pattee, 1968). Capillary tubes, although successfully utilized by Hartman and Pattee (1968) and Valland (1969) may cause spattering of inocula on the agar plates, and have only limited use on solid media. Needles and pins when used as multiple inoculators have the advantages of capability of inoculating into liquid or solid media and are inexpensive to make.

The merits and defects of conventional viable cell count procedures were recorded in Standard Methods for the Examination of Dairy Products (American Public Health Association, 1967). Use of standard loops for bacterial dilutions were reported by Lindsey (1959), Thompson et al. (1960), Noyes et al. (1960), Brumfitt and Percival (1964), and Jasper and Dellinger (1966). Subsequent plating of the diluted bacterial samples on agar showed that variations between the standard plate count and the plate loop counts were about the same as variations within the standard plate count alone (Hartman, 1968).

A Microtiter system was developed by Sever (1962) based on the spiral loop design of Takatsy (1955) and has been used for rapid dilution of virus, red blood cells, antigens and antibodies (Rosenbaum et al., 1963; Sever et al., 1964; Edwards, 1964; Lewis and Knights, 1964; Morse and Mah, 1967). Dilution of bacterial cells for the purpose of viable cell counts with the Microtiter system has not been suggested prior to this investigation.

The little tube method was developed for rapid and large scale inoculation of small volumes of bacterial cells into small quantities of reagents for biochemical tests in an attempt to increase efficiency of microbiological techniques. A list of 38 specific usages along with references were compiled by Hartman (1968). The coagulase test for staphylococci by means of the little tube was performed by Loeb (1903), Chapman et al. (1941), McClung and Weinberg (1956) and Wilson et al. (1959).

Plastic, glass and lucite trays containing many wells or depressions have been used for miniaturized microbiological studies (Takatsy, 1955; Beargie et al., 1965; Kende and Robbins, 1965). Recently, Sneath and Stevens (1967) reported the use of a commercially made, divided petri dish for culturing bacteria. Microtiter plates containing 96 wells each were used as miniaturized vessels for carbohydrate fermentation tests (Davies and Gutekunst, 1968).

B. Staphylococcal Food Poisoning

1. General incidence of staphylococci

The ubiquity of staphylococci has long been recognized. They are found on the body surfaces and respiratory passages of men and animals. Because certain strains of these Gram positive, non-motile, pigment producing cocci, under suitable conditions, can release a potent exotoxin, which causes severe

vomiting, nausea, prostration and diarrhea, when ingested by a susceptible person, staphylococci are considered potential health hazards when they are found.

Ever since staphylococci were recognized by Pasteur in 1880 and were classified by Rosenbach in 1884, food poisoning cases of staphylococcal origin have continually been described (Elek, 1959).

High protein foods primarily have been implicated in staphylococcal food poisoning outbreaks. Tanner and Tanner (1953), in a report on foods responsible for staphylococcal food poisoning, listed such foods as follows: meat products including cooked liver sausage, chicken salad, lamb's tongue, meat pie, turkey salad, canned sausage and ham; dairy products including raw milk, homemade buttermilk, pasteurized milk, cheese and ice cream; pastry products including layer cake, chocolate cream pie, eclairs, cream pie, pineapple trifle, and custard-filled cake; sandwiches including ham, egg, and olive; potato salad, bread pudding and Hollandaise sauce.

A case of food poisoning due to a yellow hemolytic staphylococcus was described by Dack et al. (1930), and using human volunteers they established a toxic agent which was present in the filtrate obtained from a pure culture of the suspected organisms. Dack (1956) reviewed several case-histories reported by investigators in the late nineteenth century and early twentieth century of staphylococcal food

poisoning due to cheese, beef, dried beef, and milk. Detailed descriptions of the responsible organisms involved and the symptoms exhibited by the victims were also made. Reported incidences of staphylococcal food poisoning have increased rapidly in recent years. This may be due, in part, to the advancement in diagnostic techniques resulting in greater frequency of clinical reports, and in part to actual increase of staphylococcal food poisoning.

Staphylococcal food poisoning accounted for 82% of all food poisoning outbreaks reported to the United State Public Health Service between 1945 and 1947 (Feig, 1950). Between 1952 and 1960, staphylococcal food poisoning ranked first on the list of outbreaks of food and water borne diseases in America (Dauer and Davids, 1960). Data made available by the National Communicable Disease Center, Atlanta, Georgia, (Dack, 1963a), showed that from 1957 to 1962 there were 206 staphylococcal food poisoning outbreaks involving 7868 cases. In 1966 in the United States 14.4% of all the food poisoning outbreaks were caused by Staphylococcus aureus, while about 59.7% of the food poisoning cases were of unknown etiology (Salmonella Surveillance, 1967a). Twenty-one confirmed outbreaks of staphylococcal food poisoning involving 2,317 cases were described in the first half of 1968 (Morbidity and Mortality Weekly Report, 1968). Chicken salad (Peavy et al., 1968), tuna fish salad (Blakey et al., 1968), ham (Pineiro

et al., 1968), and baked ham (Franzen et al., 1969) were implicated in 4 recent outbreaks involving ca. 2,000 people in the United States.

Outbreaks of staphylococcal food poisoning have also been recorded in England, Wales, Japan, and Holland. Vernon (1966) stated that staphylococcal food poisoning was rated second following salmonellosis among food poisoning of all types from 1956 to 1965 in England and Wales. Recently, Canadians reported a case-history of staphylococcal food poisoning involving a commercial barbecued chicken (Pivnick et al., 1968a). Since staphylococcal food poisoning is an unreportable disease, many more cases undoubtedly have occurred on a world wide basis.

2. Incidence of staphylococci in poultry products

Many bacterial genera including Achromobacter, Alcaligenes, Bacillus, Corynebacterium, Escherichia, Flavobacterium, Microbacterium, Micrococcus, Paracolobactrum, Proteus, Pseudomonas, Salmonella, and Staphylococcus have been found in poultry, poultry products, and processing plants (Lochhead and Landerkin, 1935; Gunderson et al., 1946, 1947, 1954; Ayres et al., 1950, 1956; Walker and Ayres, 1956, 1959; Barnes, 1960a, 1960b; Nagel et al., 1960; Kotula et al., 1962; Kraft et al., 1963; Biester and Schwarte, 1965; Bryan, 1965; Salzer et al., 1965, 1967; da Silva, 1967; Woodard, 1968; and others).

Brucella spp., Clostridium spp., Corynebacterium diphtheriae, Erysipelothrix insidiosa, Listeria monocytogenes, Mycobacterium avium, Paracolobactrum spp., Pasteurella spp., Salmonella spp., and Staphylococcus aureus were cited as bacterial agents which cause diseases and are common to both poultry and man (Sadler et al., 1965). The presence of staphylococci and Salmonella in poultry meat are of public health concern in view of increased consumption of poultry meat in the United States and many other countries.

In examining chicken giblets, cooked pot pies, and chicken a-la-king, Gunderson et al. (1954) found coagulase positive staphylococci in almost all the samples examined. Creamed chicken and creamed meat dishes, frozen poultry pies and unfrozen chicken were implicated as reservoirs for staphylococci (Buchbinder et al., 1949; Canale-Parola and Ordal, 1957; Fanelli and Ayres, 1959). Small numbers of coagulase positive staphylococci were found on dressed chicken and skin surface of poultry (Ayres et al., 1956; Walker and Ayres, 1959). The effects of freezing and holding at -29C on the survival of staphylococci on turkey meat were studied by Kraft et al. (1963). The occurrence of coagulase-positive staphylococci in turkey giblets and the effects of washing in reducing staphylococci in the edible viscera were reported by Salzer et al. (1965, 1967).

Bacteriological examination of 2,513 chicken and turkey livers by Sadler et al. (1965) revealed the presence of coliforms, streptococci, staphylococci, Lactobacillus spp., Achromobacter spp., Bacillus spp., Proteus spp., diptheroids, Pseudomonas spp., Pasteurella spp., Clostridium spp., and paracolons. The percentages of coagulase positive and coagulase negative staphylococci in chicken fryer livers, chicken hen livers, and turkey livers were 1.2 and 10.2, 3.2 and 8.1, and 0.7 and 5.3 respectively. In a related study Genigeorgis and Sadler (1966a) found 1.6% of the samples from poultry livers had coagulase positive staphylococci. Since they found 50% (7 of 14) of the staphylococci tested for enterotoxin production were positive, they deduced that ca. 0.8% of the isolates from poultry livers were staphylotoxigenic.

Following the possible "chain of contamination" of staphylococci in turkey processing plants, da Silva (1967) concluded that workers' hands and chill tank water contributed to the increase of S. aureus on the meat, equipment and final product in the further processing room. Senn and Williams (1967) reported 3 outbreaks of staphylococcal food poisoning involving market rotisseries in California. In a study of staphylococci on turkey rolls during processing and storage, the bacteria were found on raw turkey rolls before frozen storage but were not recovered in rolls after cooking (Woodard, 1968). Growth and enterotoxigenesis of 9 strains of S. aureus

on commercial barbecued chickens were studied by Pivnick et al. (1968b).

3. Media for isolation and detection of staphylococci

Many selective media have been developed for the isolation and identification of staphylococci in hospital environments as well as from foods. Some of the more common media are mannitol salt agar (Chapman, 1945), staphylococcus medium No. 110 (Chapman, 1946), staphylococcus medium No. 110 fortified with egg yolk (Herman and Morelli, 1960), tellurite glycine agar (Zebovitz et al., 1955), and egg yolk-azide agar (Hopton, 1961). Other media were developed from existing media by addition of chemicals such as polymyxin (Crisley et al., 1964) and pyruvate (Baird-Parker, 1962).

Since most workers claimed superiority of their own medium over others, comparative studies of various media were made to determine the relative efficiency of the media. Jay (1963), using coagulase positive staphylococci isolates from meat, compared the following six selective media: staphylococcus medium No. 110 fortified with egg yolk, mannitol-sorbic acid broth, mannitol salt agar, tellurite-glycine agar, polymyxin agar, and Vogel and Johnson agar (Vogel and Johnson, 1961). Jay concluded that staphylococcus medium No. 110 fortified with egg yolk was most efficient.

A comparative study was made by Crisley et al. (1965) using five selective media; the efficiency of the media in

descending order were: (1) tellurite polymyxin egg yolk agar, (2) Baird-Parker's egg-tellurite-glycine-pyruvate-agar, (3) tellurite glycine agar, (4) tellurite egg yolk agar, and (5) staphylococcus medium No. 110.

These studies did not give conclusive indications as to the superiority of any one medium. It should be noted that except for tellurite glycine agar, the media used by Jay (1963) and Crisley et al. (1965) were different.

De Waart et al. (1968) compared the efficiency of Baird-Parker's egg-tellurite-glycine-pyruvate-agar and Chapman's agar in the enumeration of S. aureus in foods. They concluded that the former medium was better. A new medium, phenolphthalein diphosphate agar with polymyxin was found to give results comparable to seven other selective media (staphylococcus medium No. 110, tellurite-polymyxin-egg-yolk agar, Baird-Parker medium, Vogel and Johnson agar, egg yolk-sodium azide-agar, Russian milk-salt-agar, and English milk-salt-agar) when tested for the isolation and enumeration of coagulase-positive staphylococci from foods (Hobbs et al., 1968).

Liquid enrichment media have also been used in conjunction with the solid plating media for greater recovery of staphylococci especially when small numbers are involved. Wilson et al. (1959) incubated aliquots of food samples (custard, ham, creamed chicken, and milk) in an enrichment broth (3.7% brain heart infusion broth, 2.0% mannitol, and 7.5% NaCl) for 24-48

hrs before plating on a confirmatory agar medium. Crisley (1964) developed a cooked meat medium with 10% NaCl added for enrichment before samples were streaked on confirmatory agar. Another combination procedure was developed by Raj (1965) which involves enrichment of organisms in mannitol-sorbic acid broth, then streaking on staphylococcus medium No. 110 agar with egg yolk and incubating at 45C. A comparative study involving 3 liquid media (cooked meat broth plus 10% NaCl, trypticase soy broth plus 10% NaCl, and sorbic acid broth) and 5 solid media (staphylococcus medium No. 110, polymyxin-B-agar, Vogel and Johnson agar, tellurite glycine agar, and mannitol egg agar) for isolating coagulase-positive staphylococci from food products was made by Baer et al. (1966). They concluded that trypticase soy broth containing 10% NaCl combined with Vogel and Johnson agar as the plating medium was the most efficient media system.

Because of conflicting reports as to the efficiency of different media for the isolation of staphylococci from food, individual investigators have to execute their own judgement in selecting a suitable medium for their specific studies. Staphylococcus medium No. 110 with added egg yolk has been successfully used to isolate staphylococci from turkey products at the Food Research Laboratory at Iowa State University (da Silva, 1967; Woodard, 1968; Kraft and Rey, personal communication, 1969) and was selected for use in the present investigation.

4. Properties and detection of staphylococcal enterotoxins

To date, four types of enterotoxins have been detected, based on their distinct immunological differences. Since the possibility exists that more enterotoxins may be discovered, a sequential lettering system was adopted for systematic nomenclature of staphylococcal enterotoxins (Casman, et al., 1963a).

Enterotoxins A, B, C, and D were detected and described by Casman (1960), Bergdoll et al. (1959a), Bergdoll et al. (1965a), and Casman et al. (1967) respectively. The purification and characterization of enterotoxin A (Chu et al., 1966), enterotoxin B (Bergdoll et al., 1959a, 1965b; Frea et al., 1963; Schantz et al., 1965; Spero et al., 1965; Wagman et al., 1965) and enterotoxin C (Avena and Bergdoll, 1967; Borja and Bergdoll, 1967) have been reported. The amino acid compositions and terminal amino acids of these enterotoxins have also been determined (Bergdoll et al., 1965b, 1967a; Spero et al., 1965; Huang et al., 1967). Physiochemical properties of enterotoxin D have not been reported at the present time.

A comparison of some physiochemical properties of enterotoxins A, B, C_1, and C_2 is presented in Table 1.

Frequency of occurrence of enterotoxins A, B, and C was studied by Hall (1968) who reported that of 149 enterotoxin producing strains of Staphylococcus aureus, 93 produced enterotoxin A, 17 produced enterotoxin B, 26 produced enterotoxin C and 13 produced a combination of A, B, or C. Casman et al.

Table 1. Comparison of enterotoxins A, B, and C[a]

Property	Enterotoxins			
	A	B	C_1	C_2
Nitrogen content (%)	16.5	16.1	16.2	16.0
Sedimentation coefficient ($S^0_{20,w}$),S	3.04	2.78	3.00	2.90
Diffusion coefficient ($D^0_{20,w}$) 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	6.8	8.6	8.6	7.0
Partial specific volume	0.726	0.726	0.732	0.742
Maximum absorption (mμ)	277	277	277	277
Extinction ($E^{1\%}_{1cm}$)	14.3	14.4	12.1	12.1
Toxicity ED_{50} μg/monkey	5	5	5	5-10
Molecular weight	34,700	30,000	34,100	34,000
C-terminal amino acid	serine	lysine	glycine	glycine
N-terminal amino acid	alanine	glutamate	glutamate	glutamate

[a]Modified from Bergdoll et al. (1967a) with the addition of C- and N-terminal amino acid information extracted from Bergdoll et al. (1965b, 1967a), Spero et al. (1965), and Huang et al. (1967).

(1967) included the occurrence of enterotoxin D in a survey of incidence of production of enterotoxins by staphylococcal isolates from food-poisoning outbreaks. Strains producing enterotoxin A were found to be most prominent (49%) followed by strains producing a mixture of enterotoxin A and another enterotoxin, usually toxin D (29%). About 8% of the isolates produced enterotoxin D. Only 3.8% produced enterotoxin B and 2.5% formed enterotoxin C. Unpublished studies of Bergdoll (Foster and Bergdoll, 1968) suggested that occurrence of enterotoxin C producing strains may be higher than the reported values.

Use of human volunteers as biological assay for staphylococcal enterotoxin detection has been described by Barber (1914), Dack et al. (1930, 1931), Davison and Dack (1942), Dolman (1943), and Armijo (1957). Raj and Bergdoll (1969), using human volunteers, recently reported that 20-25 μg of pure enterotoxin B can cause enterotoxemia in man. Although human beings provide the most reliable and useful data for enterotoxin assay, volunteers are hard to acquire because these powerful enterotoxins, although usually not fatal, act on the victims in such a manner that they wished the enterotoxins were fatal.

Other lower animals including monkeys, dogs, puppies, cats, kittens, frogs, domestic and wild mice, guinea pigs, rabbits, and canaries have been used for toxin detection with varying

degrees of success (Dack, 1956). Monkeys (Surgalla et al., 1953; Wilson, 1959; Raj and Bergdoll, 1969), cats and kittens (Dolman and Wilson, 1940; Hammon, 1941; Fulton, 1943; Matheson and Thatcher, 1955), rabbits (Bayliss, 1940), and dogs (Prohaska, 1963; da Silva, 1967) are the most reliable animals for enterotoxin assay.

Recently, McIntosh and Duggan (1965) studied the effects of staphylococcal enterotoxin B on 52 different protozoa and metozoa without finding specific responses in any organisms tested.

The use of nematodes, tissue cultures, chicken embryos, and tropical fish as assay systems for enterotoxin was discussed by Bergdoll (1963) who questioned the reliabilities of the tests.

Animals for detection of enterotoxins are impractical for large scale surveillance studies because of individual variation of response, build up of resistance after repeated exposures, and high expenses involved in operation (Casman, 1958).

Many attempts have been made to correlate enterotoxin production with certain properties of S. aureus such as the production of coagulase, phosphatase, lipase, hemolysins, gelatinase, pigments, and phage type (Genigeorgis, 1966). The usefulness of these indirect bacteriological methods are questionable because nonenterotoxigenic strains of S. aureus also may exhibit many of the same characteristics. For example,

coagulase positive strains of S. aureus do not necessarily produce enterotoxin (ca. 40-50%) and, conversely, certain coagulase negative strains were reported to produce enterotoxin (Genigeorgis and Sadler, 1966a; Bergdoll et al., 1967b; Hall, 1968).

Although Williams et al. (1953) reported that enterotoxin producing strains of staphylococci were restricted mainly to phage Groups III and IV, Munch-Peterson (1963) stated that "phage typing cannot be used to determine whether a given staphylococcus has produced or can be induced to produce enterotoxin ...." Nevertheless, Munch-Peterson (1963) also stated that the majority of staphylococci causing food poisoning belonged to Group III. Phage typing is an excellent way to study the sources and mode of contamination by staphylococci, regardless of pathogenicity, in certain given environments, such as poultry processing plants (da Silva, 1967). da Silva (1967) also observed that of 24 isolates tested, staphylococcal isolates with phage patterns 42D/47/53, 187, 83A and 53/83A, and 4 nontypable staphylococci were enterotoxigenic when dogs were tested. The largest number of da Silva's isolates belonged to Group III.

Immunological techniques for toxin detection involve the concept of antigen-antibody reactions. Since enterotoxins are simple proteins they act as antigens when injected into blood streams of animals and can elicit the production of antibodies

which can then be collected and purified (Casman and Bennett, 1964). Under carefully controlled experimental conditions, immunological techniques are able to detect not only the presence of enterotoxins in cultures and food samples but also the amount and types of toxins involved. The four existing immunological techniques for staphylotoxin detection are gel-diffusion (single-gel, double-gel, and slide tests), the fluorescent antibody technique, a flotation antigen-antibody system, and hemagglutination procedures.

The single-gel diffusion technique (Oudin, 1952) consists of layering of a solution of antigen on top of an agar column impregnated with the specific antiserum, with a visible band of precipitation formed when the antigen-antibody system migrates through the agar at a rate corresponding to the concentrations of antigen and antibody. This method and its modifications have successfully been used to detect enterotoxin B at a concentration of 1 μg/ml (Bergdoll et al., 1959b; Hall et al., 1963; Silverman, 1963; Schantz et al., 1965; Weirether et al., 1966).

Oakley's double-gel diffusion (Oakley and Fulthrope, 1953) involves 3 components; a bottom agar layer containing antiserum, a middle layer containing buffered agar and the antigens on top. The antigen and antiserum diffuse toward each other in the middle layer and form a band of precipitation at the meeting point. The position of the band depends on the

relative concentrations of the antiserum and antigen. Low concentration (0.05 μg/ml) of enterotoxin was detected when high titer of antiserum was used under prolonged incubation to several weeks (Hall et al., 1965).

The slide-Ouchterlony method (Ouchterlony, 1949), developed by Wadsworth (1957) and described by Crowle (1958, 1961), consists of a plastic template with funnel-shaped wells in contact with a thin layer of agar situated on a microscope slide. The antiserum is placed in the center well with antigen or unknown placed in the surrounding wells. Lines of precipitation are formed when specific antigen and antibody meet after diffusion in the agar. Precipitation lines formed by unknown samples with antiserum may be compared with those formed by known (purified enterotoxin) standards. The limit of sensitivity of this method is 1 μg/ml (Casman and Bennett, 1965).

Genigeorgis and Sadler (1966c) developed a fluorescent antibody technique by using antienterotoxin B serum conjugated with fluorescein isothiocyanate, and were able to detect small amounts of enterotoxin B (1-15 μg/ml) in culture media, food smears and extracts. The advantage of this method is the elimination of the cumbersome enterotoxin extraction procedure necessary in most other immunological tests.

In Hopper's flotation antigen-antibody system (1963, 1965), Rhodamine conjugated anti-serum B was mixed with food extracts or pure enterotoxin, then sparged with compressed air until a

foam was formed in the presence of a wetting agent. A gel filtration was employed in some modified procedures. The colored conjugate-toxin complex, separated from the foam layer, was then tested for agglutination of a latex polystyrene suspension. This method can detect as little as 1 μg/ml of enterotoxin B after the toxin is concentrated by centrifugation and column chromatography on Sephadex G-25 (Hopper, 1965). Although the method is rapid (2-3 hrs), food proteins, such as egg albumen, interfere with the separation of the colored complex from the foam.

The hemagglutination-inhibition concept is based on the principle that when specific antiserum is first exposed and reacted with its antigen, either as pure enterotoxin or crude enterotoxin in culture or food filtrates, it will not agglutinate erythrocytes previously sensitized with pure enterotoxin.

