A POSSIBLE METHOD FOR DETECTING ENTEROTOXIGENIC STAPHYLOCOCCI BY PAPER CHROMATOGRAPHY

by

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TABLE OF CONTENTS

INTRODUCTION

It has long been known that certain strains of staphylococci are capable of producing a substance which is highly toxic for man when ingested with his food and drink. The gastrointestinal disturbance which results is due to the poisonous metabolic product alone and living organisms need not be present in the food at the time of consumption. Death rarely, if ever, occurs as a direct result of staphylococcal food poisoning, but this does not lessen its importance from a public health standpoint. The large number of outbreaks reported annually is considered to be but a fraction of the total number. This is a logical assumption due to the ubiquitous nature of enterotoxin-producing staphylococci and the fact that nearly any food may become involved under proper conditions.

In the event of a food poisoning outbreak it is essential that it be traced to the food and persons involved in its preparation if subsequent occurrences from the same source are to be prevented. It is not an easy task to establish the presence of staphylococcus enterotoxin in a suspected food. At the present time there is but one way to be absolutely certain of its presence: feed a portion of the food to human volunteers and observe for typical symptoms of a gastrointestinal poisoning. The problems and disadvantages of this method are obvious, and for many years investigators have been searching for a simple, reliable test for enterotoxigenic staphylococci and for the toxic substance itself. As a result of these efforts, a number of tests have been developed,

but there is not one which has the desired degree of accuracy. Circumstantial evidence, then, is used to incriminate foods suspected of causing staphylococcal food poisoning.

It was felt worthwhile to investigate paper chromatography as a means of qualitatively assaying for the presence of staphylococcus enterotoxin. Since there is a reasonably close correlation between a coagulase positive organism and its ability to elaborate the poison, a comparative study was designed to show differences in the chromatographic patterns of coagulase positive and coagulase negative strains of staphylococci.

REVIEW OF LITERATURE

The ability of certain staphylococci to elaborate a substance capable of inducing food poisoning symptoms in human beings was first demonstrated by Barber (1914). Other workers at that time apparently failed to recognize the significance of Barber's initial work and it was all but forgotten during the 15 years which followed.

Interest in the field actually began when Dack, et al. (1930) presented conclusive evidence that certain of these organisms were capable of producing a filterable poison in food substances. They were successful in isolating a yellow, hemolytic staphylococcus from sponge cake which had produced an acute gastroenteritis in 11 persons. The isolated organism was cultured in a veal-infusion broth from which a cell-free filtrate was prepared. Consumption of this filtrate by human volunteers produced food poisoning similar to that which had occurred in those persons

who had eaten the cake.

Jordan (1930) expanded upon this work and showed that various staphylococcal strains of diverse origin and of diverse cultural characteristics were capable of elaborating an enterotoxic substance. In the years that followed a great deal of research concerning the enterotoxigenic nature of certain staphylococci was accomplished and this enthusiasm has continued to the present time.

An approach to the problem of enterotoxin assay is presented in this study which, to the author's knowledge, has never before been tried. In view of this fact, a review of the literature will necessarily be limited to investigations closely related to this project.

Woolpert and Dack (1933) investigated enterotoxin production in several strains of Staphylococcus aureus and reported that it appeared to be a substance separate and distinct from the hemolysins, dermotoxin, and lethal toxin which are also produced by certain pathogenic strains of staphylococci. Further evidence supporting this concept was presented by Dolman (1934), Surgalla and Hite (1945), and by Thatcher and Matheson (1955).

Physical and chemical properties of enterotoxic filtrates have been investigated and much has been learned, even though the specific gastrointestinal irritant has never been isolated in pure form. Early experiments by Jordan, et al. (1931) demonstrated the durability and stability of staphylococcus enterotoxin. They were interested in the effects of heat, cold

storage, and chlorination upon the toxicity of the substance. It was found that boiling for a period of 30 minutes failed to destroy the enterotoxic fraction in culture filtrates. Similarly, storage at low temperatures for as long as 67 days had no apparent effect and a three minute exposure to concentrated chlorine yielded analogous results. Jordan and Burrows (1933) observed that the active principle would not distill, that it was not readily dialyzable, that it was unstable to .01N sodium hydroxide, and that it was unstable to heat in the presence of .01N hydrochloric acid. The size or molecular weight of the enterotoxin molecule has not been accurately determined since the substance has yet to be obtained in pure form. However, certain limits as to its molecular size have been determined by indirect evidence. Dack (1956) in referring to Davison's work on enterotoxin stated that since enterotoxin would pass through a collodion membrane, this observation indicated that the maximum molecular weight was less than 15,000 to 20,000. Dack further stated that a minimum of 8,000 to 10,000 was indicated since the toxin would not pass through Visking cellulose sausage casing.