Robinson and Thatcher (1965) reported an indirect hemagglutination-inhibition procedure which can detect 0.04 μg/ml of enterotoxin A in 2-3 hrs.

A rapid procedure using a "reversed passive" hemagglutination concept was developed by Silverman et al. (1968) who claimed to be able to detect 0.0007 μg of toxin in a few hours.

Morse and Mah (1967), utilizing the rapid Microtiter technique, devised a Microtiter hemagglutination-inhibition assay method which can detect 0.4 μg/ml of enterotoxin B in culture filtrates within 6 hrs. Kollias (1967) used this

procedure to detect enterotoxin B in milk with limited success. Because of the speed and sensitivity, this method was tested in the present study for the detection of all four enterotoxins in culture filtrate as well as in poultry products.

5. Detection of enterotoxins in cultures and foods

Production of toxin by enterotoxigenic strains of Staphylococcus aureus in complex and defined media has been the subject of much research. Complex media such as a casein hydrolysate medium (Favorite and Hammon, 1941; Bergdoll, 1962; Kato et al., 1966; Peters, 1966), semi-solid and solidified brain heart infusion agar (Casman and Bennett, 1963) and brain heart infusion plus 10% NaCl (Genigeorgis and Sadler, 1966b) were studied for enterotoxin production. A high yield of about 200-500 μg enterotoxin per ml of culture supernatant was found under optimal growth condition in a complex medium (Bergdoll, 1962; Kato et al., 1966). Hall et al. (1963) reported consistent enterotoxin B production by S. aureus S-6 in complex media such as brain heart infusion, tryticase soy broth, NZ-amine A, trypticase, proteose peptone, tryptone, neopeptone, casitone, and tryptose.

Enterotoxigenesis of S. aureus in synthetic media was reviewed and investigated by Fung (1967). A chemically defined medium was developed for growth and enterotoxigenesis of S. aureus S-6, which produced a detectable level of 10 μg of enterotoxin per ml of culture supernatant.

Various foods were used for the detection of staphylotoxins by either addition of known amounts of enterotoxins into the food or growing toxigenic strains of S. aureus in food before enterotoxin detection. Artificially added enterotoxins A and B were recovered with success from custard, chicken-a-la-king, meat salads, meat and fish slurries (Hall et al., 1963), milk, cheese, coconut custard pie, cooked and raw ground beef, cooked ground shrimp and turkey (Casman and Bennett, 1965; Read et al., 1965a, 1965b) by use of gel-diffusion tests. Enterotoxins A and B were detected after production by enterotoxigenic strains of S. aureus in slurries of shrimp, scallop, lobster, crabmeat, custard filling, ham and cured meat, and cheese (Kelly and Dack, 1936; Thatcher et al., 1962; Casman et al., 1963b; Hall et al., 1963; Donnelly et al., 1967; Genigeorgis et al., 1969).

C. Salmonellae Food Poisoning

1. General incidence of Salmonellae and salmonellosis

Salmonellae have long been associated with food poisoning cases; symptoms include diarrhea, cramps, nausea, vomiting, fever, headache, and prostration in victims, although fatality is low.

These Gram negative, usually motile and H_2S-producing rods are found in mollusks, arthropods, fish, reptiles, birds, mammals, various household pets, and many domestic animals

including chickens, turkeys, ducks, geese, pigeons, cattle, sheep and goats (Hinshaw and McNeil, 1951; Buxton, 1957; Ayres, 1969). Among food products as possible vehicles of Salmonellae, Bowmer (1965) listed meat and meat products, poultry and poultry products, eggs and egg products, milk and milk products, fish and shell fish, canned foods, jams, fruits, vegetables, pickles, sauces, dry powdered foods and bread.

In 1968 several different food and drug commodities (whole egg solids, egg white solids, powdered pepsin, pasteurized egg yolk solids, gelatin, egg noodles, non-fat dry milk, pancreatin, vitamin mineral powder, liver protein fraction, dry yeast, sausage and cheese pizza, cocoa mix, and smoked fish) were recalled by the Food and Drug Administration because of the presence of Salmonella (Salmonella Surveillance, 1968).

Although Bruner (1968) indicated that doses of at least 2×10^5 for virulent strains and 1×10^9 for less virulent strains of Salmonella are needed in order to produce gastroenteritis in a normal adult male, the occurrence in foods of any one of the 1,200 serotypes of Salmonellae was considered a potential hazard as a source of human disease (Sadler, 1968). Martin and Ewing (1969) reported that of the 1,300 presently known Salmonella serotype species, 33 account for ca. 90% of the isolates reported from humans and ca. 80% of the isolates from nonhuman sources. Between 1963 and 1967, human isolations of Salmonellae were around 20,000 per year and nonhuman

isolations increased from 5,389 in 1963 to 8,794 in 1967 (Ayres, 1969).

Salmonelloses of both man and animals are believed to be increasing in recent years (Galton et al., 1964; Biester and Schwarte, 1965; Edwards and Galton, 1967). This could be attributed to better and more systematic reporting procedures as well as to actual increase of incidences. The National Communicable Disease Center (Salmonella Surveillance, 1967b) reported incidences of human salmonellosis in the United States from 1942-1966 and showed a general increase from ca. 6,000 cases in 1942 to ca. 20,000 cases in 1966. A slight drop of isolations was reported in 1966 and 1965 as compared to 1964. The number of isolations for the 3 years were 20,040, 20,865, and 21,113 respectively. The numbers of reported isolations for 1967 and 1968 were 19,736, and 19,745, respectively (Salmonella Surveillance, 1967, 1968, 1969) showing essentially constant numbers of isolations of Salmonella in the past 6 years. In the beginning months of 1969, about 1,200 isolations were reported per month, which was about average as compared to other months in the past 6 years (Salmonella Surveillance, 1969).

Isolations of Salmonella were also reported in many other countries. In 1968 (Anderson, 1969) a total of 3,227 isolates were obtained in Australia, Papua and New Guinea. Belgium reported 1,905, 2,871, and 3,439 isolations for the years

1965, 1966, and 1967 (Van Oye, 1967, 1968). Canadians reported 2,910 and 2,551 Salmonella isolations in 1965 and 1966, respectively (Yurack, 1967). Von Bullian and Von Pictzsh (1968) reported 8,630 and 8,879 Salmonella isolations in Germany and West Berlin in 1966 and 1967, respectively. During the years 1964 and 1965, Gerichter (1967) reported 1,552 isolations of Salmonella in Israel. Isolations of Salmonella were also reported in Czechoslovakia (Matejovska, 1966), France (LeMinor, 1969), Great Britain (Taylor, 1967), Jamaica (Grant, 1967), Japan (Fukumi, 1965), Poland (Buczowski and Pietkiewicz, 1967), and many other countries. Salmonellosis has attracted the attention of public health workers the world over.

2. Salmonella in poultry and poultry products

The occurrence in Salmonella in poultry was reviewed in detail by Buxton (1957), Moran (1961), Bryan (1965), Hall (1965), Van Roekel (1965), and Williams (1965).

Felsenfeld et al. (1950), Edwards (1958), Galton and Steele (1961), and Kampelmacher (1963) reported that poultry constitutes large reservoirs of Salmonellae.

Salmonellae were isolated from the feces of 2.5 percent of 650 turkeys by Smith and Buxton (1951). Thirty serotypes of Salmonellae were isolated from 21 percent of 1,148 turkey poults during routine necropsy as studied by Lukas and Bradford (1954).

A cumulative total of 117 serotypes of Salmonellae were isolated from turkey and/or chickens in the United States from the early twentieth century until 1965 (Williams, 1965). Hinshaw (1965), after a comprehensive literature review, compiled a list of 102 serotypes of Salmonella isolated from turkey on a world wide basis.

It is noteworthy that 5 of the 10 most common serotypes of Salmonella (S. typhimurium, S. anatum, S. newport, S. oranienberg, and S. montevideo) were isolated from both man and poultry between 1947 and 1958, indicating the potential of poultry and poultry products as sources of Salmonella causing human salmonellosis (Quist, 1963). Quist (1963) also listed 3 major reasons why poultry products are so often involved in Salmonella outbreaks: (1) the unusual susceptibility of poultry to infection; (2) the use of eggs in the raw state, and (3) improper handling of poultry meats in kitchens where the meat is cut, mashed, stuffed, or otherwise manipulated, increasing the chances of contaminating other food products.

Reports of 5 case-histories of salmonellosis outbreaks related to poultry products (egg powder, fresh eggs, 2 cases from baby chicks, and mature birds) were presented by McCroan et al. (1963). More recently 2 outbreaks of salmonellosis of poultry origin (turkey) were reported, one affecting 98 of 116 people in a church supper (Tucker et al., 1968) and another affecting 17 of 18 people after a Thanksgiving dinner (Atman

et al., 1968). Two individuals, a 17 year old boy and a 56 year old woman, died as the result of the latter outbreak. Bowmer (1965) reviewed 10 outbreaks of salmonellosis involving meat and meat products, half of them originated from poultry and included a turkey meat and stuffing outbreak, a roast turkey outbreak, a roast pork and turkey sandwich outbreak, a chicken salad sandwich outbreak and an outbreak induced by poultry feed. A smoked turkey labeled "ready-to-eat" was cited as the vehicle of infection in a recent Salmonella outbreak affecting 11 of 17 people (Tucker et al., 1969).

In a study concerning transfer of Salmonellae from wild birds to domestic fowl, Goodchild and Tucker (1968) found 11 of 382 British wild birds carried Salmonella. Cloacal swabs of 511 apparently normal wild birds yielded only 2 Salmonella isolations. When these strains from wild birds were inoculated into domestic fowl, mortality rates ranging from 1.7% to 34.0% resulted.

The percentage isolations of Salmonella in three poultry plants in Florida were reported by Galton et al. (1955) as 5.4, 16.8, and 20.0. A lower percentage of recovery (0.5%) was reported by Morris and Ayres (1960) from samples gathered in federally inspected turkey processing plants in Iowa. Sadler et al. (1961) reported 2.4% of 2,380 turkey intestines examined were Salmonella positive. In England, Dixon and Pooley (1962) recovered Salmonella in 7.8% and 19.8% of samples obtained

from two turkey processing plants. Tucker and Gordon (1968) tested ca. 17,000 samples for Salmonella in 9 poultry packing stations and one turkey packaging station and found only 4 yielded positive results.

In a comprehensive study of the sources of Salmonella in turkey products, Bryan et al. (1968a) concluded that Salmonella were introduced to the turkeys through feed, through water and soil on the farm, and then were carried to the turkey processing plant by the birds and subsequently contaminated equipment and personnel and finally appeared in the finished product. In a related study, Bryan et al. (1968b) isolated Salmonella from 12% of chilled, eviscerated turkey carcasses, 27% of finished products and 24% of the processing equipment. A total of 23 serotypes were recovered, many of which were commonly associated with human salmonellosis. The authors implied that further-processed turkey products could directly transmit Salmonellae to humans if improperly refrigerated between the time of manufacture and consumption and if inadequately cooked by the consumer.

3. Detection of Salmonellae in foods

Detailed descriptions of the morphological, physio-biochemical and serological properties of Salmonella spp. were presented by Breed et al. (1957), Edwards (1962, 1963), Edwards and Ewing (1962a, 1962b), Topley and Wilson (1964), and Ewing and Ball (1966).

Complete biochemical studies of suspected organisms are time consuming and expensive. Therefore, screening procedures are advantageous for routine detection of Salmonellae in food.

Quantitative procedures for the detection of Salmonellae in egg products were devised by Gibbons and Moore (1944) and Ayres (1949). Since then many workers have modified these procedures and reported improved recovery of Salmonellae (North and Bartram, 1953; Byrne et al., 1955; Osborne and Stokes, 1955; Stokes and Osborne, 1955; Gillies, 1956; Rappaport et al., 1956; Silliker and Taylor, 1958; Sugiyama et al., 1960; North 1960, 1961; Hall et al., 1964).

A comparison of 4 methods and an elaboration of a new procedure for the isolation of Salmonella from foods were made by Montford and Thatcher (1961). The direct enrichment method used by the Food Hygiene Laboratory in England, and the pre-enrichment method generally used in the United States for Salmonella detection were found to be statistically comparable by Taylor et al. (1964) for 179 food samples. The World Health Organization sponsored comparative studies of Salmonella isolation in eight European laboratories, each using their own procedure, and reported that great differences existed among the various laboratories (Edel and Kampelmacher, 1968).

A detailed review of isolation methods for Salmonellae in foods and feeds was given by Galton et al. (1968). Also, the development and effectiveness of various liquid and solid media

and various procedures were discussed. A procedure similar to that of Galton et al. (1968) for the isolation of Salmonella from animal feed and feed ingredients was published by the U.S. Department of Agriculture, Agricultural Research Service (1968). Thatcher and Clark (1968) described procedures for isolation of Salmonella from dried egg products, frozen unpasteurized and pasteurized liquid eggs, frozen dried processed foods, raw meat, poultry, and powdered milk.

The "conventional" methods usually involve 5 basic steps including: (1) pre-enrichment and/or enrichment in non-selective and/or selective liquid media (18 to 24 hrs), (2) plating on selective agars (18 to 24 hrs), (3) biochemical screening (18 to 24 hrs), (4) serological screening (a few minutes), and (5) final identification (indefinite time period).

Since the time involved in performing the conventional procedures ranges from 54-96 hrs or longer, more rapid screening would be helpful to facilitate detection of Salmonella in food.

One such approach is the use of a fluorescent antibody (FA) technique for rapid detection of Salmonella (Ayres, 1967). Thomason et al. (1957, 1959) applied the FA technique of Coons et al. (1942) and detected Salmonella in pure culture with success. The FA technique was applied to detect Salmonella in eggs and egg products (Haglund et al., 1964), and meat and poultry (Georgala and Boothroyd, 1965). Insalata et al. (1967)

reported the use of the FA method for detecting Salmonella in nine food varieties without false-positive or false-negative results. An agreement of 92.1% was found between the conventional method and the FA technique in Salmonella detection of 1,013 feed and feed ingredient samples (Laramore and Moritz, 1969). The time required to perform the FA test for Salmonella detection is about 24 to 48 hrs less than the conventional method.

A reagent tablet procedure for rapid biochemical confirmation of Salmonellae after isolation on selective agar was reported by Schafer et al. (1968).

Motility of Salmonella has been exploited as a means of rapid isolation of this organism from mixed cultures. Bibb (1927) was able to differentiate Salmonellae from Shigellae and other organisms by relative motility of these organisms through capillary tubes. Nonselective semi-solid medium imbedded in a U-tube was used to differentiate Salmonella from Pseudomonas; the former, being less aerophilic, penetrated and migrated through the medium faster than the latter (Ino and Graber, 1955). Stuart and Pivnick (1965) employed a semisolid-enrichment medium in modified U-tubes to facilitate isolation of Salmonella form swine and human feces. This system required 1 to 11 days for the organisms to migrate through the medium. Incorporation of an indicator for the detection of mannitol fermentation and hydrogen sulfide production in a similar

U-tube system was developed by Banwart et al. (1966) for screening Salmonella from samples of dried egg. Use of a secondary enrichment with a special tube arrangement for the isolation of Salmonellae from abattoir swabs was reported by Harvey et al. (1966), who claimed that a selective toxic chemical was unnecessary. Recently, Banwart (1968) reported satisfactory recovery of Salmonella within 24 hrs for further biochemical and serological tests by use of a special glassware apparatus with semi-solid agar columns.

Pijper (1952) investigated the filtration and separation according to sizes and motility of bacteria and claimed that Salmonella could be differentiated from other organisms by their ability to migrate rapidly through a mass of cotton wool. Applying this concept and the FA technique, Abrahamsson et al. (1968) reported detection of Salmonella in 1 to 2 days in a single-culture technique they developed.

From the descriptions in the literature, the above procedures for Salmonella detection by motility and requiring special glassware apparatus are either time consuming or cumbersome to operate.

III. MATERIALS AND METHODS

A. Stock Cultures and Isolation of Unknowns

Frozen stock cultures stored at -20C were prepared by quick freezing 3 ml aliquots of log-phase culture at -40C. The following organisms were employed in these studies: Staphylococcus aureus 100 (toxin A producer), S. aureus 196E (toxin A producer), S. aureus S-6 (producer of toxins A and B), S. aureus 137 (toxin C producer), Salmonella typhimurium, S. anatum 53, S. infantis, S. thompson, and S. heidelberg. S. aureus S-6, and S. aureus 137 were obtained through the courtesy of the Food Research Institute, Madison, Wisconsin. The National Center for Urban and Industrial Health, Cincinnati, Ohio provided S. aureus 196E. Salmonella spp. were taken from the culture collection of the Department of Food Technology, Iowa State University, Ames, Iowa. Other test organisms including S. aureus 494 (toxin D producer and supplied by E. P. Casman, Food and Drug Administration, Washington, D.C.), Proteus spp., Escherichia coli K-12, and S. aureus strains (obtained from Department of Bacteriology, Iowa State University, Ames, Iowa), Serratia marcescens, Streptococcus durans, Pseudomonas fluorescens F-21 (from the Department of Food Technology, Iowa State University, Ames, Iowa) and P. glycinea (from H. Tachibani, U.S. Department of Agriculture, Ames, Iowa) were used for experimentation. A number of isolates

from hamburger meat and beef steaks were also used.

Unknown organisms were isolated from turkey rolls and turkey roasts by the contact plate method and the homogenate spread plate method. The contact plate method consisted of directly touching the surfaces of solidified agar (selective and non-selective) with surfaces of turkey products. The homogenate spread plate method involved homogenizing pieces of turkey meat in an Osterizer (John Oster Manufacturing Company, Racine, Wisconsin) with sterile distilled water, then spreading the homogenate in solid agar (selective and non-selective) surfaces by use of a sterile bent glass rod. Staphylococcus medium No. 110 (Difco) with egg yolk was used for isolation of Staphylococcus aureus. This medium was prepared by adding, aseptically, one egg yolk to 500 ml of sterile staphylococcus medium No. 110. The inoculated plates were incubated at 37C for 24 hrs before discrete typical colonies were picked and streaked on solidified nutrient agar (Difco) plates and slants for purification and storage.

Two groups of unknowns were isolated from turkey meat, turkey roasts and turkey rolls. The first group (100 cultures) was isolated from turkey meat suspected as the vehicle of a staphylococcal food poisoning outbreak. Three persons (one female and two male) showed symptoms of staphylococcal food poisoning, including vomiting, diarrhea, and prostration but no pyrexia, 3 hrs after eating a Thanksgiving dinner in which

the main dish was a roast turkey. The victims were fully recovered after 12 hrs of resting.

The other group of isolates was obtained from commercially frozen and thawed turkey rolls and two turkey roasts. The contact plate method was used to isolate staphylococci from two sliced sections of each turkey roll and one turkey roast. Staphylococci were also isolated from three sliced sections of the other turkey roast . A total of 72 isolates were obtained from these nine sections, eight isolates from each section.

B. Microtiter Methods for Viable Cell Counts

A rapid Microtiter method consisting of a serial dilution and spot plating procedure (McKinney et al., 1959) was used for enumeration of viable cell counts in bacterial cultures (pure and mixed) as well as in food samples (Fung and Kraft, 1968b; Fung and LaGrange, 1969). All Microtiter equipment was obtained from the Cooke Engineering Co., Alexandria, Va. A sterile automatic pipetting machine (Becton, Dickinson and Company, Rutherford, N.J.) was used to dispense 0.225 ml of sterile distilled water into each of 96 wells in a pre-sterilized, plastic Microtiter "V" or "U" plate (8 X 12 wells). Aliquots of 0.025 ml of bacterial culture or food samples (well homogenized) were introduced into the first of each series of 8 wells by pre-calibrated sterile loops. Separation of clusters or chains of cells were made by vigorous shaking

or by use of an ultrasonic probe (Biosonik 11 Ultrasonic Probe, Model BP-11, Bronwill Scientific, Rochester, N.Y.). Serial dilutions were made by rotating the loops rapidly 30 times in the appropriate first wells to ensure homogeneity of dilutions. The loops were than drain-dried by placing them on a sterile blotting paper to eliminate possible carry-over from the original culture. The drain-dried loops were then placed into the same wells and rotated rapidly 20 times before they were carefully introduced into the second dilution blanks. The first row of wells gave a 1:10 dilution of the original culture. Serial dilution, with drying of the loops between each dilution, to the eighth row of wells then gave a 10^8 dilution of the original culture. The time consumed for the entire process of dilution of six or more different strains of bacteria by this method was about 5 min.

The spot plate method of McKinney et al. (1959) was used for plating the diluted samples. Two drops of each dilution to be tested were spotted on the surface of previously poured and thoroughly dried plates of agar by means of sterile 0.025- or 0.050-ml droppers. Four to 8 drops could be spotted on the surface of one petri dish. Droppers were sterilized before use by soaking in 1% Chlorox solution for 1 hr; the sterile droppers were then rinsed in sterile distilled water. Disposable sterile droppers, Pasteur pipettes, and capillary pipettes could also be used to deliver either portions or all of the

diluted samples onto agar surfaces. The same dropper could be used for all eight dilutions of the same sample if platings were made progressively downward from the highest dilution. The spotted plates, after drying for about 30 min at room temperature, were incubated at 32 or 37C for 15-20 hrs before colonies were counted.

Spots containing an arbitrary range of 10-100 colonies were used for calculating cell density (Figure 1). A stage microscope (Spencer, American Optical Co., Buffalo, N.Y.) at a magnification of 10 times was used to facilitate counting when desired. The counts were then multiplied by the appropriate dilution factors to estimate the cell densities in the original samples.

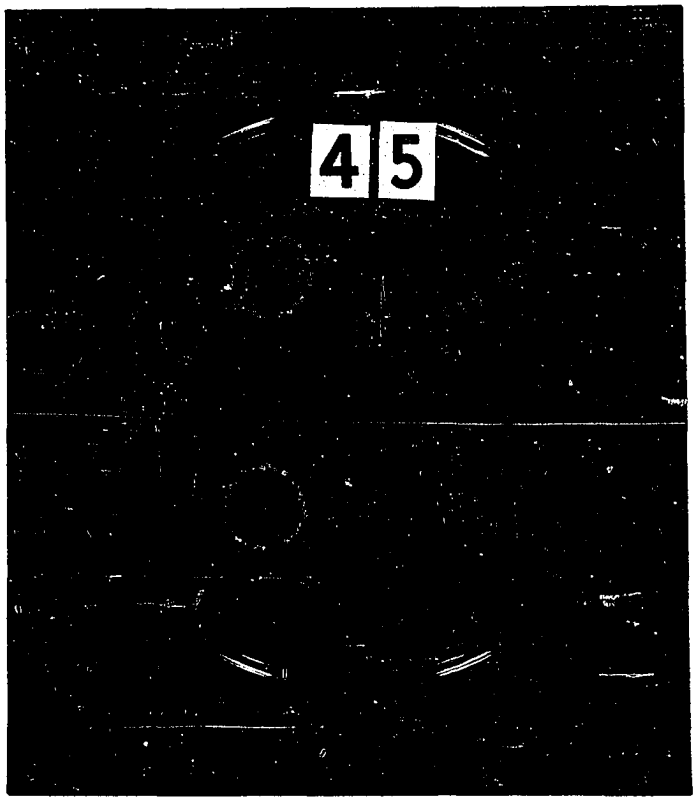
A related rapid procedure employing the Microtiter system and MPN technique to detect viable cell counts was also developed (Fung and Kraft, 1969). In this procedure wells of the Microtiter plate were filled with 0.225 ml of sterile nutrient broth instead of sterile distilled water for both dilution and growth of organisms. Three pretested loops were disinfected by dipping in alcohol and flaming and were used to deliver 0.025 ml of each of the same bacterial sample into the first well of each of three series of 8 wells. The first well was then a 1:10 dilution of the original sample. The three loops were rotated rapidly 20 times before the contents were drain dried on a piece of blotting paper and again disinfected

by alcohol and flaming. Then the loops were reintroduced to the first wells and rotated for 20 times before being carefully placed into the second series of wells. Serial dilutions to the eighth row of wells then gave a 10^8 dilution of the original sample. The alcohol flaming procedure between each dilution was necessary because as little as a single cell contaminant or an accidental carryover from the previous dilution on the loop or the stem will cause erroneous results. Twelve loops could be operated simultaneously by hand or by a commercially made micro-diluter to dilute four bacterial cultures in triplicate to a dilution of 10^8 in about 10 min. The Microtiter plate with diluted cultures was then covered with a dried sterile glass plate and incubated at 37C overnight. Presumptive data could be obtained as early as 6 hr after incubation.

Turbidity of the broth in the wells was indicative of bacterial growth and was designated as positive (Figure 2). Table 2, modified from McCrady (1918), shows the MPN of viable cells from all possible combinations of positive wells of the first 2 dilutions, each in 3 wells of the same sample. The result was reported as MPN per 0.25 ml, or MPN X 4 per ml. When higher dilutions are involved, the MPN was computed from the last 2 dilutions showing positive wells and then adjusted to viable cell counts per ml by multiplying by 4 and 10^{n-2}, where n is the positive exponential of the highest dilution used. For example, the MPN of a result such as 3+/3, 3+/3, 1+/3, and

Figure 1. Duplicate spots of different dilutions from a milk sample. The numbers 4 and 5 represent 10^{-4} and 10^{-5} dilutions. Data obtained from the 10^{-5} dilution were used to calculate cell density

Figure 2. Growth of organisms in the wells of a Microtiter plate for MPN evaluation. The viable cell count for sample A is 45 (from Table 2) X 4 X 10^{4-2}, or 45 X 4 X 10^{2}, or 1.8 X 10^{4} organisms per ml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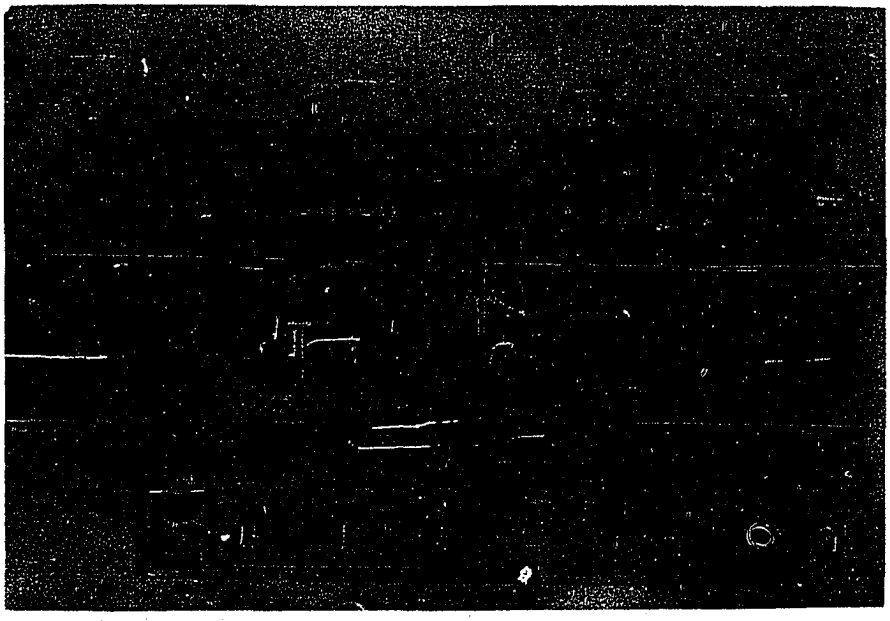

Table 2. MPN per 0.25 ml of sample[a]

Positive wells in 10^{-1} (0.025 ml)	Positive wells in 10^{-2} (0.0025 ml)	MPN/0.25 ml
0	0	0
0	1	3
0	2	6
0	3	10
1	0	4
1	1	7
1	2	12
1	3	16
2	0	9
2	1	15
2	2	20
2	3	30
3	0	25
3	1	45
3	2	110
3	3	250+

[a]Modified from McCrady (1918); instead of 100 ml of sample, 0.25 ml was used.

0+/3 of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, respectively, is 45 (from Table 2) X 4 X 10^{6-2}, or 180 X 10^{4} cells per ml of the original sample.

The Standard Plate Count and the conventional spread plate count were performed in control experiments to check the validity of the above Microtiter procedures. Standard Plate Counts were made using the procedure described in Standard Methods for the Examination of Dairy Products (1967). For the spread plate method, duplicate plates of each dilution, obtained from the dilution bottle-pipette procedure, were made

by introducing 0.5 ml of a dilution onto the dried surface of the agar in a petri dish and spreading evenly with a sterile bent glass rod. The petri dishes were incubated at 24 or 37C, depending on species and samples, for 24-48 hrs. After incubation, all inoculated plates were observed under a Quebec colony counter (Spencer; American Optical Co., Buffalo, N.Y.), and plates containing less than 300 colonies per plate were counted. The counts were then multiplied by the appropriate dilution factors to estimate the cell densities in the original samples.

By use of the Microtiter-spot plate method described above, the associative growth of Staphylococcus aureus S-6 and Pseudomonas fluorescens F-21 was studied. Log-phase cultures grown aerobically in nutrient broth at 30C were employed as inocula. Nepheloflasks (300 ml; Bellco Glass Inc., Vineland, N.J.) containing a total of 50 ml of nutrient broth were used as vessels. Two sample flasks were prepared by inoculating aseptically 1 ml of each strain individually into a flask. A third sample flask for the mixed culture was inoculated with 1 ml of each strain. All three flasks were incubated aerobically at 30C. At suitable intervals, mainly at the log phase, turbidity and viable-cell counts of the contents of each flask were measured. Turbidity was determined by use of Klett-Summerson colorimeter (green filter at 540 mμ) with the meter set at zero against a blank of uninoculated medium, and cell

counts were evaluated by the Microtiter-spot plate method. For S. aureus S-6 counts were made on nutrient agar and on staphylococcus medium No. 110 with egg yolk. For P. fluorescens F-21, the cells were counted on nutrient agar and on Pseudomonas agar F (Difco). For the mixed culture, all three media were used for counts.

A short-term storage study of bacterial counts in manufacturing-grade milk using the Microtiter-spot plate method was also made. Nine manufacturing-grade milk samples were stored at 4C and at intervals of 3, 5, and 12 days, viable cell counts were estimated.

Viable cell counts of four 10% turkey infusion samples were evaluated by the agar plate method, the Microtiter-spot plate method and the Microtiter-MPN method to give direct comparisons of the three procedures.

C. Rapid Methods for Bacterial Identification

The following procedures were developed mainly for the isolation and identification of Staphylococcus aureus from turkey meat but could also be adapted for other bacteriological studies. Eight known cultures (S. aureus 100, S. aureus 196E, S. aureus S-6, S. aureus 137, S. aureus 494, Salmonella typhimurium, S. anatum 53, S. infantis) and 172 unknown isolates were tested by these rapid methods.

Identification of organisms was preformed by using the following tests: Gram staining (Hucker's modification, Hucker and Conn, 1923), microscopic observations, coagulase test (using both the conventional method and the dye diffusion method), and biochemical tests with solid and liquid media by means of the multiple culture plate technique and the replica inoculation device in conjunction with sterile Microtiter plates.

A rectangular (250 X 105 X 15 mm) plastic ice-cube tray (Figure 3) divided into 75 square compartments was used as a multiple culture plate for culturing bacteria. The tray was sterilized by soaking in 1% Chlorox solution for 1 hr, then was placed in a large beaker of sterile distilled water for half an hour before rinsing three times with sterile distilled water. The sterile ice-cube tray was then placed in a large sterile Pyrex glass baking dish (330 X 195 X 52 mm) and stored until needed.

One ml of sterile melted selective or nonselective agar was introduced into each square compartment with a sterile 10 ml pipette. Solidification of agar occurred in about 15 min at room temperature. Inoculation of bacteria into these compartments was done by using disposable, sterile toothpicks. Time consumed for dispensing medium into the ice-cube tray and streaking 100 strains of bacteria onto the agar surface was less than an hour, as compared to 2 hrs or more when the

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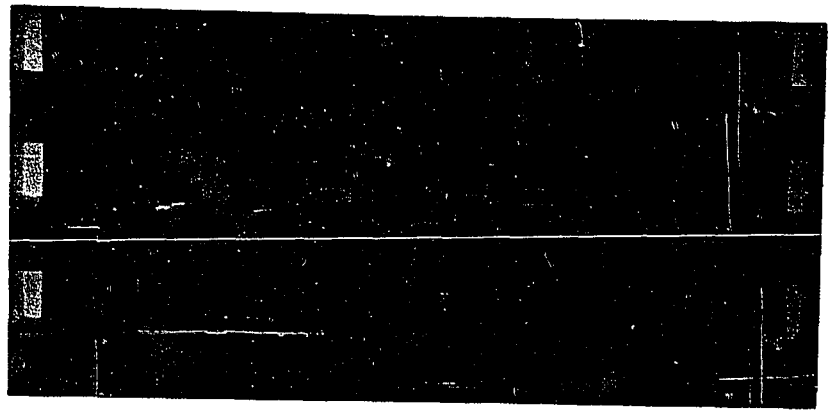
conventional loop-inoculated petri dish procedure was used for culturing bacteria.

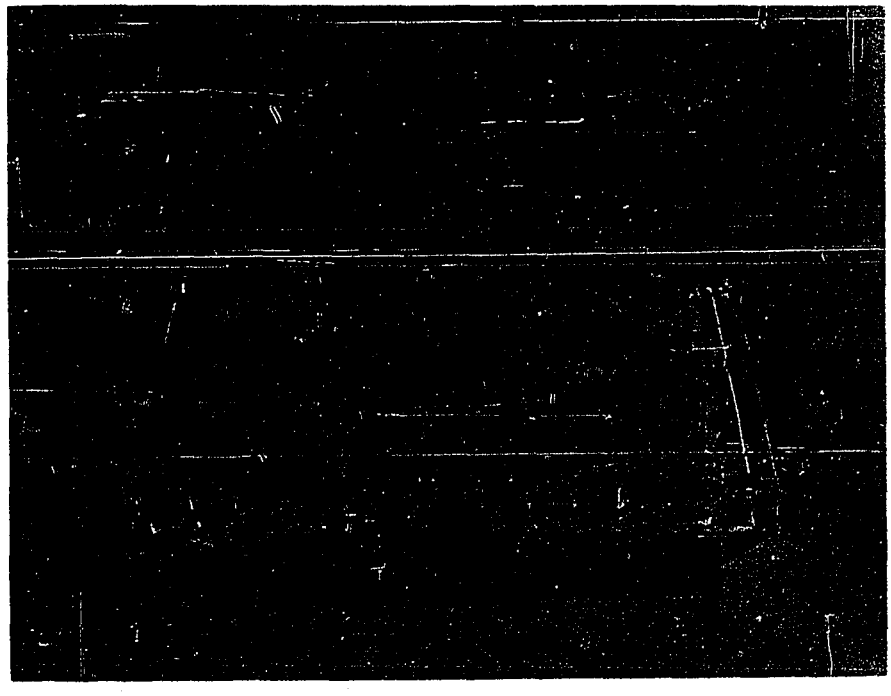
By use of the above procedure, screening of 100 suspected staphylococci from cooked turkey suspected of causing a staphylococcal food poisoning outbreak was performed by streaking each isolate in individual compartments containing nutrient agar, staphylococcus medium No. 110, mannitol salt agar, and tellurite -polymyxin- egg-yolk agar. The inoculated trays were placed in a sterile Pyrex glass baking dish. The dish was covered with a piece of sterile aluminum foil, and the entire assembly was incubated at 37C for 24 hrs. A tube coagulase test, Gram staining, and growth in PHP-NZ-amine medium for enterotoxin production of these isolates were also studied.

Another 72 isolates from 2 turkey rolls and 2 turkey roasts and 5 known strains of staphylococci and 3 known strains of Salmonella were tested by the multiple inocula device developed for rapid identification of bacteria. This device was made by inserting pins (27 mm long) into wax-filled wells of a lucite Microtiter plate (Figure 4), and was used to transfer organisms from a master culture plate to liquid or solid media. The master culture plate was prepared by introducing 4 drops (ca. 0.2 ml) of growth from 12 hr cultures in nutrient broth in small medicine bottles with droppers. Sterilization of the inoculation device was achieved by flaming the pins after they were dipped in alcohol. The sterilized inoculation device was

Figure 3. Growth of organisms on agar contained in individual compartments of a multiple culture tray. Black colonies and egg yolk reaction produced by suspected staphylococcal isolates on tellurite-polymyxin-egg-yolk-agar contained in individual compartments

Figure 4. Multipoint (96) inoculation device designed to fit the wells of a Microtiter plate. Organisms can be transferred by this device to solid or liquid media. Sterilization is achieved by dipping the device into alcohol and then flaming





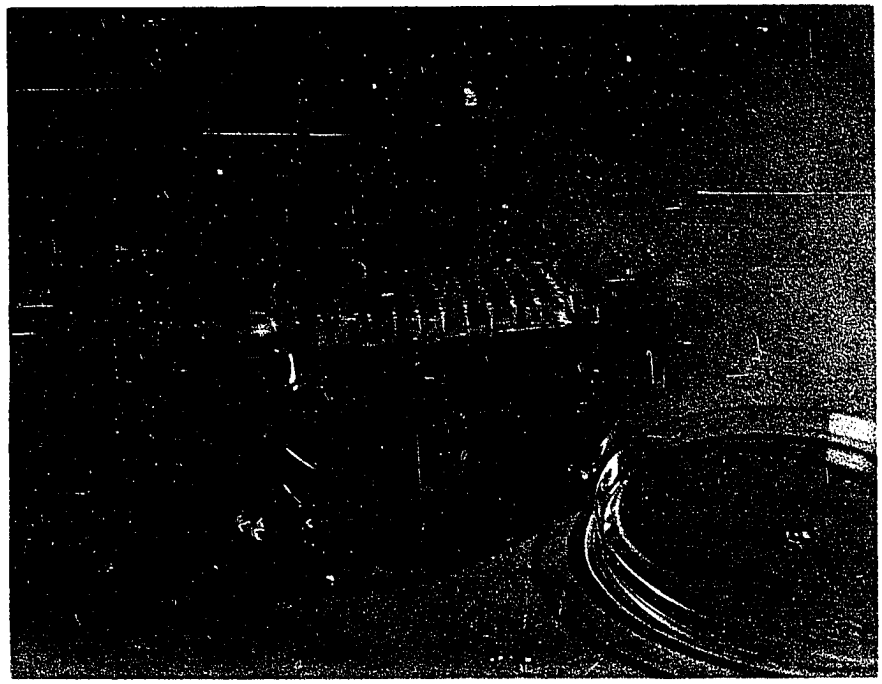
then slowly lowered into the master culture plate to remove organisms. Each pin head was ca. 2 mm in diameter and was able to deliver ca. 0.0006 ml of inoculum (Watt et al., 1966). Then the device with inocula was carefully introduced into either liquid medium in another Microtiter plate or solid agar in a large petri dish (Figures 5 and 6). The petri dish was covered and incubated in an inverted position at 37C for 24 hrs before reading of results (Figure 7). Growth of the 80 organisms on nutrient agar, staphylococcus medium No. 110, and tellurite glycine agar, was tested using the above procedure. For liquid media, a sterile glass plate was used to cover the Microtiter plate before incubation. In carbohydrate fermentation tests, 4 drops of warm-sterile Amojell (1 part of Amojell, American Oil Company, and 1 part of mineral oil) were carefully layered on top of the inoculated well by a sterile Pasteur pipette for trapping gas as well as for maintaining asepsis. With this procedure, a total of 9 carbohydrates (dextrose, dulcitol, lactose, maltose, mannitol, salicin, sorbitol, sucrose, and xylose) were tested by growing the 80 organisms separately in 0.5% carbohydrate in phenol red broth base (Difco). Change of color from red to yellow after incubation indicates carbohydrate fermentation. Gas production could be determined by observing gas bubbles trapped in the interphase of the Amojell and the liquid medium.

Other tests of the 80 organisms included Gram staining, microscopic observations, a coagulase test, and enterotoxin detection in suspected S. aureus.

Coagulase tests were performed by the dye-diffusion test of Fung and Kraft (1968a). Two drops (ca. 0.1 ml) of an 8 hr old suspect S. aureus culture were added by means of a sterile Pasteur pipette to a glass tube (5 X 15 mm) sealed at one end and containing 4 drops (ca. 0.2 ml) of reconstituted plasma solution (Difco). The tube with plasma and culture was first stirred with a sterile toothpick to ensure homogeneity, then incubated at 37 for 2 hr to permit coagulation to occur. A drop of crystal violet solution (Hucker and Conn, 1923) was added to the tube after the reaction time and allowed to diffuse for an arbitrary standard diffusion time of 1.5 hr. A basic assumption of this test is that the rate of diffusion of the dye is inversely proportional to the amount of coagulation. A scale was established to calibrate the degree of coagulation of plasma by S. aureus. From the bottom of a number of tubes, measurements were made of the length of clear zone, representing clots formed by different amounts of activity of coagulase produced (Figure 8). This eight-point scale is more meaningful and reproducible than the existing 0, +, ++, +++, ++++ designation proposed by Turner and Schwartz (1956) and is much easier to record and report. In addition to testing of coagulase of the turkey isolates mentioned above, comparisons were

Figure 5. Inoculation of organisms on agar plate by use of the multipoint inoculation device. The agar surface is flat and thoroughly dry. The Petri dish is covered before incubation in an inverted position

Figure 6. Inoculation of organisms into liquid medium in a Microtiter plate by use of the multipoint inoculation device. The Microtiter plate is covered with a sterile dry glass plate before incubation. Four drops of warm sterile Amojell solution are added for observation of gas production