Other general physical and chemical properties of staphylococcal enterotoxin have been reported in conjunction with purification studies. While it has thus far been impossible to isolate the gastrointestinal toxin in a highly purified form, certain workers have experienced moderate success and have achieved at least a partial purification. Davison and Dack (1939) found that the active principle could be removed from toxic

filtrates by adding ammonium sulfate to full saturation. They further observed that enterotoxin was not destroyed by alcohol nor was it extractable with chloroform. Partial purification of the toxin was accomplished by Bergdoll, et al. (1951). The procedure consisted of concentration in vacuo, dialysis, and precipitation with ammonium sulfate, acid, ethanol, and methanol. A material was obtained by this procedure which was inactivated by 65 per cent ethanol, heat, and by the enzyme trypsin. These investigators suspected that the enterotoxin was protein in nature. Column chromatography was employed in an attempt to isolate pure enterotoxin by Bergdoll, et al. (1952) and a 10- to 20-fold purification was reported using this technique. A substance was recovered which contained a very small amount of carbohydrate and which gave a positive ninhydrin reaction with color development equivalent to that given by a protein. These same workers subjected the isolate to electrophoretic examination and found at least two fractions to be present. The toxic fraction contained approximately 80 per cent of the total nitrogen. Paper ionophoresis has been used as another approach to the purification problem by Thatcher, et al. (1955). They first obtained a partially purified emetic fraction by precipitation methods from enterotoxic filtrates. This fraction, which contained only slight traces of the lysins, was resolved by paper ionophoresis into three protein components and one polysaccharide. Of these four fractions, only one of the proteins gave indications of enterotoxicity. Fujiwara and Sugiyama (1955) have suggested that both a protein and a polysaccharide are

necessary constituents of a fully potent staphylococcus enterotoxin.

The knowledge which has accumulated thus far concerning the physical and chemical properties of the toxic substance strongly suggests that it is primarily composed of either a low molecular weight protein or a polypeptide coupled in some manner to a carbohydrate fraction of lesser importance.

A number of workers have studied the possibility of using in vitro cultural and biochemical tests as methods of distinguishing enterotoxin-producing stains from those which lack this property. The results in general have not been too satisfactory, but a few biochemical procedures have emerged from these efforts which are used today as ways of testing enterotoxic properties in suspected organisms. Ninety-four strains of staphylococci, including a number isolated from foods, were studied by Stritar and Jordan (1935). The biochemical, hemolytic, and agglutination tests that were employed failed to establish any good criteria for differentiating staphylococci of the enterotoxic variety. It was concluded that the ability to elaborate a gastrointestinal toxin was not confined to any recognizable type. Similar results were reported by Chapman, et al. (1937) who were also interested in cultural differentiation of food-poisoning staphylococci. The toxin producers were found to possess biochemical characteristics commonly found in staphylococcal strains of infectious origin. Some of these observations included the production of yellow or orange pigment, hemolysis of rabbit blood agar, ability to coagulate human

and rabbit plasma, and fermentation of mannitol.

Stone (1935) described a cultural method for classifying staphylococci of the food-poisoning type. A variety of different strains were inoculated into a special gelatin medium and incubated for 24 hours at 37.5°C. Organisms of the enterotoxic variety apparently liquefied the gelatin so that at the end of the incubation period, the gelatin medium failed to solidify when cooled in a water bath at 21°C. He observed that non-enterotoxic strains failed to attack the gelatin as evidenced by solidification of the medium when chilled. The validity of this test was questioned by the work of Chinn (1936) who concluded that staphylococci incriminated in food poisoning cannot be differentiated from infectious strains by the use of this gelatin medium. Chinn found that about 61 per cent of his strains from food-poisoning origin gave a positive Stone reaction and that 70.6 per cent of his strains from infectious origin also yielded positive reactions. Kupchik (1937) and Hussemann and Tanner (1949) have reported observations similar to those of Chinn.

The coagulation of human or rabbit plasma, commonly called the coagulase test, provides a strong indication that the organism is an enterotoxin producer. Chapman (1944) proposed a method for the rapid presumptive examination of suspected foods based on coagulase activity. He suggested adding 0.5 ml. of the food suspended in broth to 0.5 ml. of citrated or oxalated rabbit plasma or oxalated or citrated human whole blood. The mixture was then incubated for a period of up to seven hours and examined hourly for

coagulation. A study of 32 cultures of staphylococci by Evans (1947) revealed a relationship between the production of coagulase and the ability to ferment mannitol anaerobically. Twenty-one strains of coagulase negative organisms lacked the ability to ferment mannitol under anaerobic conditions while 11 coagulase positive strains were able to ferment this carbohydrate in the complete absence of oxygen. Subsequent physiological and nutritional studies made by Evans (1948) yielded evidence that coagulase positive staphylococci constitute a rather homogeneous group. He found that they fermented mannitol, produced pigment, generally gave a positive Stone reaction, and required the vitamins thiamine and nicotinic acid. Two years later Evans and Niven (1950) made further physiological investigations and established another important relationship. Twenty-nine tests on 114 staphylococcal strains revealed that the enterotoxigenic varieties also comprised an extremely homogeneous group. They concluded that most, if not all, enterotoxic organisms are coagulase positive, but that this did not eliminate the possibility that certain coagulase negative staphylococci might produce food poisoning. That enterotoxin is not produced by all coagulase positive staphylococci was shown by Evans, et al. (1950). Wilson, et al. (1959) have recently devised a rapid test which indicates the presence of significant numbers of viable coagulase positive staphylococci in suspected foods. They suggest the value of this procedure for rapid screening purposes, but the method has the disadvantage that it is based on coagulase activity rather than demonstration of the enterotoxic material

itself.