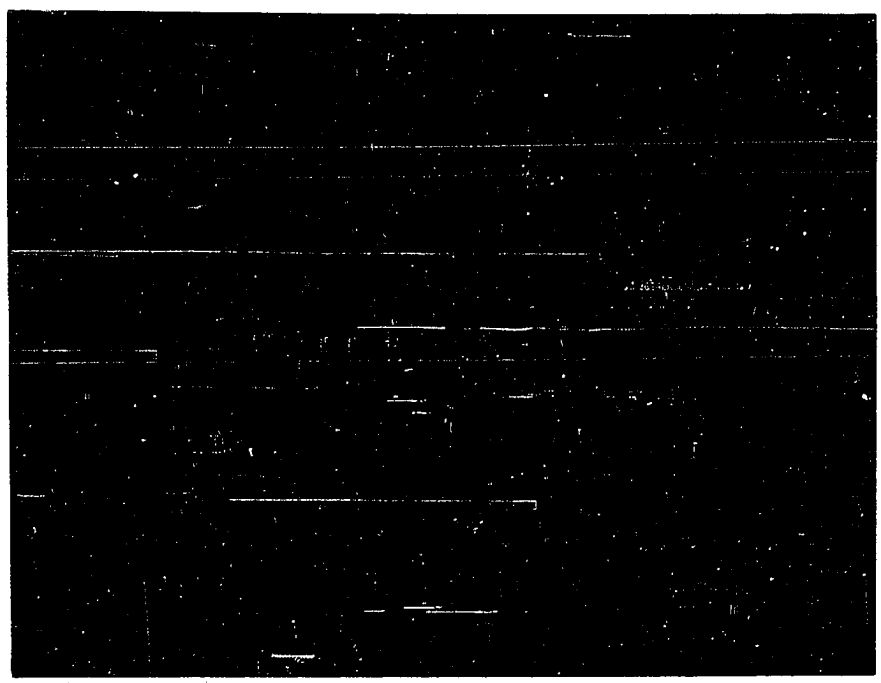
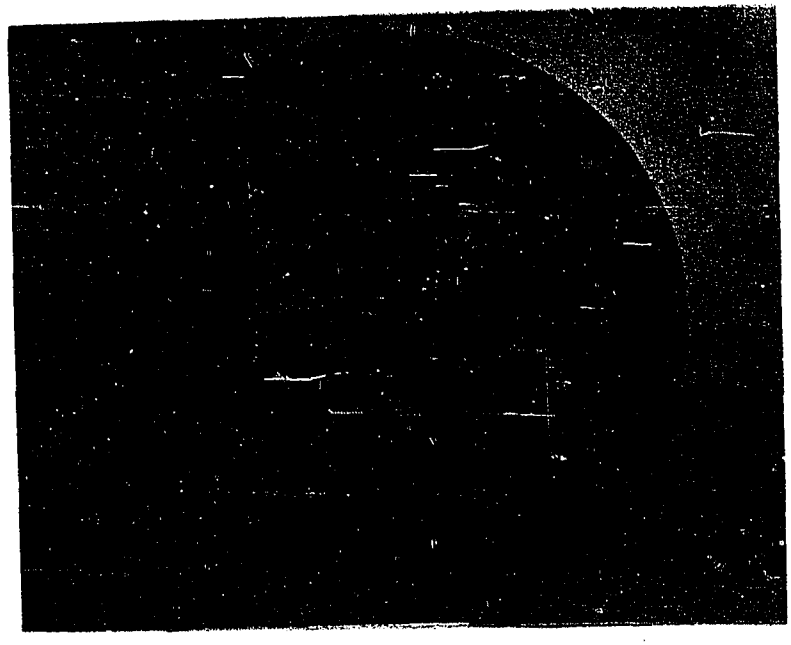
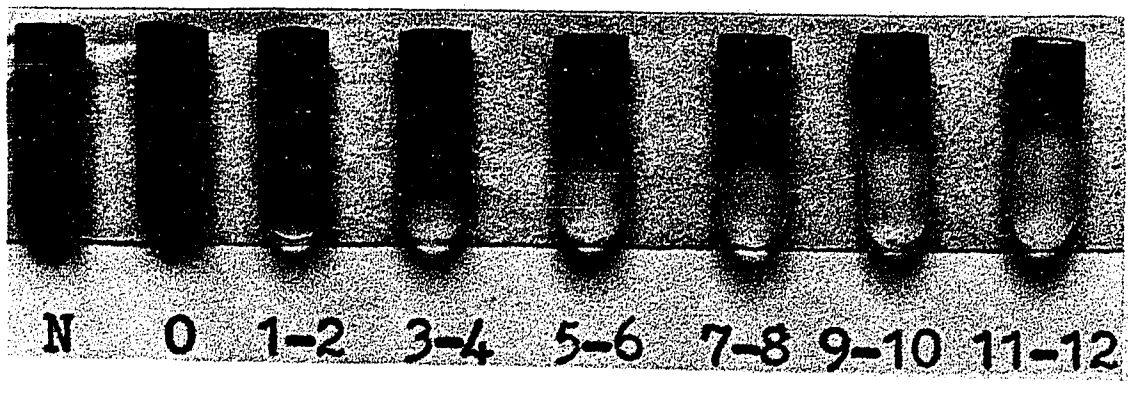


Figure 7. Growth of organisms on an agar plate inoculated with the multipoint device

Figure 8. Coagulation scale. N = negative; 0 = slightly positive. All numbers represent length of clear zones mm, measured from the bottom of the tubes



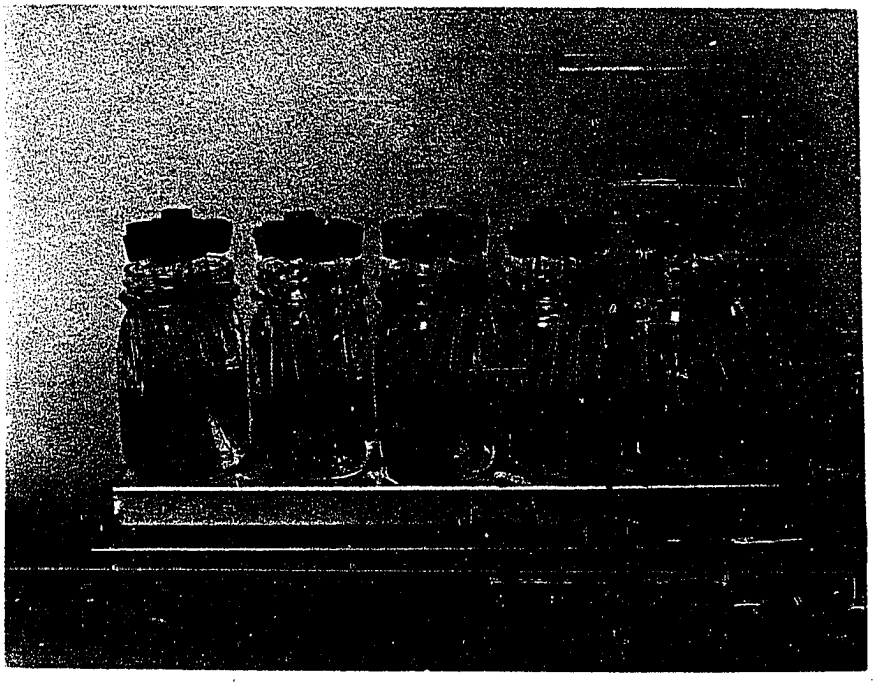

N 0 1-2 3-4 5-6 7-8 9-10 11-12

made of coagulation of plasma by 23 staphylococcal strains in different growth stages. Also, the dye-diffusion method was compared with the conventional tube method with 71 strains of staphylococci isolated from steak and hamburger.

A temperature gradient system, (Figure 9) capable of handling a reasonable number of test tubes (50) and providing a suitable temperature range (25-50C) was developed for studying the effects of temperature and the speed of migrations of Salmonella and Proteus in the multi-layer motility agar system (to be described in detail in a later section). A 10 X 40 cm hot plate, (Eberbach Corporation, Ann Arbor, Michigan), heated at one end and has a range of temperature from 25C to 50C, was used. Five 1-pint Mason jars, each filled with water, 8 test tubes and a thermometer, were placed on the hot plate for 3 hr until the temperature became constant. The temperatures of the water in the jars were adjusted to 28C, 33C, 37C, 42C, and 45C, by shifting the jars on the hot plate until appropriate locations were determined. Slight fluctuation of temperature ($\pm$0.5C) and evaporation of water were found to be insignificant for a 48 hr experiment.

Forty test tubes filled with agar layers were placed in 5 Mason jars each holding 8 tubes and water and adjusted to 28C, 33C, 37C, 42C, and 45C, respectively. Three ml each of four strains of Salmonella (S. typhimurium, S. heidelberg, S. anatum 53, and S. thompson) and four strains of Proteus (P. 2D8,

Figure 9. Temperature gradient system. The metal plate is heated from the left end. Heat conduction through the plate creates a temperature gradient on the plate and subsequently in the Mason jars containing water and test tubes (with liquid or solid medium) for bacterial culture



P. 2D13, P. 2D14, and P. 2D15) were inoculated into each of the eight tubes in one Mason jar. All 5 Mason jars were inoculated to test growth and the ability of these organisms to migrate through the agar systems and provide biochemical reactions at the different specified temperatures and time. Observations of biochemical reactions were taken at 0, 17, 24, and 36 hrs of incubation.

D. Detection of Staphylococcal Enterotoxins

A modified Microtiter hemagglutination-inhibition (HA1) assay (Morse and Mah, 1967) was developed for the detection of staphylococcal enterotoxins A, B, C, and D in culture filtrates and turkey infusions.

Staphylococcus aureus strains were grown individually or in combinations in 50 ml of a sterile complex medium containing 3% protein hydrolysate broth (PHP, Mead Johnson International, Evansville, Ind.), 3% NZ-amine NAK (Sheffield Chemical, Norwich, N.Y.) supplemented with vitamins (thiamine, niacin, and pantothenate, each at a level of 0.5 μg/ml) for 24 hrs at 37C in a gyrotory water-bath shaker (New Brunswick Scientific Company, New Brunswick, N.J.). Supernatants of cultures were collected after growth by centrifugation at 4,340 X G for 2 min, then kept frozen until needed. Food sample preparations for toxin analysis, after growth of organisms or the addition of toxins, were centrifuged at 4,340 X G for 5 min, acidified

to pH 4.5 with 0.1 M HCl, filtered through Whatman No. 42 paper, boiled for 15 min, neutralized to pH 7 with 0.1 M NaOH and were again filtered through Whatman No. 42 paper.

Crude enterotoxins A, B, and C of known concentrations, and their specific antisera were obtained through the courtesy of M. S. Bergdoll of the University of Wisconsin. Crude enterotoxin D of unknown concentration and its specific antiserum were kindly made available by E. P. Casman of the U.S. Department of Health, Education and Welfare.[1]

Formalinization of sheep erythrocytes was made following the procedure of Csizmas (1960). Sheep erythrocytes were collected in Alsever's solution at the College of Veterinary Medicine, Iowa State University. The Red Blood Cells (RBC) were washed 6 times in 10 volumes of cold 0.85% NaCl solution by centrifugation (at 1,085 X G for 2 min), decanted and resuspended between each washing step. The washed packed cells were resuspended in 8 volumes of phosphate-buffered saline solution (1.278 g Na_2HPO_4, 0.816 g KH_2PO_4, and 7.9 g NaCl) at pH 6.8. Formaldehyde solution, 40%, U.S.P., equal to 1/4 of the volume of the cell suspension, was poured into a cellophane dialysis bag, twisted and knotted at both ends. The sac and the RBC suspension were put into a beaker and agitated at room temperature for 2 hrs in a shaker at a speed which provided the most vigorous mixing action obtainable with the least amount of foaming. The contents of the sac were then

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[1]Lyophilized enterotoxin D (0.1 ml) was diluted to 10 ml with distilled water to make a 100 arbitrary units of enterotoxin D per ml of solution.

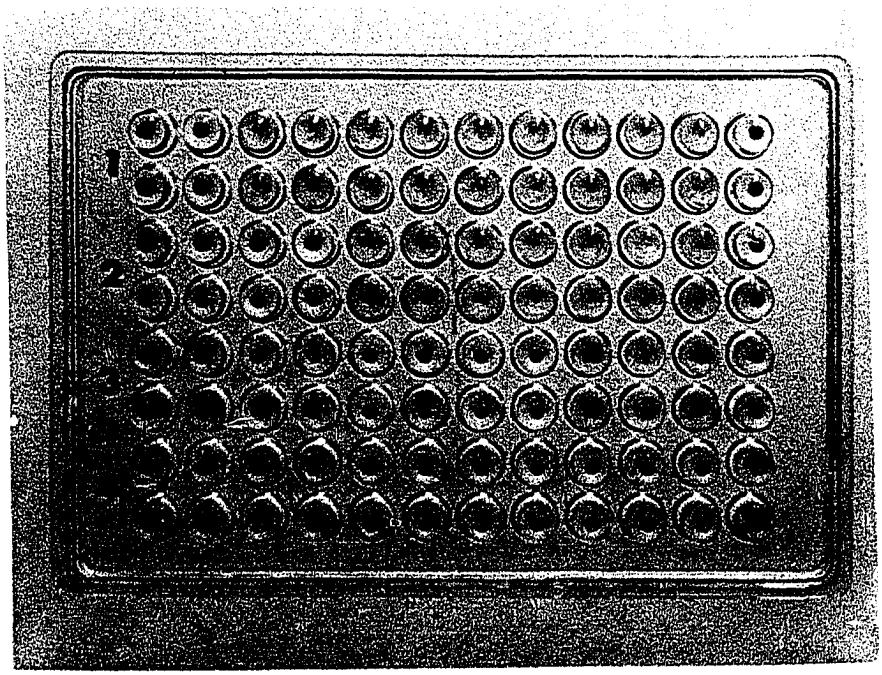
poured into the beaker and the mixture was agitated for another 12 hrs. After filtration through cheese cloth, the cell suspension was washed 6 times in saline as described before. Finally, the packed formalinized cells were resuspended to a concentration of 2.5% in saline and stored in the frozen state in 10 ml aliquots until used.

Thawed formalinized RBC were washed 4 times by centrifugation (1,085 X G for 2 min) decanted and resuspended to 2.5% concentration in saline before sensitization. This cell suspension was incubated with an equal volume of freshly prepared tannic acid (1:5,000) at 37C for 10 min. Tanned RBC were washed in Tris buffer (8.5 g of NaCl, 3.6 g of KCl, and 3.0 g of hydroxymethyl-amino-methane, in 1 liter of distilled water), pH 7.5, once. The cells were again reconstituted to a 2.5% suspension before addition of small amounts of enterotoxin A, B, or C to give a 10 μg/ml concentration of enterotoxins in the suspension. Ten arbitrary units/ml of enterotoxin D were used for sensitization as the exact concentration of the provided toxin was not known. Sensitization of RBC was performed at room temperature (28-30C) with frequent agitation for 30 min. The cells were then washed in 0.1% Bovine Serum Albumin (BSA; grade A, Calbiochem) in Tris buffer and resuspended to a 0.75% concentration. These were the freshly sensitized RBC, ready to be used in the HAI test. Lyophilized sensitized RBC were prepared by dispensing 5 ml aliquots of the sensitized RBC in

10 ml vials and freezing at -20C before lyophilization in a freeze-dryer (Virtis Company, Inc., Gardiner, N.Y.) for 24 hrs. The lyophilized cells were kept in the freezer until needed. Reconstitution was done by adding 5 ml of distilled water to each vial; this volume was sufficient to provide enough sensitized RBC for testing 96 units containing samples and their dilutions. A long range test of stability of the lyophilized sensitized-RBC was made by taking, at various intervals within one year, lyophiles out of storage and performing HAI tests. Reconstituted sensitized RBC were found to be stable after storage in the refrigerator at 4C for 2 days.

A Microtiter plate (8 X 12 wells) was used as the vessel for reaction and for holding serial dilutions. A drop (0.025 ml) of sample (prepared as described) was added to the first two wells by a 0.025 ml dropper. The same amount (0.025 ml) of 0.1% BSA in Tris buffer solution was added, as diluent, to the second well and all other subsequent wells in which dilutions were to be made. Pre-calibrated loops, delivering 0.025 ml, were used for serial dilutions of the samples starting from the second well. Then a drop (0.025 ml) of suitable antiserum (1:50, 1:800, and 1:200 dilutions of antisera A, B, and C respectively; dilution of antiserum D was not determined) was added to each well containing samples and their dilutions. These antisera dilutions were empirically established to give sensitivity factors of specific enterotoxin (Figure 10). A

Figure 10. Determination of antiserum dilutions and sensitivity factors of enterotoxin C. Rows 1, 2, 3, and 4 contain duplicate dilutions of antiserum C: 1:25, 1:50, 1:200, and 1:800, respectively. The wells in columns 1 to 11 (starting from the left) contain enterotoxin C, starting with 5.0 µg in column 1 and serially diluting 1:2 in successive columns (5.0, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.020, 0.010, and 0.005 µg/well, respectively). The last column of wells holds controls, each containing 0.05 ml of Tris buffer plus 0.1% BSA solution and 0.05 ml of sensitized RBC. HAI results in button formation similar to the settling of RBC in the controls. Sensitivity factor obtained from antiserum dilution 1:200 is 0.029 µg/well or 1.17 µg/ml. Antisera dilutions and sensitivity factors of enterotoxins A and B were determined in the similar manner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positive control was prepared by serially diluting a known amount of enterotoxin (200 μg/ml or 5 μg/well). From this control the sensitivity factors were calculated. A series of wells without sample and antiserum except 0.05 ml of 0.1% BSA in Tris buffer solution was prepared as a negative control. The Microtiter plate with samples, serially diluted and with added specific antiserum was incubated at room temperature (28-30C) for 45-60 min; then 0.05 ml of reconstituted sensitized-RBC or freshly sensitized RBC was added to each well. The plate was then sealed with a clear plastic tape or a clean glass plate and was shaken on a Thomas-Boerner (Arthur J. Thomas Co., Philadelphia, Pa.) shaker for 1 min before placing in a cold room (4C) for cells to settle. Readable results were obtainable after 1 1/2 hrs; however, clearer results could be obtained after prolonged incubation (4-5 hrs).

After incubation was completed, the wells containing toxin showed a settling of RBC at the bottom of the well (button formation) because the specific antiserum in the well bound the toxin and could not agglutinate with the sensitized RBC added at a later time. Those wells with no toxin or very low levels of toxin (less than 0.029 μg/well) showed agglutination of RBC because the excess antiserum in the well reacted with the sensitized RBC and formed a lattice. Linear interpolation of the toxin concentrations producing the highest titer of HAI and the lowest titer of hemagglutination (HA) in

the same series of dilution of the toxin control was considered as the sensitivity factor of this batch of cells. In estimating toxin concentration of unknowns, the linear interpolation of the dilutions between the highest titer of HAI and the lowest titer of HA in the same series, was used as the dilution factor. Multiplication of this factor with the sensitivity factor gave an estimate of the toxin level in question.

Klett-Summerson colorimetry and the HAI test were used to evaluate growth and toxigenesis of S. aureus S-6 in 16 different complex media (Table 3), including the PHP-NZ-amine medium already mentioned.

Production of enterotoxins and growth of S. aureus 196E, S. aureus S-6, and S. aureus 137, grown separately in Nephelo flasks in relation to time in the PHP-NZ-amine medium, was measured by turbidity of the cultures and by withdrawing small samples for enterotoxin assay at 3 hr intervals for the first 12 hrs and then for 22 1/2, 24, and 48 hrs of incubation at 37C.

Recovery of toxins A, B, and C from 33.3%, 10%, 2%, and 1% sterile turkey meat infusion (50 ml) after addition of 2 μg/ml of toxin A, 20 μg/ml of toxins B and C individually into each of the above concentrations of turkey meat infusion was tested. The twelve flasks with toxins and infusions were incubated at room temperature (28-30C) for 1 hr before samples were prepared, as described, for enterotoxin analysis using the HAI method.

Table 3. Media for growth and toxigenesis of S. aureus S-6

2%	NZ-amine type A[a]
2%	NZ-amine B
2%	NZ-amine E
2%	NZ-amine HD
2%	NZ-amine NAK
2%	NZ-amine S
2%	NZ-amine YT
2%	NZ-case
2%	Edamine
2%	Edamine S
2%	HY-case
2%	HY-case amine
2%	Pepticase
2%	Soy peptone
2%	PHP + thiamine, niacin, and pantothenate
3%	PHP + 3% NZ-amine NAK + thiamine, niacin and pantothenate

[a]All media obtained from Sheffield Chemical, Norwich, N.Y., except for PHP media, which were obtained from Mead Johnson International, Evansville, Indiana.

A competitive toxin production study of S. aureus 196E, S. aureus S-6, and S. aureus 137 was made by growing these three enterotoxigenic strains individually and in factorial combinations in 50 ml of PHP-NZ-amine medium and 10% sterile turkey meat infusion at 37C in a gyrotory water-bath shaker for 48 hrs. Qualitative and quantitative estimation of the three enterotoxins were made by the HAI test after sample preparations.

Detection of enterotoxin A, B, and C productions by 172 isolates from turkey products, grown in the PHP-NZ-amine medium was also made.

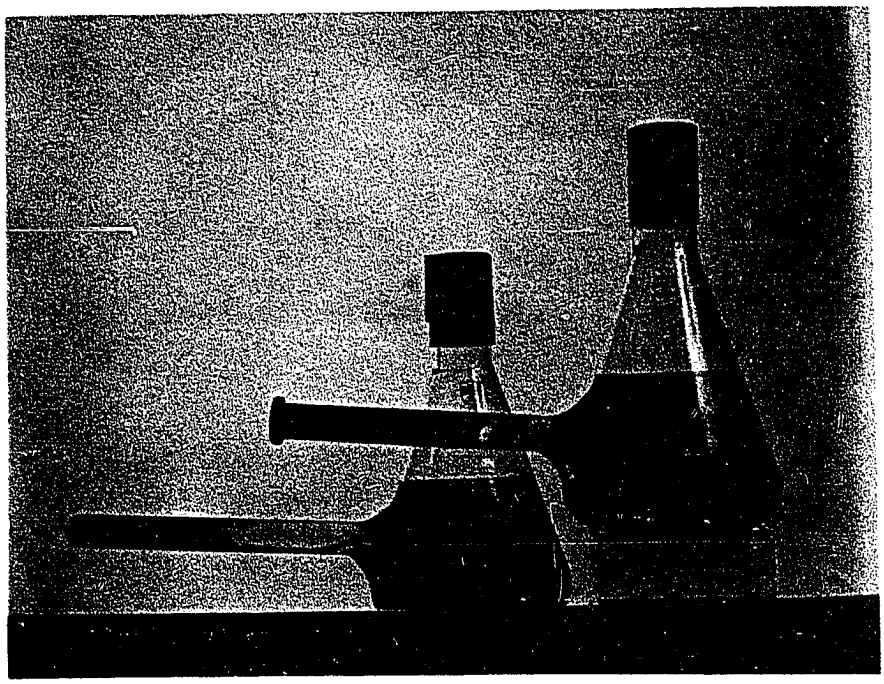
Sensitization of RBC by various units (5, 10 and 100) of enterotoxin D for HA and HAI tests using varying dilutions of antiserum D (from undiluted to 1:800) was performed to test the applicability of the HAI test for this enterotoxin.

E. Detection of Salmonella

The motility of Salmonella in semisolid agar with selective toxic material is the basis of the following multi-layer motility agar system for the detection of Salmonella in culture and in foods.

A nepheloflask was the vessel of choice for growth of Salmonella in selective liquid media or food homogenates for detection of motility and biochemical reactions. For the latter, solid media in the side arm was used (Figure 11A). When isolation of organisms was desired, a modified nepheloflask with the tip of the side arm cut and capped with a rubber stopper was used so that motile organisms could be withdrawn with a sterile syringe (Figure 11B). A commercially made modified nepheloflask was also used for this purpose. The tip of the side arm was threaded to fit a screw cap which protects the rubber stopper from contamination and from dislodging. Cultures could be drawn through the rubber stopper from the side arm after the cap was removed, and streaked directly on brilliant green agar (Figure 12A,B).

Figure 11. Biochemical reactions of *Salmonella* in multi-layer motility agar flask. Flasks A and B are uninoculated. Flasks C and D show presumptive and confirmed biochemical reactions of motile-H_2S positive *Salmonella*, respectively



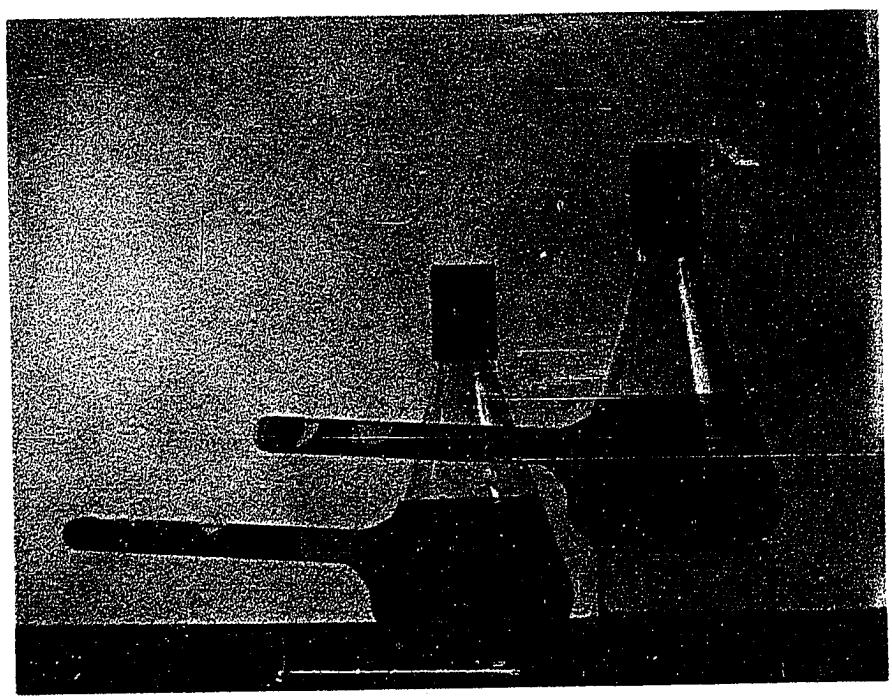
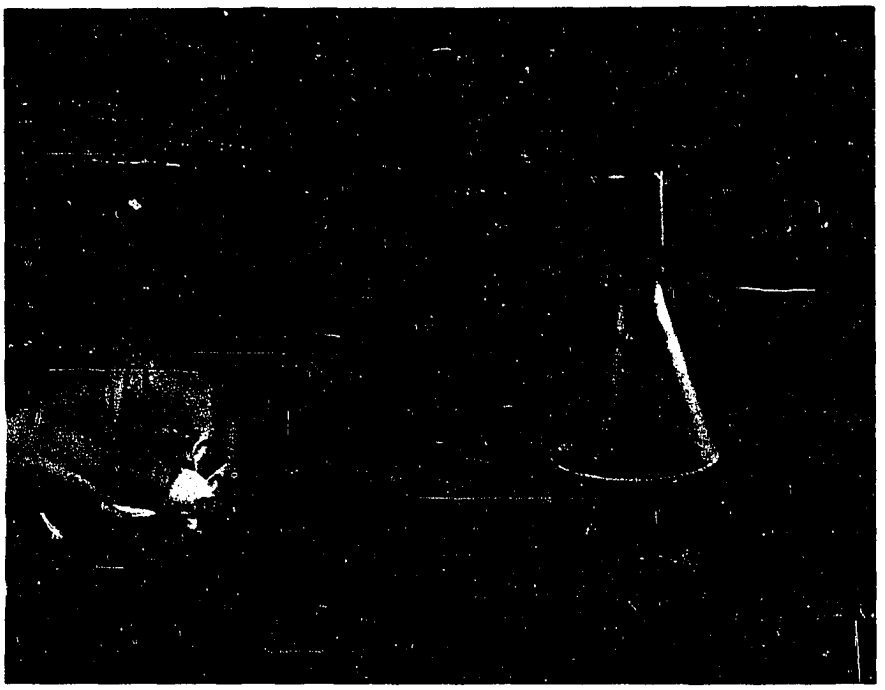
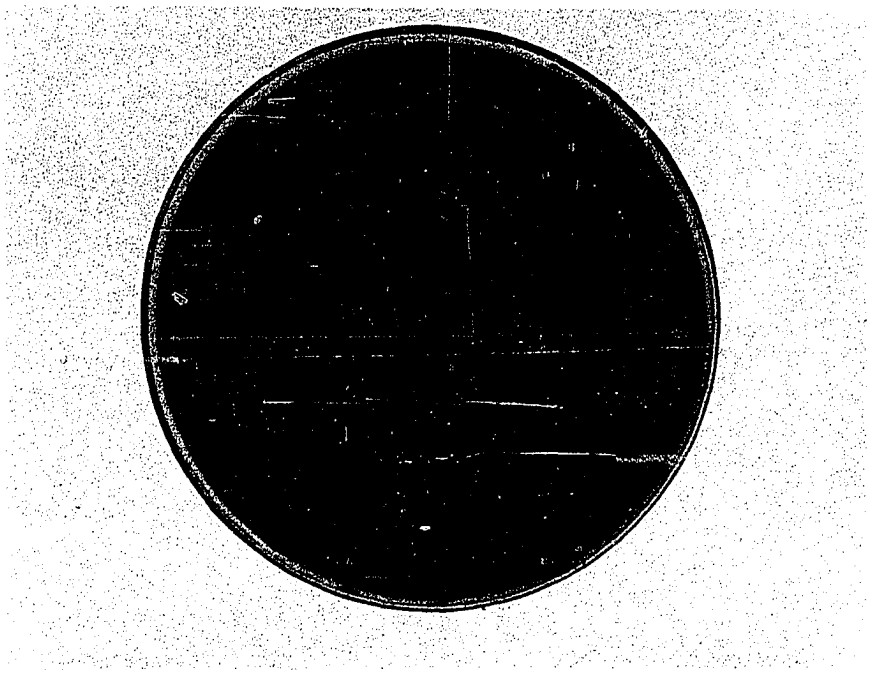


Figure 12. Testing for Salmonella from a nepheloflask culture

(A) Top: Extraction of organisms from the side arm of a modified nepheloflask by use of a sterile syringe. (B) Bottom: Growth of organisms streaked by syringe on brillian green agar plate





Sterile and melted triple sugar iron agar (TSI, Difco, 3 ml), carbohydrate agar (0.5% mannitol or dulcitol, 1% agar in Difco phenol red base broth, 3 ml), and selenite cystine agar (Difco selenite cystine broth, SIM agar, and 1% agar, 3 ml) were introduced into the side arm (placed perpendicularly in a test-tube rack) of a pre-sterilized nepheloflask. The flask was positioned so that the side arm was upright and the agars were added in succession after solidification between each addition. After solid media preparation, the nepheloflask was placed in a normal position so that the side arm was parallel to the bench top before 250 ml of sterile lactose broth (Difco) and 1% inoculum were added.

Turbidity of the lactose broth indicated growth of organisms in the flask. Motile Salmonella migrated through the selenite cystine agar layer and fermented the mannitol or dulcitol causing change of color from red to yellow with gas production in the carbohydrate agar layer. Other motile organisms were either inhibited or delayed in the selenite cystine layer and did not give a typical Salmonella reaction in the carbohydrate agar. This was considered as a presumptive biochemical test for Salmonella which occurred after 12 hr of incubation. When motile Salmonella migrated to the TSI agar layer, carbohydrates were fermented and the color of the agar changed from red to yellow with the production of gas (Figure 11C). Excessive gas production sometimes forced the selenite

cystine agar layer out of the side arm into the flask. After 4-6 more hrs, blackening developed at the tip of the side arm due to hydrogen sulfide production under anaerobic conditions. This stage was regarded as a confirmed biochemical test for the presence of motile-H_2S producing Salmonella (Figure 11D). Serological reactions could be performed by picking organisms from the tip using a sterile syringe and streaking the inoculated needle on brilliant green agar for Salmonella to grow (Figure 12B). A slide agglutination test for Salmonella was performed by making a smear of typical 12-hr Salmonella cultures from brilliant green agar, and mixing with commercially prepared polyvalent O-serum.

Using the motility flask system described, an attempt was made to detect Salmonella in a mixed population containing Proteus sp. 2D20, Pseudomonas aeruginosa 2F40, and Escherichia coli 2B5. S. typhimurium was used as a representative organism in this study unless specified. Four control motility flasks were prepared by aseptically inoculating a 1% inoculum of each strain individually into a flask. Viable cell counts of each test organisms were also made. Two other control flasks were prepared, one was inoculated with 1% Salmonella and the other with 1% inoculum of all four test organisms. At the end of the experiment, cultures were taken from the tip of the side arm of both flasks and streaked on differential media to detect the presence of Salmonella. Seven additional

motility flasks were prepared; each flask was inoculated with a 1% concentration of Proteus sp. 2D20, P. aeruginosa 2F40, and E. coli 2B5 and successive decimal dilutions of Salmonella. Thus, the concentration of Salmonella in the seventh flask was a 10^{-6} dilution of the bacteria in the first flask. All 13 flasks were incubated at 37C for 36 hrs with observations made at 0, 17, 20, 24, 30, and 36 hrs.

Preliminary experiments showed the ability of Proteus sp. 2D20 and E. Coli 2B5 to travel through the above agar layers and produce atypical Salmonella reactions. In order to further eliminate these competitive organisms in this system, Sodium lauryl Sulphate (SLS, Jameson, 1961) was incorporated into the selenite cystine agar layer. This was done by adding appropriate percentages of sterile SLS into sterile and melted selenite cystine agar mixture. A series of motility flasks were prepared as described previously except for addition of SLS (0.1, 0.15, 0.2, 0.3, 0.5, and 1%) in the selenite cystine agar, and substitution of mannitol for dulcitol in the carbohydrate layer for all subsequent experiments. The flasks were inoculated at a level of 1% with cultures of Proteus sp. 2D15, E. coli 2B5, and Salmonella individually to determine the effects of different concentrations of SLS on the migration of these organisms in this multi-layer motility agar system. The 18 flasks were incubated at 37C for 36 hrs with readings taken at 0, 17, 24, 30, and 36 hrs.

Another attempt was made to detect Salmonella in mixed cultures containing Proteus sp. 2D20 and E. coli 2B5 by using the multi-layer motility agar system, with the incorporation of 0.15% SLS in the selenite cystine agar, and the conventional method. The multi-layer motility agar procedure was identical to the previous experiment on detection of Salmonella in mixed cultures. The conventional method included: (1) growing pure or mixed cultures in 200 ml of sterile lactose broth for 8 hrs at 37C, (2) transferring 5 ml of the lactose broth culture into 100 ml of selenite cystine broth and incubating for 24 hrs at 37C, (3) streaking the selenite cystine broth cultures on brilliant green agar and incubating for 24 hrs at 37C, (4) stabbing 3 typical pink colonies into 3 TSI slants and incubating for 24 hrs at 37C, and (5) slide polyvalent 0 serum agglutination tests of all cultures showing typical biochemical reactions in TSI slants.

Five known strains and 20 unknown Salmonella isolates were tested in this multi-layer motility agar system by introducing 1% inoculum into 250 ml lactose broth contained in multi-layer motility agar flasks. The selenite cystine agar was fortified with 0.15% SLS. The flasks were incubated at 37C for 42 hrs with readings taken at 0, 17, 24, 36, and 42 hrs of incubation. The Salmonella isolates were previously obtained from chicken by use of the conventional procedures including polyvalent 0 serum agglutination tests.

Fifty grams of turkey rolls were homogenized aseptically with 100 ml each of sterile distilled water and sterile lactose broth in an Osterizer, then introduced into a multi-layer motility agar flask. For this test a total of ten flasks containing turkey roll homogenate were prepared. One control flask was inoculated with Salmonella, and another with Proteus. Eight test flasks were inoculated with constant numbers of competitive organisms (Escherichia, Proteus, and Pseudomonas) and decimally diluted Salmonella. A similar experiment, with no competitive organisms, was performed by inoculating, in duplicate, three dilutions (at low concentrations) of five known strains of Salmonella. Inoculations were made in 30 motility agar flasks containing turkey roll homogenate and another 30 motility agar flasks containing turkey roast homogenate. The flasks were incubated at 37C and readings were taken at 0, 24, and 36 hrs of incubation.

For the detection of Salmonella in whole egg, one whole egg was aseptically introduced into a motility flask containing sterile lactose broth to give a total volume of 250 ml. The egg shell was disinfected by dipping the egg in alcohol, draining and flaming the air-space end of the egg. A small portion of the egg shell was then cracked and the contents were carefully emptied into the flask. One set of flasks was prepared with one egg and lactose broth added to each flask. The mixture was then inoculated with a constant number of

competitive organisms and serial dilutions of Salmonella. Another set of motility flasks was prepared with the first flask only containing one egg and lactose broth with Salmonella. Decimal dilutions of Salmonella from this flask were made to the next three flasks each of which contained 250 ml of sterile lactose broth. A constant number of competitive organisms was added to all four flasks. Detection of Salmonella in whole egg was also performed by preparing twelve motility flasks with one egg added aseptically to each flask but without added inocula. The flasks were incubated at 37C with readings taken at suitable intervals up to 40 hrs.

Similar experiments were performed using commercial egg yolk solids, egg white solids and whole egg solids in order to test the applicability of this system for detection of Salmonella in processed egg products. Fifty grams of egg solids were added aseptically to a series of motility flasks and dispersed in sterile lactose broth to give a total liquid volume of 250 ml. Again the Salmonella culture was decimally diluted in the flask with constant numbers of competitive organisms. Viable cell counts of all inocula were made for each experiment. The flasks, prepared and inoculated, were incubated at 37C for a maximum of 48 hrs with readings usually taken at 0, 17, 20, 24, and 36 hrs.

Detection of Salmonella in chicken wings purchased from a local store and stored at 4C for 2 to 12 days was made by

rinsing each chicken wing, individually, in 200 ml of sterile lactose broth; and adding the broth aseptically to a multi-layer motility agar flask for Salmonella detection. A total of 29 chicken wings was tested by the new method as well as the conventional method to evaluate the efficiency of the two methods for Salmonella detection.

MPN evaluation of Salmonella was made by inoculating known concentrations of Salmonella in a multi-layer motility agar flask containing 250 ml of sterile lactose broth, then diluting in triplicate, to 10^{-2} and 10^{-4} dilutions in 6 other flasks. The seven flasks were incubated at 37C for 36 hrs after which the reactions in the side arms were recorded. The 3 tube table of McCrady (1918) was consulted for MPN estimation. To reduce the number of flasks to be prepared, 10^{-1} and 10^{-3} dilutions were not tested. Since the 10^{-4} dilution was made from the corresponding 10^{-2} dilution, a positive result in the 10^{-4} dilution flask also implied a positive result in the 10^{-3} dilution flask provided the 10^{-2} dilution flask was also positive. Conversely, if the 10^{-2} dilution flask was positive and the 10^{-4} dilution negative, the 10^{-3} could either be positive or negative. In such a case the MPN was reported as a range between the two limits. For the above experiment, organisms were picked from the tips of modified nepheloflasks and streaked on brilliant green agar for serological tests of Salmonella.

IV. RESULTS AND DISCUSSION

A. Bacteriological Techniques

Bacteriological and biochemical reactions of the 180 isolates and cultures on various solid and liquid media by use of the conventional procedures (Gram staining and microscopic examination) and the rapid methods are reported in the Appendix, Tables 27 and 28.

Two bacteriological techniques are discussed in this section. Except for 10 staphylococcal isolates and 5 known cultures, the isolates and cultures used in these two sets of experiments were different from the 180 organisms mentioned above.

Coagulation of plasma by 23 staphylococcal strains in different growth stages (Table 4) was tested by the dye diffusion method. Also, the dye-diffusion method was compared with the conventional tube method with 71 strains of staphylococci (Table 5). These organisms were grown in trypticase soy broth (BBL), brain heart infusion (Difco), or nutrient broth (Difco) in a water-bath shaker at 37C for different time intervals.

The data showed good correlation of the two methods with strongly positive cultures (Table 5). With weakly positive cultures, the dye-diffusion method facilitated measurement of coagulase activity. Also, the dye method made use of less plasma (about one-fourth that needed in the conventional tube

Table 4. Comparison of degrees of coagulation by S. aureus strains

Organism	Source	Degree of coagulation			
		1.5-hr cells		24-hr cells	
		2 hr[a]	4 hr[a]	2 hr[a]	4 hr[a]
S. aureus 3A2	Hartman[b]	10	10	10	10
S. aureus 3A3		5	6	6	6
S. aureus 3A12					
S. aureus 3A15		10	10	10	10
S. aureus 3A17		10	10	10	10
S. aureus 3A19		9	10	10	10
S. aureus 3A23		11	11	11	11
S. aureus 3A30		9	9	9	9
S. aureus 3A31		8	9	9	9
S. aureus S-6	Bergdoll[c]	11	11	11	11
S. aureus 6536P	Kraft[d]				
S. aureus 293	Hall[e]				
S. aureus 196E	Hall[e]	8	8	8	8
Isolate D-1	Fung[f]	9	10	10	10
Isolate D-2		9	10	10	10
Isolate D-3		9	10	10	11
Isolate D-4		11	11	11	11
Isolate D-5		11	11	11	11
Isolate D-6		8	9	9	9
Isolate D-7		11	11	11	11
Isolate D-8		10	10	10	10
Isolate D-9		10	10	11	11
Isolate D-10		10	10	10	10

[a]Incubation time.

[b]Department of Bacteriology, Iowa State University, Ames, Iowa.

[c]University of Wisconsin.

[d]Department of Dairy and Food Industry, Iowa State University, Ames, Iowa.

[e]National Center for Urban and Industrial Health, Cincinnati, Ohio.

[f]Isolated from turkey meat suspected to have caused staphylococcal food poisoning.

Table 5. Comparison of dye-diffusion method and conventional tube method

No. of staphylococcal strains[a]	Degree of coagulation: Dye-diffusion method (length of clear zone)	Degree of coagulation: Conventional method
	mm	
4	11	4+
10	10	4+
4	9	4+
6	8	4+
1	7	4+
1	5	4+
2	5	
3	4	
2	1	
9	0	
29	Negative	
Total tested, 71	Total coagulase-positive, 42	Total coagulase-positive, 26

[a]Isolates from beef steak and hamburger.

technique), representing a saving of materials for large-scale screening tests. The same principle and reasoning in this test can be applied to other tests in which coagulation or increased viscosity is the parameter under scrutiny.

Data for the temperature gradient study are presented in Table 6. The temperature range providing fastest (17 hrs) completed Salmonella reactions in this system is between 37-42C. Temperatures of 28 and 33C showed slight delay of complete reactions, while 45C did not support growth at all. This system did not allow Proteus to give typical Salmonella

Table 6. Effects of temperature on migration and biochemical reactions of *Salmonella* and *Proteus* in the multi-layer motility agar system

Organisms	Temperatures															
	28C				33C				37C				42C				45C			
	0[a]	17	24	36	0	17	24	36	0	17	24	36	0	17	24	36	0	17	24	36
S. typhimurium	-[b]	±	+	+	-	±	+	+	-	+	+	+	-	+	+	+	-	-	-	-
S. heidelberg	-	±	+	+	-	±	+	+	-	+	+	+	-	+	+	+	-	-	-	-
S. anatum 53	-	±	+	+	-	±	+	+	-	+	+	+	-	+	+	+	-	-	-	-
S. thompson	-	±	+	+	-	±	+	+	-	+	+	+	-	+	+	+	-	-	-	-
Proteus 2D8	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
Proteus 2D13	-	A	A	A	-	A	A	A	-	-	-	A	-	-	-	-	-	-	-	-
Proteus 2D14	-	-	A	A	-	A	A	A	-	-	-	A	-	-	-	-	-	-	-	-
Proteus 2D15	-	A	A	A	-	A	A	A	-	-	A	A	-	-	A	A	-	-	-	-

[a]Time of incubation in hrs.

[b]- signifies no reaction in the agar layers,
± signifies *Salmonella* reaction with only change of color and gas production,
+ signifies typical *Salmonella* reaction including gas and H_2S production,
A signifies atypical *Salmonella* reaction with no gas formation and a dull yellow color.

reactions. The data also showed that at 37, 42, and 45C, biochemical activities for Proteus spp. in this system were inhibited or retarded. At lower temperatures the rate of reaction for Proteus was faster. This set of data demonstrated the usefulness of the simple temperature gradient system in studies of this nature.

B. Identification and Characterization of Staphylococcal Isolates

All the isolates (100) from the turkey meat suspected of causing food poisoning grew well on nutrient agar, staphylococcus medium No. 110 with egg yolk, and in nutrient broth and the PHP-NZ-amine medium. Ninety-seven of the isolates were Gram positive cocci aggregated in clusters, and which grew well on mannitol salt agar (except one isolate) and produced typical black colonies on tellurite-polymyxin-egg-yolk agar (except for three cultures). Coagulase activity ranged from 2 to 12 on the dye-diffusion scale and was distributed rather evenly among the 97 strains. From the above information, these 97 strains were identified as coagulase positive Staphylococcus aureus.

A total of 37% of the isolates were enterotoxigenic as determined by the HAI test. Of the three enterotoxins tested only enterotoxin A was detected. Twelve strains produced 1.77 µg/ml, 5 strains produced 1.18 µg/ml, 15 strains produced 0.89

µg/ml and 5 strains produced 0.59 µg/ml of enterotoxin A in PHP-NZ-amine medium. All the enterotoxigenic strains were coagulase positive in varying degrees (ranging from 3 to 11). The other 60 strains of S. aureus were also coagulase positive but nontoxigenic. The other three isolates were Gram positive and coagulase negative rods and did not grow in mannitol salt agar and tellurite-polymyxin-egg-yolk agar. The identities of these three isolates were not determined. These experiments demonstrated the usefulness of the multiple culture plate, the dye diffusion coagulase test, and the HAI test for the isolation, detection and characterization of S. aureus from turkey meat as well as detection of enterotoxin both qualitatively and quantitatively by the isolates.