Bacteriophage typing has been applied to the differentiation of staphylococcal strains and an excellent discussion of this has been presented by Blair and Carr (1953). Using this typing technique it has been possible to incriminate foods suspected of having caused a staphylococcal poisoning.

The obvious disadvantage of using susceptible human volunteers as a means of assaying for the presence of staphylococcus enterotoxin led a number of investigators on a search for a suitable laboratory animal. In the years that followed, two animals, the kitten and monkey, received the most attention. Certain workers have suggested the use of other animals such as the frog and the pig, but these have apparently not been widely accepted as being suitable for assay work.

Enterotoxic filtrates known to produce symptoms in human beings were fed to young monkeys by Jordan and McBroom (1931) in an attempt to find a satisfactory laboratory animal. Their results in general were not too successful. Thirteen monkeys were administered toxin in amounts ranging from 5 to 20 ml. through a rubber catheter introduced into the stomach. Five of the animals showed symptoms similar to those observed in humans. Three monkeys showed slight symptoms and the remaining five were not visibly affected. Minett (1938) made some analogous observations and concluded that feeding tests on monkeys, dogs, and cats were unsatisfactory for detecting the presence of enterotoxin. He attributed this to varying susceptibility of the test animals

utilizing oral adminstration of the toxic filtrates. The possibility of assaying for the active principle by parenteral injection of both monkeys and kittens was studied by Davison, et al. (1938). It was felt by these workers to be a better method and one that appeared to offer certain advantages over the feeding technique. It was found to be a more delicate test and required only a minute amount of test material. In addition, susceptible animals could be used repeatedly. Surgalla, et al. (1953) made some extensive observations on the monkey feeding test over a four and one-half year period. A wide variation in susceptibility was found to exist after approximately 1000 monkeys were administered staphylococcal enterotoxin intragastrically. It was noted that animals previously fed toxin were less susceptible to a second feeding and that the occurrence of unprovoked emesis was quite low. Recently, Sugiyama, et al. (1958) showed that the incidence of vomiting in monkeys fed enterotoxic filtrates could be greatly increased if the animal was first treated with subemetic doses of dihydroergotamine methanesulfonate (DHE-45). Two groups of monkeys were fed equivalent amounts of enterotoxin and the incidence of vomiting in the (DHE-45) treated animals was twice that of the untreated group.

In 1936 Dolman, et al. described the use of kittens in the assay of staphylococcus enterotoxin and the main features of this early work were reviewed by Dack (1956). Potent enterotoxic filtrates were first treated with a 0.3 per cent solution of formaldehyde to inactivate other toxic materials elaborated by certain

staphylococci. They observed that 0.5 ml. of this treated material was sufficient to produce a reaction in young kittens when injected intraperitoneally. It was also found that filtrates from non-enterotoxigenic strains as well as uninoculated formalized broth failed to produce typical symptoms in the animals. Adult cats could apparently be used in this test too, but they had certain disadvantages. Results of antigenic studies on staphylococcus enterotoxin in connection with the intraperitoneal kitten test were presented by Dolman and Wilson (1938). They showed that the enterotoxic fraction was antigenic and would produce an active immunity in kittens given a series of injections. That immunized rabbit and horse serum conferred passive protection in the kittens was another factor noted by these workers. Rigdon (1938) studied the effects of the toxin on both puppies and kittens and found that typical symptoms could be produced by injection of culture medium alone. It was mentioned earlier that Davison, et al. (1938) attempted to assay enterotoxin by parenteral injection of monkeys and kittens and reported certain advantages using this technique. Phatak and Pentler (1940) tried to improve upon the kitten test by anesthetizing the animals with an intraperitoneal injection of sodium pentobarbitol prior to the administration of toxic filtrates. Staphylococcus enterotoxin was indicated if a vomiting reaction occurred 20 to 90 minutes after injection of the filtrate. Fulton (1943) made a study of enterotoxin in relation to the intraperitoneal kitten test and concluded that it was a test which could not be

relied upon to detect either the presence or absence of toxin. In direct opposition to this conclusion, Matheson and Thatcher (1955) found that the Dolman kitten test provided a high degree of positivity for the detection of a gastrointestinal irritant in staphylococcal filtrates if conditions were carefully controlled and if decisive vomiting was used as the sole criterion of a positive reaction.

The use of the frog (Rana pipiens) as an assay tool for enterotoxin has been suggested by Robinton (1949, 1950). She was able to demonstrate that filtrates which induced vomiting in the kitten test were also capable of producing spasms or antiperistalsis when fed to frogs. Doubt as to the validity of the frog test was expressed by Eddy (1951) who concluded that the spasm reaction was non-specific and could be induced by factors other than staphylococcus enterotoxin.