The second group of isolates (72) grew well on nutrient agar. All 72 isolates obtained from staphyloccus medium No. 110 with egg yolk were Gram positive cocci, grew well on tellurite glycine agar and mannitol salt agar (14 grew but did not ferment mannitol). Sixty of these isolates were coagulase positive (ranging from 0-12 on the coagulase scale). The carbohydrates fermented by these 60 isolates (with few exceptions) were dextrose, lactose, maltose, mannitol, sorbitol, sucrose, and xylose. The two carbohydrates not fermented were

dulcitol and salicin. From the above results, these 60 Gram positive and coagulase positive isolates were identified as S. aureus strains. The other 12 isolates, being Gram positive cocci, coagulase negative, and not fermenting mannitol, were identified as S. epidermidis strains. These 12 strains fermented (with some exceptions) dextrose, lactose, maltose, sucrose, and did not ferment dulcitol, mannitol, salicin, sorbitol, and xylose.

Twenty-seven isolates (37.5% of the staphylococcal isolates) produced enterotoxins A and/or C but not B. Thirteen isolates produced enterotoxin A alone and 7 isolates produced both enterotoxins A and C. Another 7 isolates produced only enterotoxin C. The amount of enterotoxin produced by each enterotoxigenic isolate was about 0.59 to 0.89 μg/ml. All the toxigenic isolates were coagulase positive. It is interesting to note that the percentage of enterotoxigenic isolates in the first group (37%) and the second group (37.5%) was almost identical. The only difference was that the first group produced only enterotoxin A while the second group produced enterotoxins A and C. da Silva (1967) found 33% of the organisms isolated from a turkey plant and tested for enterotoxin were enterotoxigenic. He used the dog test for enterotoxin detection.

C. Rapid Microtiter Techniques for Viable Cell Counts

Results of the evaluation of viable cell counts of known cultures as well as food samples by the two Microtiter procedures and the conventional agar plate method are discussed below.

Enumeration of viable cells for six bacterial types (Staphylococcus aureus, Streptococcus durans, Serratia marcescens, Pseudomonas glycinea, Escherichia coli, and mixed cultures from turkey meat) were made by the Microtiter-spot plate method and the agar plate method to give a direct comparison of the two methods. The 24-hr growth data for S. aureus S-6 and S. durans with and without ultrasonic treatment and both the agar plate method and the Microtiter-spot plate method are tabulated in Table 7. The data indicated that for S. aureus S-6 both the agar plate method and the Microtiter-spot plate method agreed closely with or without prior ultrasonic treatment of cultures. For S. durans, a 5- to 10-fold difference in counts existed between the two methods. However, the parallel data of the two methods after ultrasonic treatment essentially was in agreement.

The 24-hr growth data of S. marcescens, P. glycinea, E. coli K-12, and mixed bacteria from turkey meat are also tabulated in Table 7. Statistical analyses of the eight sets of data for these cultures collectively showed a correlation coefficient of 0.976 (see Appendix, Table 26) between the

Table 7. Comparison of viable-cell counts[a]

Organism	Agar plate method counts/ml	Microtiter-spot plate method counts/ml
Staphylococcus aureus		
Without sonic vibration	2.80×10^6	3.40×10^6
With sonic vibration	5.80×10^7	8.30×10^7
Streptococcus durans		
Without sonic vibration	1.78×10^6	12.0×10^6
With sonic vibration	11.30×10^5	6.50×10^5
Serratia marcescens	4.05×10^7	4.20×10^7
Pseudomonas glycinea	8.40×10^6	7.10×10^6
Escherichia coli	5.10×10^6	5.70×10^6
Mixed cultures from turkey meat	2.10×10^5	1.40×10^5

[a]The numbers were calculated from counts obtained from duplicate plates or spots of appropriate dilutions of each sample. Correlation coefficient of these methods is 0.976.

conventional agar plate method and the newly developed Microtiter-spot plate method.

The viable cell counts of the 34 manufacturing-grade milk samples and the 13 Grade A milk samples determined by the two methods are tabulated in Table 8 and Table 9, respectively. With the exception of eight samples (Nos. 4, 6, 7, 13, 26, 28, 29, and 34) of manufacturing-grade milk, the viable cell counts obtained by the Standard Plate Count were higher than those obtained by the Microtiter-spot plate method. However, among the Grade A milk samples, six (Nos. 37, 38, 39, 44, 45, and 46) provided higher counts for Standard Plate Count and the other six revealed higher counts for the Microtiter-spot plate method; No. 36 gave essentially the same count by both methods.

Table 8. Comparison of viable-cell counts in manufacturing-grade milk[a]

Sample number	Standard plate counts/ml 10^6	Microtiter-spot plate method counts/ml 10^6
1	6.20	2.00
2	3.10	2.50
3	6.00	1.20
4	0.91	2.00
5	19.00	10.00
6	20.00	62.00
7	0.27	2.00
8	8.50	3.80
9	0.03	0.02
10	35.00	8.00
11	15.00	7.00
12	0.74	0.16
13	12.00	49.00
14	0.48	0.17
15	0.72	0.02
16	350.00	80.00
17	18.00	11.00
18	20.00	13.00
19	0.20	0.01
20	2.80	2.00
21	3.00	1.10
22	1.20	0.31
23	11.00	3.20
24	3.10	1.10
25	16.00	4.10
26	30.00	89.00
27	33.00	8.20
28	8.50	13.00
29	3.60	7.90
30	69.00	20.00
31	0.03	0.01
32	48.00	19.00
33	3.20	0.27
34	1.60	1.60

[a]The numbers were calculated from counts obtained from duplicate plates or spots of appropriate dilutions of each sample. Correlation coefficient of these methods is 0.604.

Table 9. Comparison of viable-cell counts in Grade A milk[a]

Sample number	Standard plate counts/ml 10^4	Microtiter-spot plate method counts/ml 10^4
35	0.48	2.00
36	0.39	0.38
37	0.50	0.22
38	0.67	0.38
39	0.64	0.48
40	3.40	10.60
41	0.62	1.30
42	0.78	2.40
43	0.86	2.40
44	1.10	0.11
45	1.30	0.10
46	1.80	0.22
47	0.30	5.20

[a]The numbers were calculated from counts obtained from duplicate plates or spots of appropriate dilutions of each sample. Correlation coefficient of these methods is 0.651.

Statistical analyses of the data of the 34 manufacturing-grade milk samples showed a correlation coefficient of 0.604 between the Standard Plate Count and the Microtiter-spot plate method. Since the critical value at 1% level for 32 degrees of freedom is 0.436, this correlation coefficient is significant at the 1% level (Snedecor and Cochran, 1967). The same statistical analysis on the data obtained from Grade A milk samples showed a correlation coefficient of 0.651, which is significant at the 5% level with 11 degrees of freedom and a critical value of 0.553. The combined coefficient correlation is 0.623, which is significant at 1% level with 45 degrees of freedom and a

critical value of 0.372 (see Appendix, Table 26).

The results of the storage study are tabulated in Table 10. Although no comparison of methods was made, the Microtiter method demonstrated increases in bacterial numbers in milk samples during storage, as may be expected.

Table 10. Microtiter-spot plate method count of manufacturing-grade milk in cold storage[a]

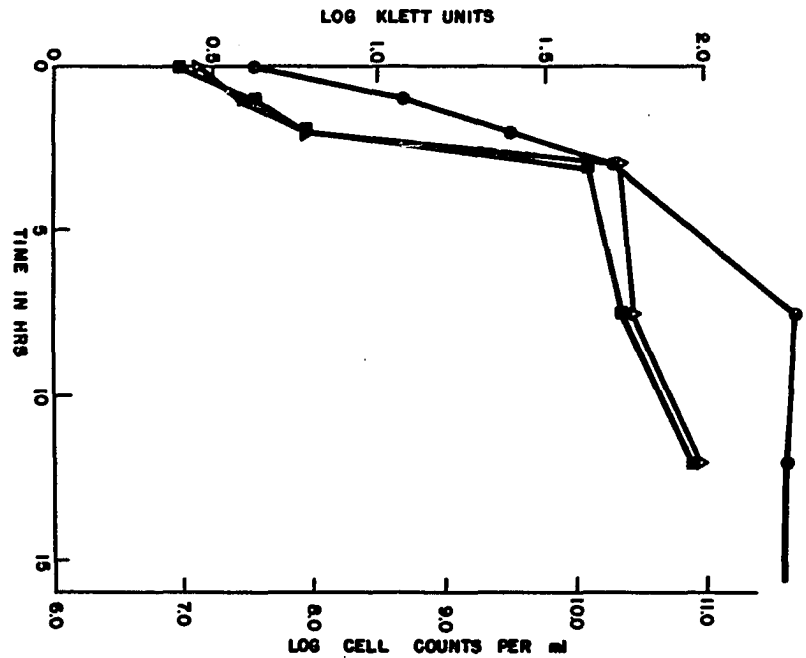
Sample number	Days of storage		
	3	5	12
1	1.50×10^5	2.00×10^6	4.60×10^6
2	1.10×10^6	2.50×10^6	5.60×10^7
3	4.80×10^5	1.20×10^6	3.80×10^7
4	7.20×10^5	2.00×10^7	3.00×10^7
5	2.70×10^6	1.00×10^7	0.80×10^7
6	1.30×10^6	6.20×10^7	1.70×10^7
7	1.90×10^6	2.00×10^6	3.80×10^7
8	7.90×10^4	3.80×10^6	4.00×10^6
9	5.00×10^3	2.40×10^4	3.80×10^7

[a]At refrigerated temperature of 4C.

The results of the associative growth study are presented in Figures 13, 14, and 15. Figure 13 represents the turbidity, counts on nutrient agar and on Pseudomonas Agar F for P. fluorescens F-21. Figure 14 represents the turbidity, cell counts on nutrient agar and on Staphylococcus medium 110 with egg yolk for S. aureus S-6. These data demonstrated the expected logarithmic growth of these two strains of bacteria individually in nutrient broth. Figure 15 shows turbidity and growth of the mixed culture on all three media. Logarithmic

Figure 13. Growth of *P. fluorescens* F-21 in nutrient broth and other media at 30C. Symbols: ● , Klett units; ■ , growth on nutrient agar; ▲ , growth on *Pseudomonas* Agar F

Figure 14. Growth of *S. aureus* S-6 in nutrient broth and other media at 30C. Symbols: ● , Klett units; ■ , growth on nutrient agar; ▲ , growth on Staphylococcus medium No. 110 with egg yolk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1.0
1.5
2.0
0
5
10
15
TIME IN HRS
6.0
7.0
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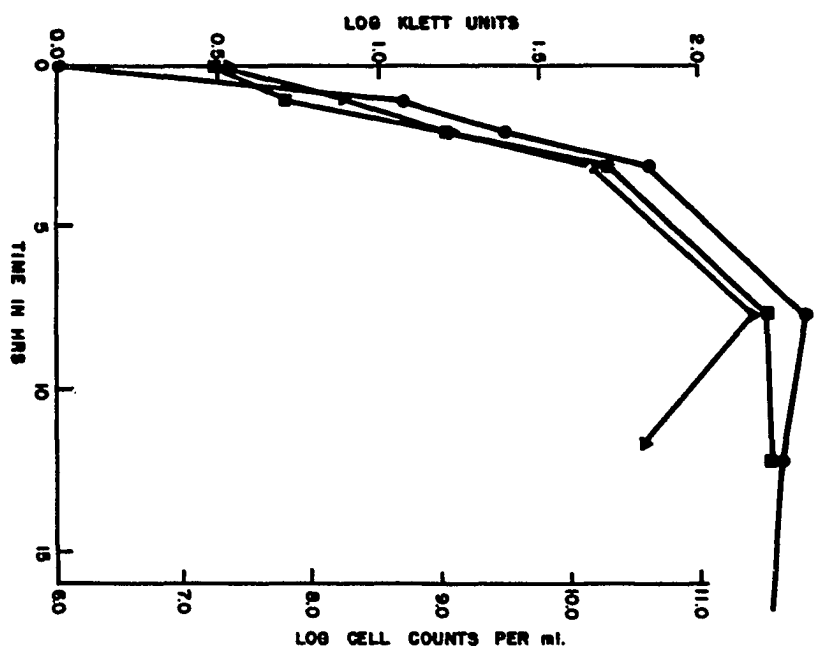
10.0
11.0
LOG CELL COUNTS PER ml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0.5
1.0
1.5
2.0
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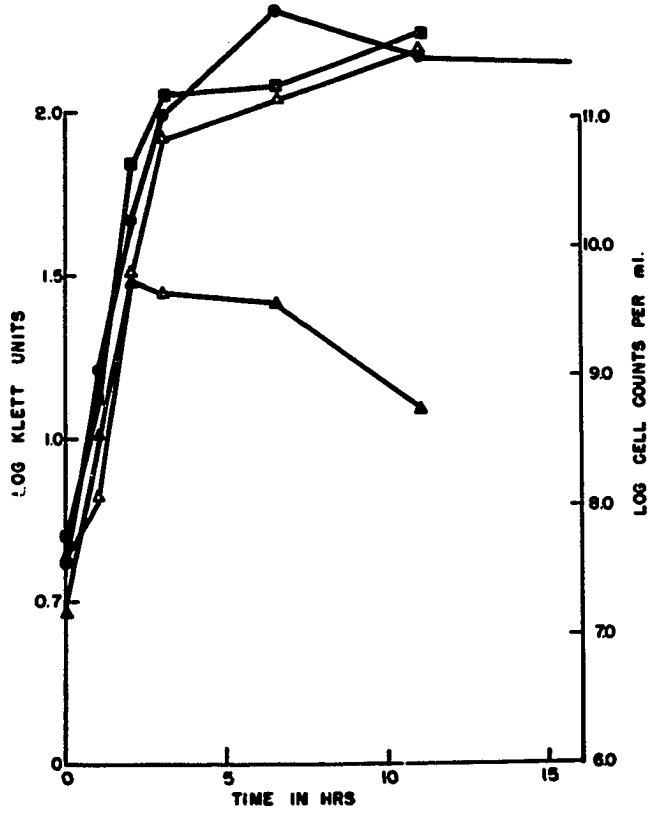
5
10
15
TIME IN HRS
6.0
7.0
8.0
9.0
10.0
11.0
LOG CELL COUNTS PER ml.

Figure 15. Growth of mixed culture of *P. fluorescens* F-21 and *S. aureus* S-6 in nutrient broth and other media at 30C. Symbols: ● , Klett units; ■ , growth on nutrient agar; △ , growth on *Pseudomonas* Agar F; ▲ , growth on Staphylococcus medium No. 110 with egg yolk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LOG CELL COUNTS PER ml.
TIME IN HRS
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1.5
1.0
0.7
0
11.0
10.0
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8.0
7.0
6.0
0
5
10
15

growth, as measured by turbidity and counts on nutrient agar, occurred as expected. The counts on the selective medium, Pseudomonas Agar F showed the same pattern as the counts of the mixed culture on nutrient agar and the counts for P. fluorescens F-21 when grown individually. However, the growth pattern on Staphylococcus medium 110 with egg yolk was logarithmic for the first 2 hr and then was stationary; the stationary period was followed by a rapid decrease. This pattern was different from the pattern of the mixed culture and the pattern obtained from S. aureus S-6 when grown in association with P. fluorescens F-21 and also showed the applicability of the Microtiter-spot plate method for studies when mixed flora occurring on foods are to be estimated.

The Microtiter-spot plate method has been found in this study to be at least 12 times more efficient than the Standard Plate Count method in terms of time necessary to complete the procedures. Also, readable results could be obtained approximately 15 hrs earlier.

The viable cell counts of the 12 samples obtained from the Microtiter-MPN technique and the conventional agar plate method are presented in Table 11. The results show close correlation between the data of the two methods with a correlation coefficient of 0.801, which was significant at the 1% level (see Appendix, Table 26).

Table 11. Comparisons of viable cell counts

Samples	Microtiter-MPN counts/ml 10^7	Agar plate counts/ml 10^7
Milk 1	1.8	4.8
Milk 2	10.0	13.0
Milk 3	0.2	0.6
Milk 4	10.0	52.0
S. typhimurium	0.1	0.3
S. heidelberg	1.0	0.9
S. anatum 53	1.8	2.4
S. infantis	1.8	1.1
S. aureus S-6	1.0	5.9
S. aureus 100	1.0	1.0
S. aureus 137	1.0	7.3
S. aureus 196E	1.0	1.4

Correlation coefficient of these methods is 0.801.

The viable cell counts of the four samples of turkey infusion by the agar plate method, the Microtiter-spot plate method and the Microtiter-MPN method showed close agreement among the three methods (Table 12). Generally, the agar plate method provided slightly higher counts compared to the two Microtiter methods. Analysis of variance of the three methods (treatments) showed no significant differences of these treatments at the 1% level of significance (see Appendix, Table 29).

The comparison of the data of the two Microtiter methods and the conventional method for enumeration of viable cells in pure cultures as well as food samples showed that the Microtiter methods are comparable to the conventional agar plate

Table 12. Comparison of viable cell counts by three procedures

10% Turkey meat infusion	Agar plate counts/ml 10^4	Microtiter-spot plate counts/ml 10^4	Microtiter-MPN counts/ml 10^4
A	3.9	3.4	1.1
B	5.1	2.6	1.1
C	3.4	2.3	4.5
D	5.7	2.6	2.5

method in accuracy and precision of counting viable cells of bacterial cultures. Statistical analyses showed that the correlation between the two Microtiter methods and the agar plate method are highly significant. Moreover, the efficiency of the microtiter method in terms of economy of space, time, and material far exceeded the agar plate method. The Microtiter-spot plate has the following advantages over the agar plate method: (1) utilization of one Microtiter plate instead of many dilution bottles; (2) utilization of one petri dish instead of several dishes for duplicate plating of several dilutions; (3) utilization of one set of loops and droppers instead of many pipettes; (4) occupying only a very small area for operation and storage; (5) ease of sterilization and cleaning; and (6) ease and saving of time in enumeration of colonies. The Microtiter-MPN method has the following advantages over both the agar plate method and the conventional MPN

method: (1) utilization of one Microtiter plate instead of many dilution bottles and test tubes; (2) utilization of one set of loops instead of many pipettes; (3) occupying only a small area of operation and incubation; and (4) savings of time in obtaining data.

From these studies, the practical applicability of the Microtiter-spot plate method was demonstrated by: (1) growth of mixed cultures of S. aureus S-6, and P. fluorescens F-21, (2) determinations of the bacterial content of stored milk, and (3) evaluation of spore survival after heat treatment by Baldock et al. (1968).

D. Detection of Staphylococcal Enterotoxins

Growth of Staphylococcus aureus S-6 in 16 different media and detection of enterotoxins A, B, and C by the HAI test are presented in Table 13. The two media most suitable for enterotoxin production by S. aureus S-6 were 2% PHP plus vitamins, yielding 2.34 µg/ml of enterotoxin A and 68.96 µg/ml of enterotoxin B, and 3% PHP + 3% NZ-amine NAK plus vitamins, producing 2.34 µg/ml of enterotoxin A and 200-250 µg/ml of enterotoxin B. The other media supporting production of detectable amounts of enterotoxin B were Edamine S, Soy peptone powder, and pepticase (1.17 µg/ml of enterotoxin B). Enterotoxins A, B, and C were not produced by S. aureus S-6 in other media. The amount of growth of S. aureus S-6 in these media in

terms of Klett units and enterotoxin production were not directly related. S. aureus S-6 was found to have produced 10 μg/ml of enterotoxin B in an acid hydrolyzed casein medium with no appreciable increase in Klett units after 24 hrs of incubation at 37C.[1] The PHP-NZ-amine medium was used for further enterotoxin detection studies.

Growth and toxigenesis in the PHP-NZ-amine medium, in relation to time, by S. aureus 196E, S. aureus S-6, and S. aureus 137 are recorded in Figure 16. All three strains started to release their respective enterotoxins into the medium at the late log phase and early stationary phase, and continued to release enterotoxins until maximum production was reached after 24 hrs of incubation. Prolonged incubation to 48 hrs resulted in no further increase of enterotoxin yield. The time for S. aureus S-6 to release enterotoxin B and the amount released in this experiment were in good agreement with the findings of Markus and Silverman (1969) who used the single-diffusion technique of Weirether et al. (1966) to quantify enterotoxin B.

Stability of the lyophilized sensitized RBC in long term storage was measured by comparing the HA and HAI patterns of these cells with the patterns obtained from freshly sensitized

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[1]Unpublished work of the author at The University of North Carolina, Chapel Hill, North Carolina, 1967.

Table 13. Growth and toxigenesis of S. aureus S-6 in different media[a]

Media[b]	Klett units	Enterotoxin production µg/ml A	B	C
NZ-amine type A	325	-	-	-
NZ-amine B	290	-	-	-
NZ-amine E	290	-	-	-
NZ-amine HD	129	-	-	-
NZ-amine NAK	270	-	-	-
NZ-amine S	106	-	-	-
NZ-amine YT	375	-	-	-
NZ-case	150	-	-	-
Edamine	410	-	-	-
Edamine type S	170	-	1.17	-
HY-case	126	-	-	-
HY-case amine	300	-	-	-
Pepticase	95	-	1.17	-
PHP	300	2.34	168.96	-
3% PHP + 3% NZ-amine NAK[c]	450	2.34	200-250	-
Soy peptone	360	-	1.17	-

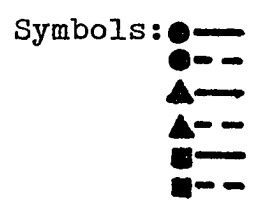
[a]Incubation at 37C for 24 hr in a gyrotory water-bath shaker (150 rev/min), 50 ml of media in a 250-ml Erlenmeyer flask. One percent inculum was used after washing of cells in sterile distilled water 4 times.

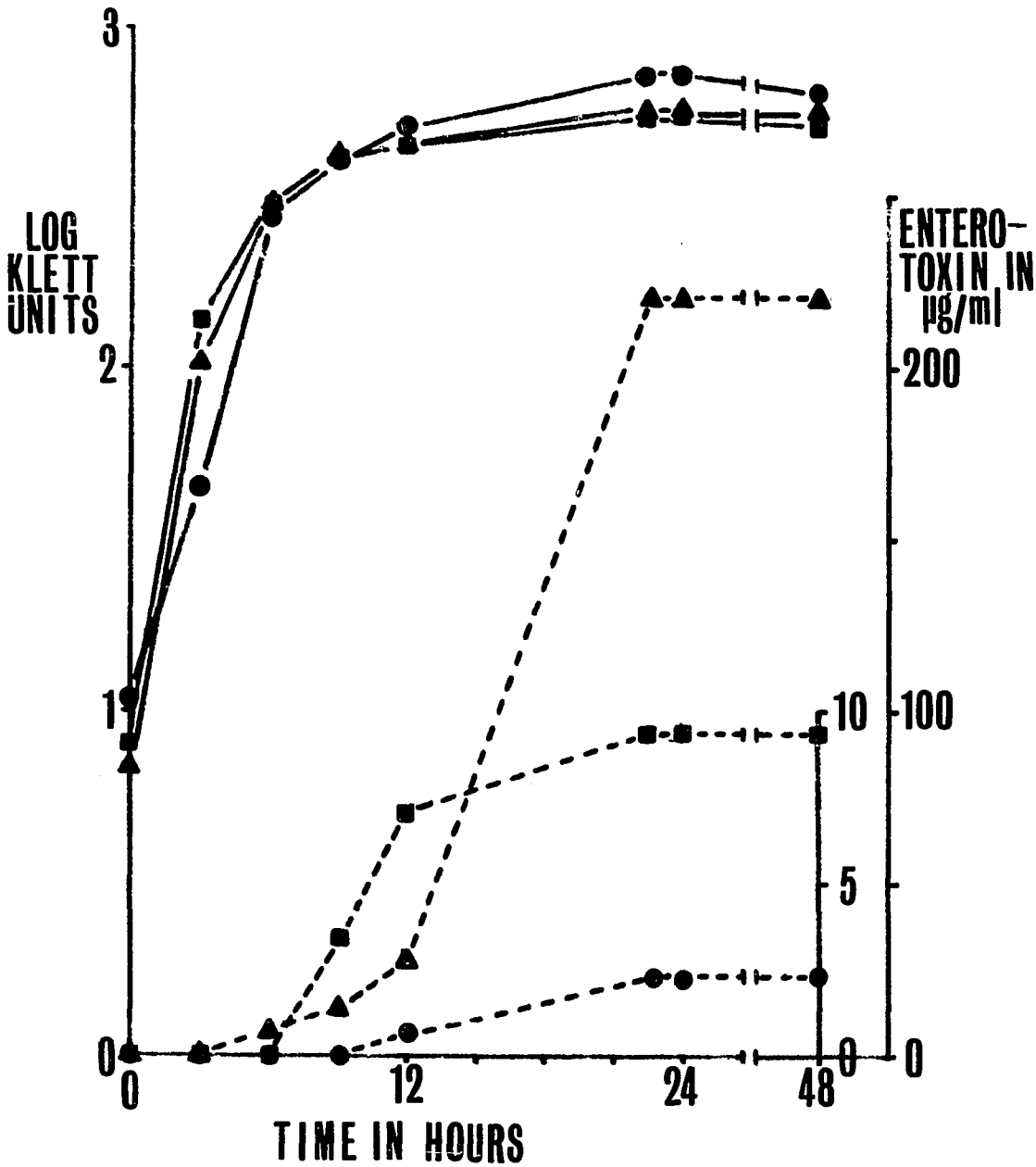
[b]All media were made as a 2% solution in distilled water except where indicated.

[c]Supplemented with thiamine, niacin, and pantothenate, each at a level of 0.5 µg/ml.

RBC. Positive enterotoxin controls (200 µg/ml or 5 µg/well) in each experiment served to check the pattern of HA and HAI of the lyophilized sensitized RBC as well as providing suitable sensitivity factors for calculation of enteroxin in samples. The factors obtained from freshly sensitized RBC of enterotoxins A, B, and C were between 0.59 to 1.17 µg/ml. The

Figure 16. Growth and toxigenesis of 3 strains of S. aureus in relation to time

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100
10
5
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0
12
24
48
TIME IN HOURS

sensitivity factors of enterotoxins B and C obtained from lyophilized sensitized RBC after one year of storage were unchanged. However, lyophilized enterotoxin A sensitized RBC were unstable after 3 months of storage; the reason was unknown. The stability of lyophilized sensitized RBC for enterotoxins B and C is of practical importance because reconstitution of RBC is far more convenient than sensitization of RBC every time the HAI test is performed. Also, consistent results could be obtained from the same batch of lyophiles.

Percentage recovery of enterotoxins A, B, and C after known amounts were added to four different concentrations of sterile turkey infusion by use of the simplified enterotoxin extraction procedure and HAI test are presented in Table 14. Relatively good recoveries (56.0 to 88.5%) were obtained for all three enterotoxins at low concentrations of turkey infusion (1.0 to 10.0%). However, at a 33.3% concentration of turkey infusion, no enterotoxin was recovered. Further extraction of enterotoxins, including dialysis and column chromotography are needed in order to obtain toxins from undiluted turkey samples or meat slurry (Satterlee, L. D. and Kraft, A. A., Iowa State University, Ames, Iowa, personal communication, 1969).

Quantitative and qualitative evaluations of enterotoxins produced by factorial combinations of S. aureus 196E, S. aureus S-6, and S. aureus 137 in PHP-NZ-amine medium and 10% turkey meat infusion showed a general decrease of enterotoxin

Table 14. Recovery of enterotoxins from turkey infusion

Enterotoxins added	Percentages of turkey infusion	Enterotoxins recovered (μg/ml)	Percentage recovery
Enterotoxin A[a] 2 μg/ml	33.3	0	0
	10.0	1.18	59.0
	2.0	1.77	88.5
	1.0	1.77	88.5
Enterotoxin B 20 μg/ml	33.3	0	0
	10.0	13.94	69.7
	2.0	13.94	69.7
	1.0	13.94	69.7
Enterotoxin C 20 μg/ml	33.3	0	0
	10.0	11.2	56.0
	2.0	11.2	56.0
	1.0	11.2	56.0

[a]Enterotoxin A was evaluated with freshly prepared sensitized RBC, while enterotoxins B and C were evaluated by lyophilized sensitized RBC.

production by all three strains when grown in these combinations (Table 15). Production of enterotoxin B by S. aureus S-6 was reduced when the organism was grown with other strains. Enterotoxin C production by S. aureus 137 was suppressed or reduced in the presence of other S. aureus strains. S. aureus S-6 and S. aureus 196E produced enterotoxin A in the presence of S. aureus 137 in 10% turkey meat infusion. However, in PHP-NZ-amine medium, no enterotoxin A was produced by combined growth of S. aureus 196E and S. aureus S-6, or all three strains together. The reason for such irregular enterotoxin production was not determined. Pivnick et al. (1968) observed

Table 15. Competitive toxin production

Strains of *S. aureus*	Expected enterotoxin production	PHP-N-Z amine medium toxin production, µg/ml			Turkey meat infusion toxin production, µg/ml		
		A[a]	B	C	A	B	C
196E	A	4.68	0	0	3.54	0	0
S-6	A + B	3.45	112.32	0	3.54	40.80	0
137	C	2.34	0	7.02	0	0	7.02
196E + S-6	A + B	0	3.51	0	3.54	14.00	0
196E + 137	A + C	2.34	0	0.59	1.77	0	0
S-6 + 137	A + B + C	0.59	14.04	0	3.54	7.02	0
196E + S-6 + 137	A + B + C	0	4.68	0	3.54	4.68	0
None	None	0	0	0	0	0	0

[a]Enterotoxin A was evaluated with freshly prepared sensitized RBC, while enterotoxins B and C were evaluated by lyophilized sensitized RBC.

the inability of four enterotoxigenic strains of S. aureus to produce enterotoxin when grown together with 5 non-toxigenic strains in barbecued chicken after 24 hrs of incubation. However, they could detect enterotoxin after 2.5 days of incubation by the double gel diffusion method. Competition of nonenterotoxigenic strains with enterotoxigenic strains was listed as one of the reasons for the inability of mixed cultures of S. aureus to produce enterotoxin after 24 hrs of incubation. The other causes may be insufficient incubation and anaerobiosis. Since the present study was conducted for 48 hrs with ample aeration, the latter explanations were not applicable in accounting for the reduction of enterotoxin production. Competition among enterotoxigenic strains for nutrients or enterotoxin precusors, mutual suppression of enterotoxin formation and utilization of energy for competition instead of for production of secondary metabolites such as enterotoxins (McLean et al., 1968) are some of the possible reasons for reduced enterotoxin production when S. aureus strains were grown together.

All attempts made to detect enterotoxin D in the crude state as well as in culture filtrates by the HAI method failed. The reasons probably are because "the antiserum is relatively low in titer and the enterotoxin is very crude" (Casman, E. P., U.S. Department of Health, Education and Welfare, personal communication, 1969).

The above experiments on staphylococcal enterotoxin detection demonstrated the ability of the HAI test to detect enterotoxins A, B, and C in culture as well as in food samples. The fact that lyophilized sensitized RBC were stable and usable in the HAI test helped reduce the time and labor involved in the assay procedure to a minimum. Detection of 24 samples including assay procedures and incubation time could be achieved in 4-5 hrs. Although RBC sensitized with enterotoxin A were not stable after prolonged storage in the lyophilized state, they were stable for at least one month. Reconstituted RBC provided more distinct settling of cells in the wells compared to freshly sensitized RBC, thus facilitating ease in data collection. Amounts and kinds of enterotoxins produced by various strains of S. aureus detected by the HAI test were comparable to similar studies reported in the literature in which gel-diffusion tests were used (Kato et al., 1966; McLean et al., 1968; Markus and Silverman, 1969; Morse et al., 1969).

The advantages of the HAI test include ease of operation, short assay time (4-5 hrs), and sensitivity (ability to detect as little as 0.010-0.039 μg of toxins). Disadvantages include ambiguity of the end-point determination when toxin samples were not properly prepared and inability to ascertain the exact amount of toxin between dilutions.

The HAI test, with lyophilized sensitized-RBC, in conjunction with the simplified sample preparation steps has the

potential as a rapid procedure for staphylococcal enterotoxin detection in bacterial cultures and foods.

E. Detection of *Salmonella*

The results for detection of *Salmonella* in mixed cultures are presented in Table 16. Typical complete *Salmonella* reactions were obtained in the *Salmonella* control flask as early as 17 hrs of incubation. Other control flasks showed no typical *Salmonella* reactions. The *E. coli* 2B5 control demonstrated acid production but no gas formation. The *Proteus* sp. 2D20 control showed acid but no gas formation after 24 hrs incubation while the flask containing only *Pseudomonas* gave no biochemical changes. In the modified nepheloflasks, typical *Salmonella* reactions were obtained in about 20 hrs of incubation. *Salmonella* only was recovered when organisms were drawn from the tip of the side arm by a sterile syringe and streaked on differential media (brilliant green agar, EMB agar, and *Pseudomonas* P agar).

In the test flasks, *Salmonella* was present in small numbers (2.1×10^{-1} to 2.1×10^{5} per flask) compared with the competitive organisms (3.9×10^{7} per flask). Typical *Salmonella* reactions progressed in direct relation to the initial numbers of these organisms. The greatest inoculum of *Salmonella* initially showed typical complete reactions first. Of significance was the observation that flask No. 10 showed complete *Salmonella* reactions after 36 hrs with initially only

Table 16. Detection of *Salmonella* in mixed cultures

Flask	Incubation time in hr						No. of organisms in flask	
	0	17	20	24	30	36	*Salmonella*	Others
1	R	B	B	B	B	B	2.1×10^5	---
2	R	R	R	R	R	R	---	3.8×10^7 (*P. aeruginosa* 2F40)
3	R	R	R	Y	Y	Y	---	1.1×10^5 (*Proteus* sp. 2D20)
4	R	Y	Y	Y	Y	Y	---	1.1×10^4 (*E. coli* 2B5)
5	R	G	B	B	B	B	2.1×10^5	3.9×10^7 (Combined)
6	R	G	B	B	B	B	2.1×10^4	3.9×10^7
7	R	G	G	B	B	B	2.1×10^3	3.9×10^7
8	R	G	G	G	B	B	2.1×10^2	3.9×10^7
9	R	G	G	G	B	B	2.1×10^1	3.9×10^7
10	R	R	Y	G	G	B	2.1×10^0	3.9×10^7
11	R	R	Y	Y	Y	Y	2.1×10^{-1}	3.9×10^7

R - red mannitol layer and red TSI layer
Y - yellow in both layers
G - gas formation in agar layer(s) - presumptive biochemical test
B - blackening in TSI layer - confirmed biochemical test

2.1 Salmonella present in 250 ml of medium in the presence of 3.9×10^7 competitive organisms. No typical Salmonella reactions were demonstrated in the next dilution flask (0.21 Salmonella calculated per 250 ml) after 36 hrs. Prolonged incubation to 96 hrs also yielded negative results.

Effects of sodium lauryl sulfate (SLS) on motility of organisms are presented in Table 17. Data for E. coli 2B5 were not included because all 6 concentrations of SLS showed inhibitory effects. Proteus sp. 2D15 was delayed at the 0.10% SLS level and gave atypical Salmonella reactions (no acid and gas in dulcitol layer) in the side arm after 36 hrs of incubation. At an SLS level of 0.15% or more, Proteus sp. 2D15 was completely inhibited even after 36 hrs of incubation. Salmonella, conversely, were not inhibited by SLS concentrations up to 0.30%. However, 0.50% and 1.00% SLS produced delayed or inhibitory effects, respectively. Thus, 0.15% SLS was chosen to be used in future experiments to produce a higher degree of selectivity for Salmonella in this system.

Comparison of the multi-layer motility agar method and the conventional method for the detection of low numbers of Salmonella in mixed cultures, as reported in Table 18, showed direct correlation of the two procedures. Low levels (calculated at 4-40 cells per flask) of Salmonella were detected in the presence of high numbers of competitive organisms (2.5×10^6 cells per flask). In this experiment duplicate samples

Table 17. Effects of SLS concentrations

Percentage of SLS	Incubation time in hrs: Salmonella typhimurium 0	17	24	30	36	Proteus sp. 2D15 0	17	24	30	36
0.10	R	G	B	B	B	R	R	Y	Y	B
0.15	R	Y	B	B	B	R	R	R	R	R
0.20	R	Y	G	B	B	R	R	R	R	R
0.30	R	Y	G	B	B	R	R	R	R	R
0.50	R	R	R	G	B	R	R	R	R	R
1.00	R	R	R	R	R	R	R	R	R	R

R - Red dulcitol layer and red TSI layer
Y - Yellow agar layer(s)
G - Gas formation in agar layers - presumptive biochemical test
B - Blackening in TSI layer - confirmed biochemical test

Table 18. Comparison of two methods for Salmonella detection in culture

	MLAS[a]	Conventional[b]	Organisms per flask: Salmonella	Others	
1	+[c]	+	40	-	
2	-	-	-	1.7×10^3	(Proteus)
3	-	-	-	2.5×10^6	(Escherchia)
4	+	+	40	2.5×10^6	(Combined)
5	+	+	4	2.5×10^6	(Combined)
6	-	-	.4	2.5×10^6	(Combined)
7	-	-	.04	2.5×10^6	(Combined)
8	-	-	.004	2.5×10^6	(Combined)

[a]MLAS - Multi-layer agar system including serological tests from organism picked from the tip of the modified nepheloflasks.

[b]Conventional - Modification of Galton et al. (1968) procedure including serological tests.

[c]Duplicate analyses for each sample.

were tested. Also, organisms were picked from the tip of the modified nepheloflask after 36 hrs of incubation, streaked on brilliant green agar, and serological tests were performed on typical colonies.

Tests of twenty-five strains of Salmonella in this system are recorded in Table 19. The five known Salmonella strains gave positive results as early as 17 hrs of incubation with the production of acid, gas, and H_2S. Eighteen of the twenty isolates gave positive results after 24 hrs of incubation with production of gas and acid but no H_2S. After 36 hrs of incubation one additional isolate (No. 2) provided a positive result. The other isolate (No. 1) did not show typical reactions after prolonged incubations. These isolates were found to be H_2S-negative by the conventional procedure. Thus, of twenty-five strains of Salmonella tested, twenty-four gave positive results in the specified time limit of 36 hrs. It is possible that the organism giving negative results may have been non-motile.

Data for the detection of Salmonella in the turkey roll homogenate are presented in Table 20. The Salmonella control showed a confirmed Salmonella reaction in 24 hrs while the Proteus control remained unchanged at 36 hrs. Control flasks for Escherichia and Pseudomonas were not prepared because these organisms were previously shown not to penetrate the selenite cystine agar layer. Confirmed biochemical Salmonella reactions were obtained in all flasks containing Salmonella

Table 19. Test of different strains of *Salmonella*

Organisms	Incubation time in hrs				
	0	17	24	36	42
S. typhimurium	R	B	B	B	B
S. hiedelberg	R	Y	B	B	B
S. anatum 53	R	Y	B	B	B
S. infantis	R	B	B	B	B
S. thompson	R	B	B	B	B
Isolate 1	R	R	R	R	R
Isolate 2	R	R	R	G	G
Isolates 3 to 20	R	R	G	G	G

Table 20. Detection of *Salmonella* in turkey roll homogenate

Flask	Incubation time in hrs			No. of organisms in flask	
	0	24	36	*Salmonella*	Others
1	R	B	B	7.5×10^{6}[a]	---
2	R	R	R	---	1.5×10^{7} (*Proteus*)
3	R	G	B	7.5×10^{6}	6.5×10^{8} (combined)
4	R	G	B	7.5×10^{5}[b]	6.5×10^{8}
5	R	Y	B	7.5×10^{4}	6.5×10^{8}
6	R	Y	B	7.5×10^{3}	6.5×10^{8}
7	R	R	B	7.5×10^{2}	6.5×10^{8}
8	R	R	B	7.5×10^{1}	6.5×10^{8}
9	R	R	B	7.5×10^{0}	6.5×10^{8}
10	R	R	R	7.5×10^{-1}	6.5×10^{8}

[a]Initial level of inoculum.

[b]Concentrations calculated by serial dilutions.

(at concentrations of 7.5 to 7.5 X 10^6 cells per flask) grown in the presence of large numbers of competitive organisms (6.5 X 10^8 cells per flask). Of significance was the observation that flask No. 9 showed a confirmed biochemical Salmonella reaction after 36 hrs with only 7.5 Salmonella present initially in the turkey roll homogenate, although the level of competitive bacteria was high (6.5 X 10^8 cells per 250 ml). No reactions were observed in the next dilution flask (0.75 Salmonella calculated per 250 ml turkey roll homogenate) after 36 hrs.

The data for the detection of the five strains of Salmonella in turkey roast and turkey roll homogenates are presented in Table 21. Again low levels of all five Salmonella strains were detected in both turkey products within 36 hrs. The fact that positive results were obtained from some flasks containing theoretically less than one Salmonella may be the result of dilution variations within the limit of experimental error.

Table 22 shows results for detection of Salmonella in whole egg and egg yolk solids. Flasks 1 to 8 demonstrated the ability of this system to detect as few as 3 Salmonella per egg. Complete reactions of the first series (flasks 1 to 4) with one egg in each flask, were delayed for several hours compared to the second series (flasks 5 to 8) which had one egg in the first flask only and serial dilutions of egg in the

Table 21. Detection of different strains of *Salmonella* in turkey products

Organisms	No. of organisms in flask[a]	Incubation in hrs: Turkey roast homogenate 0	24	36	Turkey roll homogenate 0	24	36
S. typhimurium	30	R	B	B	R	B	B
	3	R	G	B	R	G	B
	0.3	R	R	R	R	R	R
S. hiedelberg	33	R	G	B	R	G	B
	3.3	R	G	B	R	G	B
	0.33	R	R	R	R	R	R
S. anatum 53	9.5	R	G	B	R	G	B
	0.95	R	G	B	R	G	B
	0.095	R	R	R	R	R	R
S. infantis	8.5	R	G	B	R	G	B
	0.85	R	G	B	R	G	B
	0.085	R	R	R	R	R	R
S. thompson	5	R	G	B	R	G	B
	0.5	R	Y	B	R	G	B
	0.05	R	R	R	R	R	R

[a]Duplicate analyses for each dilution.

Table 22. Detection of *Salmonella* in whole egg and egg yolk solids

Flask	Incubation time in hrs: 0	17	24	30	36	40	No. of organisms in flask: Salmonella	Others	Amount of egg
1	R	R	R	G	B	B	3000	1.2×10^9	1
2	R	R	R	Y	Y	B	300	1.2×10^9	1
3	R	R	R	Y	B	B	30	1.2×10^9	1
4	R	R	R	R	Y	B	3	1.2×10^9	1

Table 22. (Continued)

Flask	Incubation time in hrs 0	17	24	30	36	40	No. of organisms in flask Salmonella	Others	Amount of egg
5	R	R	G	G	B	B	3000	1.2×10^9	1
6	R	Y	G	G	B	B	300	1.2×10^9	0.1
7	R	Y	Y	Y	B	B	30	1.2×10^9	0.01
8	R	Y	G	G	B	B	3	1.2×10^9	0.001
9	R	R	Y	G	B	B	3000	---	1
									Amount of egg yolk solids
10	R	R	Y	Y	Y	G	12000	1.7×10^8	50 gm
11	R	R	Y	G	G	G	1200	1.7×10^8	50 gm
12	R	R	R	G	G	G	120	1.7×10^8	50 gm
13	R	R	R	G	G	G	12	1.7×10^8	50 gm

next three flasks. These results may have been caused by more complete dispersion of Salmonella in the lower concentrations of egg material and possible dilution of antibacterial agents in the second series. The Salmonella control (flask 9) demonstrated a complete reaction after 36 hrs of incubation. Similar data were obtained for egg yolk solids (flask 10 to 13) in which small numbers of Salmonella (12 to 1.2×10^4 cells per flask) were detected in the presence of large numbers of competitive organisms (1.7×10^8 cells per flask). No reaction was observed after 36 hrs of incubation of the twelve flasks containing one whole egg each without inocula,

indicating the absence of Salmonella in the eggs tested.

Results for detection of Salmonella in whole egg solids and egg white solids are presented in Table 23. Again, small numbers of Salmonella (3.7 to 37 cells per flask) in both products were detected in the presence of large numbers of competitive organisms (2.1×10^7 cells per flask).