Hopkins and Poland (1942) experimented with the young suckling pig and the results of their work suggested that this animal might prove useful for toxin assay. Fifty-three of 57 pigs vomitted when injected intraperitoneally with potent filtrates. The response was evidently highly specific since the animals did not react when injected with autoclaved filtrates from enterotoxic strains, filtrates from non-enterotoxic strains, or filtered uninoculated medium.

The effects of enterotoxin on isolated rabbit gut were studied by Richmond, et al. (1942) and by Anderson (1953). Each suggested the possibility of using an in vitro test such as this for

demonstrating the toxin. Kelsey and Hobbs (1954) were unable to repeat Anderson's work and concluded that isolated rabbit gut was unlikely to be of value as an indicator for the presence of staphylococcus enterotoxin.

EXPERIMENTAL, PART I. DEVELOPMENT OF CHROMATOGRAPHIC METHOD FOR DETECTING ENTEROTOXIGENIC STAPHYLOCOCCI

At the onset of this study a search was made for a solvent system which could be used in separating various constituents in staphylococcus culture supernatants by paper chromatographic methods. The results of several preliminary experiments indicated that a pyridine tert-amyl alcohol system might be effective in achieving this separation. An effort was then made to determine similarities and differences in the chromatographic patterns of coagulase positive and coagulase negative strains of staphylococci.

Materials and Methods

Cultures. Cultures of Staphylococcus species used in this portion of the work (Table 1) were obtained from the Kansas State Board of Health and from the collection of Dr. P. S. Rajulu. The coagulase reaction of each strain was verified with the use of human plasma.

Culture Medium. A liquid culture medium for optimum enterotoxin production was described by Surgalla, et al. (1951). This medium was chosen for this work because it is easily prepared and has certain advantages over solid culture media in chromatographic work. One modification in the preparation of the medium was

Table 1. Staphylococcus species studied.

Strain number	Coagulase reaction	Strain number	Coagulase reaction
I[1]	Positive	KBH 702[2]	Positive
II	Positive	KBH 703	Positive
III	Positive	KBH 437-3	Negative
IV	Positive	KBH 438-2	Negative
V	Positive	KBH 438-3	Negative
VI	Positive	KBH 725	Negative
VII	Positive	KBH 1031-1	Negative
VIII	Positive	KBH 1032	Negative
IX	Positive	KBH 1037	Negative
X	Positive	KBH 1064	Negative
XI	Positive	KBH 1069	Negative
XII	Positive	KBH 2299	Negative
XIII	Positive	KBH 2306	Negative
XIV	Positive	KBH 2315	Negative
XV	Positive	KBH 2320	Negative
XVI	Positive	KBH 2321	Negative
XVII	Positive	KBH 2325	Negative
XVIII	Positive	KBH 2327	Negative
XIX	Positive	KBH 2328	Negative
XX	Negative	KBH 2390	Negative

[1]Strains with Roman numeral designations were obtained from Dr. P. S. Rajulu.

[2]KBH strains were obtained from the Kansas State Board of Health.

necessary due to the unavailability of Amigen (Mead Johnson). An identical amount of Bacto-Tryptone (Difco) was substituted since it, like Amigen, consists of a pancreatic digest of casein. The composition of the medium in grams per liter is as follows:

Bacto-Tryptone (Difco)	15.0 grams
Nicotinic acid (Merck)	0.00123 grams
Thiamine hydrochloride (Research Laboratories)	0.00005 grams
Glucose (anhydrous) (Pfanstiehl)	2.5 grams
Distilled water	Q. S. 1000 ml.

After adjusting the medium to pH 7.6, 25 ml. aliquots were placed in 300 ml. prescription bottles, plugged with cotton, and autoclaved at 121°C. for 20 minutes.

Incubation. Each bottle of medium was inoculated with a loopful of an 18- to 24-hour culture of staphylococci and allowed to incubate at 37°C. for three days under an atmosphere of approximately 20 per cent carbon dioxide and 80 per cent air in an aluminum anaerobic jar. Maximum gas exchange was enhanced by placing the bottles in a horizontal position and by using cotton stoppers rather than screw caps.

Preparation of Supernatants. At the end of the three-day incubation period the liquid cultures were centrifuged for 30 to 40 minutes at 2600 revolutions per minute. Enough cells were removed in this manner to produce a clear supernatant. The supernatants were removed and stored in the refrigerator at 3°C.

Solvent System. The pyridine tert-amyl alcohol solvent system which was used throughout this study was prepared by mixing 70 ml. of pyridine (Fisher) with 70 ml. of tert-amyl alcohol (Fisher) and 60 ml. of deionized water (Block, et al., 1952).

Chromatographic Development. Ascending chromatography was used throughout this study. The chamber consisted of a Fisher American Medical Museum Jar size number 11 which had the following internal dimensions: 24.7 cm. (width) x 24.7 cm. (height) x 11.6 cm. (depth). Large sheets of Whatman number 1 chromatography paper were cut into smaller sheets 21 cm. wide and of sufficient length to be supported at the top of the chamber. Culture supernatants

were applied in 5 µl. amounts on a line approximately 2.5 cm. from one end of the paper. Not more than five such spots were placed on a single sheet. Enough solvent was placed in the chamber to cover the bottom completely (150 to 200 ml.). The atmosphere inside the chamber was equilibrated with the solvent for about one hour at which time the paper was introduced and allowed to develop in the dark for four hours at 37°C. At the conclusion of the development period, the chromatograms were removed from the chamber and oven dried at 110°C. for a period of three to five minutes.

Detection of Substances on the Chromatogram. Each chromatogram was sprayed lightly with a 1.0 per cent solution of triketohydrindene hydrate (ninhydrin) in n-butanol and oven dried at 110°C. for three minutes. Spots became apparent and varied from red to reddish purple in color. R_f values were computed for each spot observed using the following equation:

$$R_f = \frac{\text{Distance spot traveled}}{\text{Distance solvent front traveled}}$$

Controls. Uninoculated culture medium was analyzed chromatographically as a control on the composition of the medium.

Results and Interpretation

Distinct differences were noted in the chromatographic patterns of the coagulase positive and coagulase negative strains tested. The R_f values for the series of spots that appeared in each case are summarized in Table 2. Slight deviations in the R_f values of similar spots were to be expected under experimental conditions.

Table 2. Coagulase test results and R_f values of spots separated from supernatants of staphylococcus cultures using pyridine tert-amyl alcohol.

Supernatant from culture	Coagulase reaction	Spots separated and their R_f values						
		1	2	3	4	5	6	7
I	Positive	.12	-	-	.36	.45	.54	.66
II	Positive	.12	-	-	.36	.45	.54	.66
III	Positive	.13	-	-	.36	.45	.53	.65
IV	Positive	.13	-	-	.36	.45	.53	.65
V	Positive	.12	-	-	.37	.45	.53	.65
VI	Positive	.12	-	-	.37	.45	.53	.65
VII	Positive	.12	-	-	.37	.45	.53	.65
VIII	Positive	.11	-	-	.34	.41	.50	.62
IX	Positive	.14	-	-	.38	.45	.53	.65
X	Positive	.14	-	-	.38	.45	.53	.65
XI	Positive	.14	-	-	.38	.45	.53	.65
XII	Positive	.14	-	-	.38	.45	.53	.65
XIII	Positive	.13	-	-	.36	.44	.53	.64
XIV	Positive	.13	-	-	.36	.44	.53	.64
XV	Positive	.13	-	-	.36	.44	.53	.64
XVI	Positive	.13	-	-	.36	.44	.53	.64
XVII	Positive	.11	-	-	.34	.41	.50	.62
XVIII	Positive	.11	-	-	.34	.41	.50	.62
XIX	Positive	.11	-	-	.34	.41	.50	.62
KBH 702	Positive	.12	-	-	.36	.45	.54	.66
KBH 703	Positive	.13	-	-	.36	.45	.53	.65
XX	Negative	.12	-	-	.36	-	.54	.66
KBH 437-3	Negative	.10	.16	-	.36	-	.50	.61
KBH 438-2	Negative	.10	.16	-	.36	-	.50	.61
KBH 438-3	Negative	.10	.16	-	.36	-	.50	.61
KBH 725	Negative	.10	.18	.27	.37	-	.48	.58
KBH 1031-1	Negative	.10	.16	-	.36	-	.50	.61
KBH 1032	Negative	.10	.16	-	.35	-	.50	.61
KBH 1037	Negative	.10	.16	-	.35	-	.50	.61
KBH 1064	Negative	.10	.16	-	.35	-	.50	.61
KBH 1069	Negative	.10	.16	-	.35	-	.50	.61
KBH 2299	Negative	.10	.18	.27	.34	-	.48	.58
KBH 2306	Negative	.09	.19	.28	.38	-	.51	.61
KBH 2315	Negative	.09	.19	.28	.38	-	.51	.61
KBH 2320	Negative	.10	.18	.27	.34	-	.48	.58
KBH 2321	Negative	.10	.18	.27	.34	-	.48	.58
KBH 2325	Negative	.10	.18	.27	.34	-	.48	.58
KBH 2327	Negative	.09	.19	.28	.38	-	.51	.61
KBH 2328	Negative	.09	.19	.28	.38	-	.51	.61
KBH 2390	Negative	.09	.19	.28	.38	-	.51	.61
Control on medium	-	.12	.23	.30	.36	-	.54	.66

Similar R_f values have been placed in the same column. A minus sign indicates that a spot did not appear on the chromatogram in question in that position. Spots were observed in positions one, four, six, and seven regardless of which supernatant was tested. The control (uninoculated medium) as well as the coagulase positive cultures and coagulase negative cultures showed spots in these four positions. This was probably due to the appearance of amino acid and peptide constituents of the original culture medium. These substances were probably attacked only slightly, if at all, by the staphylococci. A spot failed to appear in positions two and three in every instance where a coagulase positive culture was tested. It is logical to assume that these failed to appear because the substances were utilized in the normal metabolism of the organisms. A similar explanation accounts for the lack of a spot in position three for certain of the coagulase negative cultures. In each of the coagulase positive culture supernatants which were tested, a distinct spot with an R_f value ranging from 0.41 to 0.45 appeared in position five. This was noticeably lacking in all 19 of the coagulase negative culture supernatants which were chromatographed. The fact that this particular spot also failed to appear in the control suggests that coagulase positive staphylococci possess a synthetic ability which coagulase negative strains lack. Since staphylococcus enterotoxin is known to be composed of proteinaceous material, it is not illogical to postulate that this spot might possibly consist, at least in part, of enterotoxin.