Fifteen of 17 chicken wings stored at 4C for 2 days showed positive results for Salmonella by the multi-layer motility agar method while one less positive (14 samples) was recorded by using the conventional procedure (Table 24). The same phenomenon was observed when 12 chicken wings stored at 4C for 12 days were tested for Salmonella; i.e., 1 more positive was detected by the multi-layer motility agar method (8/12) compared to the conventional method (7/12). This discrepancy may be due to one of two results; either the multi-layer motility agar system was more sensitive than the conventional method or it provided false positive results. The latter seemed unlikely because serological tests of Salmonella picked from the tip of the modified nepheloflask showed positive Salmonella reactions. The many transfer steps involved in the conventional method may be the reason for losing low levels of Salmonella in the process. It should be noted that biochemical reactions of Salmonella in the side arms of the nepheloflask were distinct and complete in 30 hrs in testing the chicken wings, compared to 80-100 hrs needed for the

Table 23. Detection of Salmonella in whole egg solid and egg white solid

No. of organisms in flask		Incubation time in hrs					
		Whole egg solid			Egg white solid		
Salmonella	Others	0	24	36	0	24	36
37	2.1×10^7	R	G	B	R	G	B
3.7	2.1×10^7	R	Y	B	R	Y	B
0.37	2.1×10^7	R	R	R	R	R	R
---	---	R	R	R	R	R	R

Table 24. Comparison of two methods for Salmonella detection in chicken wings

Chicken wings	Storage time(days)/temp.	MALS	Conventional
1 to 14	2d/4C	+	+
15	2d/4C	+	-
16 to 17	2d/4C	-	-
18 to 24	12d/4C	+	+
25	12d/4C	+	-
26 to 29	12d/4C	-	-

conventional method. Also, some of the biochemical reactions in TSI slants and subsequent serological tests of "typical" Salmonella colonies picked from brilliant green agar were partially (1 or 2) positive of the triplicate samples tested. The multi-layer motility agar system not only served as a barrier to competitive organisms but a purification step for further serological tests after growth of colonies picked from

the tip of the modified nepheloflask on brilliant green agar.

Prolonged storage (12 days) of chicken wings resulted in lower percentage (66.6%) of Salmonella detection compared to 2 day old chicken wings (88.5%). This is probably because competitive organisms outgrew Salmonella in prolonged storage at low temperature.

As shown in Table 25, results of the estimation of viable cell counts of Salmonella by the MPN-motility agar system compared favorably to the numbers of Salmonella inoculated into the system ($0.2\text{-}1.1 \times 10^4$ organisms/ml recovered compared to 2.9×10^4 organisms/ml added). Again serological tests of Salmonella picked from the tip of the modified nepheloflasks helped confirm the data of the motility system. Comparison of the motility agar method and the conventional method for viable cell counts was not performed. The demonstration of the presence of Salmonella in any food sample was considered sufficient in this work to serve as a control procedure. This set of experiments, however, demonstrated that a rough estimation of viable cell count by this new procedure could be made if necessary.

Table 25. MPN estimation of viable Salmonella counts using the motility agar system

Dilutions	MALS	Organisms per flask	
		Salmonella	Escherchia
0	+	7.3×10^4	6×10^5
10^{-2}	+	7.3×10^2	6×10^5
10^{-2}	+	7.3×10^2	6×10^5
10^{-2}	+	7.3×10^2	6×10^5
10^{-4}	+	7.3	6×10^5
10^{-4}	+	7.3	6×10^5
10^{-4}	-	7.3	6×10^5

Salmonella inoculum was 2.9×10^4 organisms/ml

MPN-MALS estimation was 0.2×10^4 to 1.1×10^4 organisms/ml

V. CONCLUSIONS

The following conclusions may be made with regard to the rapid methods for the detection of staphylococcal enterotoxins and Salmonella in turkey products.

1. The Microtiter-spot plate method and the Microtiter-MPN method are applicable for rapid evaluation of viable cell counts in bacterial cultures and in turkey meat. Multiple inoculation procedures may be used with the Microtiter method for characterizing bacteria.
2. The dye-diffusion test is a simple and useful procedure for semi-quantification of coagulase production by Staphylococcus aureus.
3. The HAI test is applicable for detection of enterotoxins A, B, and C in culture and in food after suitable sample preparations are made.
4. Competitive inhibition of enterotoxin production by mixed cultures of S. aureus exists, but the mode of action of inhibition was not determined.
5. Salmonella may be detected in naturally contaminated food as well as in artificially contaminated samples by use of a multi-layer motility agar system. The method is applicable to small numbers of Salmonella in the presence of large numbers of competitive organisms.

VI. SUMMARY

A number of rapid procedures were developed in this study for the enumeration, isolation, detection, and characterization of Staphylococcus aureus, staphylococcal enterotoxins, and Salmonellae from poultry products as well as from artificially contaminated samples.

Two Microtiter procedures were developed for rapid evaluation of viable cell counts of bacterial cultures and for bacteria in food samples. The first Microtiter method involved loop dilutions and spot-plating of samples. The second Microtiter procedure made use of the MPN concept and rapid dilution of bacteria in nutrient broth in a sterile Microtiter plate.

Statistical analyses revealed that the estimation of bacteria by the conventional agar plate method and the Microtiter procedures were comparable and had significant positive correlation. The practical applications of the Microtiter procedures included evaluation of numbers of bacteria in turkey meat, estimates of associative growth of mixed cultures, changes in bacterial populations of stored milk, and heat destruction of spores. The advantages of the Microtiter procedures are savings of time, space, labor, and money in evaluating large numbers of samples.

A convenient multipoint inoculation device was developed to facilitate inoculation of 96 strains of bacteria simultaneously into liquid and onto solid media. This device was

made by impregnating 96 straight pins into wax-filled wells of a lucite Microtiter plate. Distinct and discrete growth of each strain of bacteria on solid media was observed. Change of color or turbidity of liquid media in the Microtiter wells inoculated with organisms by the inoculation device were indicative of growth of the bacteria.

Another device for multiple culture of bacteria consisted of a tray having 75 compartments for culturing 75 strains of organisms on solid agar medium contained in each compartment. Growth of organisms and related biochemical changes of media in the compartments could be seen. Diffusion of bacterial metabolites and mixing of motile organisms could not occur because of the presence of partitions in the tray. The tray is inexpensive and easy to wash and sterilize.

A semi-quantitative evaluation of coagulase activity of S. aureus strains was developed by a dye-diffusion method. Small amounts of S. aureus cultures were incubated with double the amount of plasma in a little (5 X 15 mm) tube. After a determined reaction time, a drop of dye was applied to the tube. The rate of diffusion of dye was inversely proportional to the degree of coagulation of plasma. An eight point scale was established by measuring, in mm, the length of the clots where the dye did not penetrate after an arbitrary standard diffusion time of 1 1/2 hrs.

A temperature gradient system was developed for convenient culturing of bacteria at different temperatures. The system utilized a metal plate heated at one end. Temperature fluctuation in the system was slight (± 0.5C). A total of 8 strains of organisms grown at 5 different temperatures could be studied simultaneously in this system.

Staphylococcal enterotoxins A, B, and C were detected by the hemagglutination-inhibition (HAI) test with success in culture filtrates and food samples. The HAI concept is based on the principle that when specific antiserum is first reacted with its antigen, either as pure enterotoxin or crude enterotoxin in culture or food filtrates, it will not agglutinate erythrocytes previously sensitized with pure enterotoxin. The Microtiter system was used for rapid assay. A procedure including centrifugation, acidification, filtration, and boiling was developed for sample preparation prior to the HAI test of enterotoxins. Enterotoxin production by S. aureus strains in a complex medium in relation to time was evaluated by the HAI test. Also, percentage recovery of enterotoxins from turkey meat and competitive production of enterotoxins of mixed enterotoxigenic strains of S. aureus were studied by use of the HAI test. Quantitative and qualitative determinations of enterotoxins were made by the test. Time needed for assay procedure and data collection was about 4-5 hrs.

Another phase of this work involved development of rapid methods for detection of Salmonellae. A multi-layer motility agar system was devised which was able to detect low numbers of Salmonellae in culture as well as in turkey meat in the presence of large numbers of competitive organisms. Three types of differential agars were layered in the side arm of a nepheloflask. The first agar layer contained inhibitory chemicals such as selenite and sodium lauryl sulfate, and acted as a barrier for nonmotile organisms while providing a medium for migration of motile organisms. Salmonella only could readily migrate into the first layer. The second and third agar layers included carbohydrates, phenol red indicator, and iron. When Salmonellae reached these layers, changes typical of these bacteria occurred in the media and thus facilitated Salmonellae detection. The presence of Salmonellae in poultry products including chicken, turkey rolls, turkey roasts, whole egg, egg yolk solids, egg white solids, and whole egg solids was tested by the multi-layer motility agar system; results were comparable with a conventional method for Salmonella detection.

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IX. APPENDIX

Table 26. Correlation coefficients

From Table No.	S_x	S_y	S_{xy}	r
7	58.095	76.990	4365.541	0.976**
8	344.600	129.900	27041.773	0.604**
9	10.300	2.880	19.296	0.651*
8 and 9	351.200	135.500	47587.600	0.623**
11	48.060	11.680	449.550	0.801**

**Significant at 1% level.

*Significant at 5% level.

Table 27. Culture characteristics and enterotoxin production of 100 isolates[a] from turkey meat

Isolates	Enterotoxin A in µg/ml[b]	Coagulase scores	Gram stain and morphology	MSA[c]	TPEY[d]
1	1.77	11	+ Cocci	+[e]	+
2	1.77	10	+ Cocci	+	+
3	1.77	9	+ Cocci	+	+

[a]All isolates were obtained from typical colonies on staphylococcus medium No. 110 with egg yolk.

[b]Detection of enterotoxins B and C were also performed with negative results for all isolates.

[c]MSA = Mannitol salt agar.

[d]TPEY = Tellurite-polymyxin-egg-yolk-agar.

[e]Growth = +, no growth = - .

Table 27. (Continued)

Isolates	Enterotoxin A in µg/ml	Coagulase scores	Gram stain and morphology	MSA	TPEY
4	1.77	9	+ Cocci	+	+
5	1.77	8	+ Cocci	+	+
6	1.77	7	+ Cocci	+	+
7	1.77	6	+ Cocci	+	+
8	1.77	6	+ Cocci	+	+
9	1.77	5	+ Cocci	+	+
10	1.77	5	+ Cocci	+	+
11	1.77	5	+ Cocci	+	+
12	1.77	4	+ Cocci	+	+
13	1.18	11	+ Cocci	+	+
14	1.18	10	+ Cocci	+	+
15	1.18	10	+ Cocci	+	+
16	1.18	10	+ Cocci	+	+
17	1.18	4	+ Cocci	+	+
18	.89	11	+ Cocci	+	+
19	.89	10	+ Cocci	+	+
20	.89	10	+ Cocci	+	+
21	.89	9	+ Cocci	+	+
22	.89	9	+ Cocci	+	+
23	.89	8	+ Cocci	+	+
24	.89	7	+ Cocci	+	+
25	.89	7	+ Cocci	+	+
26	.89	5	+ Cocci	+	+
27	.89	5	+ Cocci	+	+
28	.89	4	+ Cocci	+	+
29	.89	3	+ Cocci	+	+
30	.89	3	+ Cocci	+	+
31	.89	3	+ Cocci	+	+
32	.89	3	+ Cocci	+	-
33	.59	12	+ Cocci	+	-
34	.59	11	+ Cocci	+	+
35	.59	11	+ Cocci	+	+
36	.59	10	+ Cocci	+	+
37	.59	10	+ Cocci	+	+
38	-	12	+ Cocci	+	+
39	-	11	+ Cocci	+	+
40	-	10	+ Cocci	+	+
41	-	10	+ Cocci	+	+
42	-	10	+ Cocci	+	+
43	-	10	+ Cocci	+	+
44	-	10	+ Cocci	+	+
45	-	10	+ Cocci	+	+
46	-	10	+ Cocci	+	+

Table 27. (Continued)

Isolates	Enterotoxin A in µg/ml	Coagulase scores	Gram stain and morphology	MSA	TPEY
47	-	10	+ Cocci	+	+
48	-	10	+ Cocci	+	+
49	-	10	+ Cocci	+	+
50	-	10	+ Cocci	+	+
51	-	10	+ Cocci	+	+
52	-	10	+ Cocci	+	+
53	-	10	+ Cocci	+	-
54	-	9	+ Cocci	+	+
55	-	8	+ Cocci	+	+
56	-	8	+ Cocci	+	+
57	-	8	+ Cocci	+	+
58	-	8	+ Cocci	+	+
59	-	8	+ Cocci	+	+
60	-	8	+ Cocci	+	+
61	-	8	+ Cocci	+	+
62	-	8	+ Cocci	+	+
63	-	8	+ Cocci	+	+
64	-	8	+ Cocci	+	+
65	-	8	+ Cocci	+	+
66	-	8	+ Cocci	+	+
67	-	8	+ Cocci	+	+
68	-	7	+ Cocci	+	+
69	-	7	+ Cocci	+	+
70	-	7	+ Cocci	+	+
71	-	7	+ Cocci	+	+
72	-	7	+ Cocci	+	+
73	-	6	+ Cocci	+	+
74	-	6	+ Cocci	+	+
75	-	6	+ Cocci	+	+
76	-	6	+ Cocci	+	+
77	-	6	+ Cocci	+	+
78	-	6	+ Cocci	+	+
79	-	6	+ Cocci	+	+
80	-	5	+ Cocci	+	+
81	-	5	+ Cocci	+	+
82	-	5	+ Cocci	+	+
83	-	5	+ Cocci	+	+
84	-	5	+ Cocci	+	+
85	-	5	+ Cocci	+	+
86	-	5	+ Cocci	+	+
87	-	4	+ Cocci	+	+
88	-	4	+ Cocci	+	+

Table 27. (Continued)

Isolates	Enterotoxin A in µg/ml	Coagulase scores	Gram stain and morphology	MSA	TPEY
89	-	4	+ Cocci	+	+
90	-	3	+ Cocci	+	+
91	-	3	+ Cocci	+	+
92	-	3	+ Cocci	+	+
93	-	3	+ Cocci	+	+
94	-	3	+ Cocci	+	+
95	-	3	+ Cocci	+	+
96	-	3	+ Cocci	-	+
97	-	2	+ Cocci	+	+
98	-	-	+ Rod	-	-
99	-	-	+ Rod	-	-
100	-	-	+ Rod	-	-

Table 28. Culture characteristics and enterotoxin production of 72 turkey isolates and 8 known cultures[a]

Organisms	Enterotoxin in µg/ml[b] A	C	MSA	Coagulase score	Mannitol	Dextrose	Dulcitol	Lactose	Maltose	Salicin	Sorbitol	Sucrose	Xylose
1	.59	.89	+	5	+	+	-	+	+	-	+	+	+
2	-	-	+	4	+	+	-	+	+	-	+	+	+
3	-	-	+	6	+	+	-	+	+	-	+	+	+

[a]All isolates were obtained from typical colonies on staphylococcus medium No. 110 with egg yolk. Isolates 1-16 were obtained from two sections of turkey roll 1; Isolates 17-32 were obtained from two sections of turkey roll 2; Isolates 33-48 were obtained from two sections of turkey roast 1; Isolates 49-72 were obtained from three sections of turkey roast 2. All isolates and known Staphylococcus were Gram positive cocci and all Salmonella were Gram negative rods.

[b]Detection of enterotoxin B was also performed with negative results for all organisms except S. aureus S-6.

Table 28. (Continued)

Organisms	Enterotoxin in µg/ml A	Enterotoxin in µg/ml C	MSA	Coagulase score	Mannitol	Dextrose	Dulcitol	Lactose	Maltose	Salicin	Sorbitol	Sucrose	Xylose
4	.59	-	+	6	+	+	-	+	+	-	+	+	+
5	.89	-	+	9	+	+	-	+	+	-	+	+	+
6	.89	-	+	8	+	+	-	+	+	-	+	+	+
7	.59	.89	+	12	+	+	-	+	+	-	+	+	+
8	.59	.59	+	4	+	+	-	+	+	-	+	+	+
9	-	-	+	6	+	+	-	+	+	-	+	+	+
10	-	-	+[c]	-	-	+	-	+	-	-	+	+	-
11	.59	-	+	5	+	+	-	+	+	-	+	+	+
12	-	-	+	7	+	+	-	+	+	-	+	+	+
13	.89	.59	+	6	+	+	-	+	+	-	+	+	+
14	-	-	+	8	+	+	-	+	+	-	+	+	+
15	-	-	+	11	+	+	-	+	+	-	+	+	+
16	-	-	+	12	+	+	-	+	-	-	+	+	+
17	.89	-	+	5	+	+	-	+	+	-	+	+	+
18	-	-	+	5	+	+	-	+	+	-	+	+	+
19	-	-	+	4	+	+	-	+	+	-	+	+	+
20	-	-	+[c]	-	-	+	-	+	+	-	+	+	+
21	-	-	+	5	+	+	-	+	+	-	+	+	+
22	.89	-	+	5	+	+	-	+	+	-	+	+	+
23	-	.89	+	5	+	+	-	+	+	-	+	+	+
24	-	.89	+	6	+	+	-	+	+	-	+	+	+
25	-	-	+	7	+	+	-	+	+	-	+	+	+
26	-	-	+[c]	7	-	+	-	+	+	-	+	+	+
27	-	-	+	8	+	+	-	+	+	-	+	+	+
28	.89	-	+	7	+	+	-	+	+	-	+	+	+
29	-	.59	+	7	+	+	-	+	+	-	+	+	+
30	-	-	+	9	+	+	-	+	+	-	+	+	+
31	-	.89	+	7	+	+	-	+	+	-	+	+	+
32	-	.59	+	6	+	+	-	+	+	-	+	+	+
33	-	.89	+	5	+	+	-	+	+	-	+	+	+
34	-	-	+[c]	-	-	+	-	+	+	-	+	-	-
35	-	-	+	4	+	+	-	+	+	-	+	+	+
36	-	-	+	5	+	+	-	+	+	-	+	+	+
37	-	-	+	6	+	+	-	+	+	-	+	+	+
38	-	-	+	4	+	+	-	+	+	-	+	+	+
39	-	-	+	5	+	+	-	+	+	-	+	+	+

[c]Weakly positive with no acid production on mannitol salt agar.

Table 28. (Continued)

Organisms	Enterotoxin in µg/ml A	Enterotoxin in µg/ml C	MSA	Coagulase score	Mannitol	Dextrose	Dulcitol	Lactose	Maltose	Salicin	Sorbitol	Sucrose	Xylose
40	.89	.89	+	5	+	+	-	+	+	-	+	+	+
41	-	.89	+	6	+	+	-	+	+	-	+	+	+
42	-	-	+[c]	-	-	+	-	+	+	-	-	+	+
43	-	-	+	7	+	+	-	+	+	-	+	+	+
44	-	-	+[c]	7	-	+	-	+	-	-	+	+	+
45	-	-	+	8	+	+	-	+	+	-	+	+	+
46	-	-	+	4	+	+	-	+	+	-	+	+	+
47	-	-	+	5	+	+	-	+	+	-	+	+	+
48	.89	.89	+	11	+	+	-	+	+	-	+	+	+
49	-	-	+[c]	-	-	+	-	+	-	-	-	+	-
50	-	-	+[c]	-	-	+	-	+	+	-	-	-	-
51	-	-	+[c]	-	-	+	-	+	+	-	-	+	-
52	.59	-	+	6	+	+	-	+	+	-	+	+	+
53	.89	-	+	7	+	+	-	+	+	-	+	+	+
54	.59	-	+	5	+	+	-	+	+	-	+	+	+
55	-	-	+	4	+	+	-	+	+	-	+	+	+
56	-	-	+	9	+	+	-	+	+	-	+	+	+
57	-	-	+[c]	-	-	-	-	+	+	-	-	-	+
58	-	-	+[c]	-	-	-	-	+	+	-	-	+	+
59	-	-	+	4	+	+	-	+	+	-	+	+	+
60	-	-	+	5	+	+	-	+	+	-	+	+	+
61	-	-	+	8	+	+	-	+	+	-	+	+	+
62	-	-	+	8	+	+	-	+	+	-	+	+	+
63	-	-	+	9	+	+	-	+	+	-	+	+	+
64	-	-	+	12	+	+	-	+	+	-	+	+	+
65	.59	.59	+	6	+	+	-	+	+	-	+	+	+
66	.89	-	+	8	+	+	-	+	+	-	+	+	+
67	.89	-	+	8	+	+	-	+	+	-	-	+	+
68	-	-	+[c]	-	-	+	-	+	+	-	-	+	-
69	-	-	+[c]	-	-	-	-	+	-	-	+	-	-
70	.59	-	+	9	+	+	-	+	+	-	+	+	+
71	-	-	+[c]	-	-	-	-	+	+	-	-	+	-
72	-	-	+	12	+	+	-	+	+	-	+	+	+
S. aureus 100	-	-	+	11	+	+	-	+	+	-	+	+	+
S. aureus 196E	2.36	-	+	12	+	+	-	+	+	-	+	+	+
S. aureus S-6	2.36	220(B)	+	12	+	+	-	+	+	-	+	+	+

Table 28. (Continued)

Organisms	Enterotoxin in µg/ml A	Enterotoxin in µg/ml C	MSA	Coagulase score	Mannitol	Dextrose	Dulcitol	Lactose	Maltose	Salicin	Sorbitol	Sucrose	Xylose
S. aureus 137	-	7.02	+	10	+	+	-	+	+	-	+	+	+
S. aureus 494	-	-	+	11	+	+	-	+	+	-	+	+	+
S. typhimurium			-		G[d]	+	G	-	G	-	G	+	+
S. anatum 53			-		G	+	G	-	G	+	G	+	+
S. infantis			-		G	+	G	-	+	+	G	+	+

[d]G = acid and gas production determined by the Microtiter plate-Amojell procedure.

Table 29. Analysis of variance of three methods for viable cell count

Source	d.f.	S.S.	M.S.	
among trt.	2	111.62	55.81	$F_{2,9} = 4.258$
within trt.	9	117.95	13.11	
Total	11	229.57		