EXPERIMENTAL, PART II. CHROMATOGRAPHIC STUDY OF STAPHYLOCOCCUS SPECIES ISOLATED FROM NATURAL SOURCES TO DETERMINE POSSIBLE ENTEROTOXIGENICITY

Materials and Methods

In an effort to substantiate the results which appear in Part I of this study, strains of staphylococci were isolated from a variety of natural sources and tested chromatographically. The coagulase test using human plasma was then performed on each isolate and the results were examined to see whether or not parallelism existed between coagulase activities and chromatographic patterns.

Cultures. With the exception of eight strains which were supplied by Professor V. D. Foltz, all of the staphylococcus cultures used in this portion of the work were freshly isolated from natural sources. Each specimen was streaked on Staphylococcus Medium 110 (Difco) from which the original isolation was made. The numerical designation and source of each strain are presented in Table 3. Strains preceeded by the letter F were obtained from Professor Foltz.

Production of Supernatants. Supernatants were prepared by the method described in Part I with the exception that 50-ml. prescription bottles containing 4 ml. aliquots of medium were used instead of the larger bottles to conserve space in the carbon dioxide jars.

Chromatography. The chromatographic method employed in this portion of the work was identical to that described in Part I.

Table 3. Numerical designation and source of staphylococcus isolates.

Strain number	Source	Strain number	Source
93-A	Dog (nose)	23-A	Human (nose)
96-A	Dog (ear)	25-A	Human (nose)
97-A	Dog (nose)	26-A	Human (arm)
97-B	Dog (nose)	27-A	Human (nose)
98-A	Dog (nose)	29-A	Human (nose)
99-A	Dog (nose)	31-A	Human (nose)
100-A	Kitten (nose)	33-C	Human (nose)
103-A	Dog (nose)	34-A	Human (arm)
106-A	Dog (nose)	35-A	Human (nose)
107-A	Horse (ear)	37-A	Human (nose)
108-B	Horse (ear)	37-B	Human (nose)
109-A	Horse (nose)	39-A	Human (nose)
109-B	Horse (nose)	39-B	Human (nose)
111-A	Horse (nose)	41-A	Human (nose)
113-A	Horse (nose)	41-B	Human (nose)
114-A	Calf (ear)	43-A	Human (nose)
115-A	Dog (nose)	45-A	Human (nose)
F-117-A	Cow (milk)	46-A	Human (arm)
F-120-A	Cow (milk)	47-A	Human (nose)
F-121-A	Cow (milk)	47-B	Human (nose)
F-125-A	Cow (milk)	48-A	Human (arm)
F-125-B	Cow (milk)	49-B	Human (nose)
127-A	Dog (eye)	51-A	Human (nose)
131-A	Dog (eye)	53-A	Human (nose)
81-A	Potato salad	53-B	Human (nose)
88-A	Pie	55-A	Human (nose)
89-A	Pie	56-A	Human (arm)
1-A	Human (nose)	57-A	Human (nose)
1-B	Human (arm)	59-A	Human (nose)
2-A	Human (nose)	61-A	Human (nose)
3-A	Human (nose)	63-A	Human (nose)
3-2	Human (arm)	65-B	Human (nose)
5-A	Human (nose)	66-A	Human (arm)
5-1	Human (nose)	66-B	Human (arm)
7-A	Human (nose)	67-A	Human (nose)
7-1	Human (arm)	67-B	Human (arm)
7-B	Human (nose)	69-A	Human (nose)
8-A	Human (arm)	69-B	Human (arm)
9-A	Human (nose)	70-B	Human (arm)
9-1	Human (nose)	71-A	Human (nose)
9-B	Human (arm)	73-A	Human (nose)
11-1	Human (nose)	75-A	Human (nose)
13-A	Human (nose)	76-A	Human (arm)
13-1	Human (nose)	77-A	Human (nose)
14-1	Human (arm)	77-B	Human (nose)
15-A	Human (nose)	79-A	Human (nose)

Table 3. (concluded).

Strain number	Source	Strain number	Source
17-A	Human (nose)	80-B	Human (arm)
18-A	Human (arm)	F-140	Human (skin)
19-A	Human (nose)	F-141	Human (skin)
20-A	Human (arm)	F-142	Human (throat)
21-A	Human (nose)		

Mannitol Fermentation. Each staphylococcus isolate was tested for its ability to ferment mannitol by making a stab inoculation into mannitol brom cresol purple agar as described by Lord (1959). The medium had the following composition:

Beef extract	3 grams
Peptone	5 grams
Agar Agar	10 grams
Distilled water	1000 ml.
Mannitol	5 grams
Brom cresol purple (1% alcoholic sol.)	1 ml.

All ingredients except the brom cresol purple were combined, brought to boil to dissolve, and adjusted to pH 7.0. The brom cresol purple was added and the complete medium was tubed in 5 ml. amounts and autoclaved at 121°C. for 15 minutes. Each stab culture was observed for acid production within 48 hours. A yellow color change in any part of the culture was considered to be a positive test for mannitol fermentation.

Results and Interpretation

The supernatants of each of the staphylococcus isolates were chromatographed and checked for the presence or absence of a spot in position five (R_f 0.40 to 0.45). These observations together with the corresponding coagulase reactions and mannitol fermentation results are summarized in Table 4.

Of the 101 strains which were tested, 32 were found to be coagulase positive and 69 coagulase negative. In every case the supernatants prepared from negative cultures failed to produce a spot in position five when chromatographed. This represented a 100 per cent correlation. Twenty-eight of the positive supernatants produced a definite spot in the appropriate R_f range while four did not. The correlation in this case was 88 per cent. It is possible that these four strains which were able to ferment mannitol as well as coagulate human plasma lacked the ability to elaborate the product being elicited by spot number five (enterotoxin?). Another factor may account for the observed discrepancies. Human plasma was used for all of the coagulase tests performed in this study and it is known that certain staphylococci will coagulate plasma from one source but not another. With the exception of one case, all of the cultures which produced a spot in position five and coagulated plasma were also able to ferment mannitol. All cultures producing a spot in position number five were coagulase positive.

Table 4. R_f values of spots appearing in position five with corresponding coagulase and mannitol fermentation results.

Strain number	R_f value of spot #5	Coagulase reaction	Mannitol fermentation	Strain number	R_f value of spot #5	Coagulase reaction	Mannitol fermentation
93-A	.42	P[1]	N[2]	5-A	-	N	N
96-A	-	N	P	5-1	-	N	N
97-A	.42	P	P	7-A	-	N	N
97-B	.41	P	P	7-1	-	N	P
98-A	.40	P	P	7-B	-	N	N
99-A	.45	P	P	8-A	-	N	N
100-A	.45	P	P	9-A	-	N	N
103-A	.45	P	P	9-1	-	N	N
106-A	.41	P	P	9-B	-	N	N
107-A	-	N	P	11-1	-	N	N
108-B	-	N	P	13-A	-	N	N
109-A	-	P	P	13-1	-	N	N
109-B	.44	P	P	14-1	-	N	P
111-A	.42	P	P	15-A	.41	P	P
113-A	.44	P	P	17-A	-	N	N
114-A	-	N	P	18-A	-	N	P
115-A	.42	P	P	19-A	-	N	N
F-117-A	.42	P	P	20-A	-	N	N
F-120-A	-	P	P	21-A	-	N	N
F-121-A	-	P	P	23-A	-	N	N
F-125-A	-	P	P	25-A	-	N	N
F-125-B	.42	P	P	26-A	-	N	N
127-A	.45	P	P	27-A	-	N	N
131-A	.42	P	P	29-A	-	N	N
81-A	-	N	P	31-A	-	N	P
88-A	-	N	P	33-C	.41	P	P
89-A	-	N	N	34-A	-	N	N
1-A	-	N	N	35-A	-	N	N
1-B	-	N	N	37-A	-	N	N
2-A	.40	P	P	37-B	-	N	N

Table 4. (concluded).

Strain number	R_f value of spot #5	Coagulase reaction	Mannitol fermentation	Strain number	R_f value of spot #5	Coagulase reaction	Mannitol fermentation
3-A	-	N	N	39-A	-	N	N
3-2	-	N	N	39-B	-	N	N
41-A	-	N	N	66-A	-	N	N
41-B	-	N	P	66-B	.44	P	P
43-A	-	N	N	67-A	-	N	N
45-A	-	N	N	67-B	-	N	N
46-A	-	N	P	69-A	-	N	N
47-A	-	N	N	69-B	-	N	N
47-B	-	N	N	70-B	-	N	N
48-A	-	N	N	71-A	-	N	N
49-B	-	N	N	73-A	-	N	N
51-A	.42	P	P	75-A	.40	P	P
53-A	-	N	N	76-A	-	N	N
53-B	-	N	N	77-A	-	N	N
55-A	-	N	N	77-B	-	N	N
56-A	-	N	N	79-A	.42	P	P
57-A	-	N	N	80-B	-	N	N
59-A	.45	P	P	F-140	.44	P	P
61-A	-	N	N	F-141	.42	P	P
63-A	-	N	N	F-142	.41	P	P
65-B	.45	P	P	Control	-	-	-

[1]The letter P indicates a positive reaction
[2]The letter N indicates a negative reaction
[3]A negative sign indicates no spot in position five

DISCUSSION AND CONCLUSIONS

The results which have been observed in this study indicate that paper chromatography may prove useful as a method of assay for the presence of staphylococcus enterotoxin by inference. With a few exceptions, consistent differences were found in the chromatographic patterns of coagulase positive and coagulase negative culture supernatants. This does not mean, however, that an absolutely specific test for enterotoxin has been developed. There are a number of major problems which must be resolved before this goal can possibly be achieved.

If, for example, the substance which appeared in position five (R_f 0.40 to 0.45) with the coagulase positive culture supernatants is found not to contain a toxic fraction, the value of this chromatographic procedure as a method of enterotoxin assay is questionable. If further investigation indicates this to be the case, other possibilities exist. With modifications and refinements, the chromatographic method outlined in this study may provide a convenient and accurate test for predicting pathogenicity or non-pathogenicity of a suspected staphylococcus isolate. If the three-day incubation period used in this study to prepare the supernatants could be shortened to approximately 24 hours, the length of time necessary to answer the question of pathogenicity would be the same, and in some cases less, than the time required for certain tests which are at present used routinely.

Undoubtedly the best method for determining the presence or absence of toxin in the spot appearing at position five would be

to elute the fraction from the paper and feed portions of it to human volunteers or susceptible laboratory animals. If this procedure yielded results which were consistently positive, the chromatographic procedure described would have much in its favor. The basic problem, however, would remain unsolved even after the presence of enterotoxin had been established.

A chromatographic test for staphylococcus enterotoxin would be of most value if it could be applied directly to the suspected food material. The proteinaceous material which occurs in natural foods is many times more complex than the amino acid and peptide constituents of the simple liquid medium used in this work. The difficulties which might be encountered in chromatographing liquid food extracts for enterotoxin are obvious.

It is felt that this study is worthy of further investigation. A simple, rapid, accurate chromatographic test for either the presence of staphylococcus enterotoxin or for determining pathogenicity in staphylococci would be invaluable.

SUMMARY

A method has been described for differentiating coagulase positive and coagulase negative strains of staphylococci with the use of paper chromatography. Supernatants prepared from staphylococcus cultures grown in tryptone broth were chromatographed with a pyridine tert-amyl alcohol solvent system. The separated fractions on each chromatogram were detected with ninhydrin reagent. This method was tested on a total of 141 staphylococcus

strains obtained from a variety of sources. A spot with an R_f value ranging from 0.40 to 0.45 was found to appear in 49 of 53 (92 per cent) of the coagulase positive strains tested, but it failed to appear when any of the coagulase negative culture supernatants were chromatographed. Although not all of the coagulase positive strains produced a spot in the appropriate R_f range, all of the cultures which produced the spot were coagulase positive. The possibilities of adapting this procedure to the qualitative assay of staphylococci presumed to produce enterotoxin and to the determination of pathogenicity based on coagulase activity of staphylococci are discussed.

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A POSSIBLE METHOD FOR DETECTING ENTEROTOXIGENIC STAPHYLOCOCCI BY PAPER CHROMATOGRAPHY

by

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Since staphylococcal food poisoning presents a major public health problem, it is essential that every outbreak be traced to its source and corrective action taken to prevent subsequent occurrences. To incriminate a suspected food is not always an easy task. A completely satisfactory test for either the presence of the toxic substance or for staphylococci capable of producing it does not exist at the present time. Therefore, a study was initiated with the idea of developing anassay procedure for the qualitative determination of staphylococcus enterotoxin. It was felt that paper chromatography might provide a useful tool in the solution of this problem.

The material to be chromatographed was prepared by growing strains of staphylococci in a simple medium composed of Bacto-tryptone, niacin, thiamine hydrochloride, anhydrous glucose, and distilled water for three days at 37°C. under an atmosphere of approximately 20 per cent carbon dioxide. At the end of the incubation period the cultures were centrifuged and the supernatants removed. Five µl. portions of the supernatants were applied to sheets of Whatman number 1 chromatography paper. The chromatograms were developed for four hours at 37°C. with the use of a pyridine tert-amyl alcohol solvent system. Spots were made apparent by spraying the chromatograms with a one per cent solution of ninhydrin in n-butanol and drying in the oven at 110°C. for three minutes.

During the initial phase of this study, the supernatants from 21 coagulase positive cultures were compared chromatographically

with 19 coagulase negative strains. Without exception, a spot with an R_f value ranging from 0.41 to 0.45 was observed with the coagulase positive supernatants. This spot was noticeably lacking in each of the coagulase negative chromatograms.

A study of 101 staphylococcus strains isolated from natural sources were tested and showed essentially the same results. Of 32 coagulase positive strains, 28 produced a spot with an R_f value in the range 0.40 to 0.45. This represented an 88 per cent correlation. The 69 coagulase negative cultures all failed to show a spot within this R_f range.

It is suggested that the substance or substances which appeared at R_f 0.40 to 0.45 might consist, at least in part, of enterotoxin. In addition, there are strong indications that this technique may prove useful as a method of determining the pathogenicity or non-pathogenicity of suspected staphylococcus isolates.