USE OF THE DOG AS A TEST ANIMAL FOR DETECTING STAPHYLOCOCCAL ENTEROTOXIN

by

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INTRODUCTION

The role of Staphylococcus aureus as an etiological agent in food poisoning has been established beyond question, and is of considerable importance. The prevalence of this organism in the nose and throat and on the hands of people is great; it is of equal importance in many foods of animal origin. The resistance to antibiotics which some strains have developed in recent years has caused this organism to assume greater importance in many varied fields.

Although the cause of staphylococcal food poisoning has been recognized for many years, its incidence shows no tendency to decrease. On the contrary, more and more outbreaks and cases appear to be reported each year. The most recent information available shows that the number of outbreaks has increased for the past two years. In 1959 there were more cases of staphylococcal food poisoning than all other types combined where the causative agent could be determined. There were 89 outbreaks reported in the United States, involving 4,138 persons (26). Undoubtedly there were many more outbreaks which occurred but were not reported.

The mechanism by which some strains of staphylococci are able, under the proper conditions, to elaborate a substance which causes gastro-intestinal disturbances in human beings is not known. To quote the words of Van Heyninger (93), "progress in research on staphylococcal enterotoxin is stifled

almost to the point of extinction by the difficulties in testing the toxin." Human volunteers have been used many times for the detection of toxic agents in suspected foods and in filtrates prepared from strains of Staphylococcus aureus. The use of human volunteers is by necessity very limited, since it is difficult to find an appreciable number of persons willing to subject themselves to an experience of this nature.

Monkeys are regarded as the best substitute for human volunteers, but these are expensive, difficult to maintain, and are somewhat variable in response. These animals are not readily available to many people who would undertake research on staphylococcal food poisoning. Cats have been used with varying success by some investigators, but the difficulty in securing sufficient numbers and in handling again presents a problem.

An alternate test animal for staphylococcal enterotoxin would be most helpful. The availability of dogs, together with the simplicity of their maintenance and handling, would appear to make them an ideal test animal. A few reports of attempts to use dogs as test animals have appeared in the literature, with the consensus of opinion being that dogs are not suitable for this purpose. A report by Tekse in 1951 indicated that the use of dogs as test animals has not been thoroughly investigated. He found that dogs reacted with

emesis when an enterotoxic filtrate was administered intravenously, and that they reacted negatively when a non-toxic filtrate was administered. A search of the literature has revealed no further work on this subject.

The purpose of the research reported herein is to determine the reliability of dogs as test animals in detecting staphylococcal enterotoxin by the intravenous injection of sterile heated filtrates produced from certain strains of Staphylococcus aureus.

REVIEW OF LITERATURE

Properties of Enterotoxin

The outstanding property of staphylococcal enterotoxin is its ability to cause severe gastro-intestinal upsets in human beings; in fact the name "enterotoxin" was given to this substance because it exerts its most conspicuous action on the gastro-intestinal canal or enteron (32). The symptoms produced following ingestion of this substance by susceptible persons include retching, vomiting, chills, fever, and semi-shock.

No attempt will be made to describe a typical train of symptoms in man, however, the pathological changes caused by the action of this substance are well worth noting. These changes were described by Palmer (63) who examined 42 hospitalized patients suffering from acute gastroenteritis of proven staphylococcal origin. Examination of the gastric mucosa by gastroscopy was made at intervals during 280 hours, with the first examinations being performed five hours after initiation of symptoms. Acute exogenous gastritis was observed on the initial examination and with decreasing frequency until the 80th hour, after which the examinations revealed essentially normal tissue. Biopsies were performed in 24 patients; a short-lived progression of pathological changes was noted up to the 92nd hour after appearance of symptoms.

All tissue taken after that hour was normal and no permanent morphological abnormalities were produced in the gastric mucosa.

The effect of enterotoxin on various test animals is discussed in later sections.

The physical properties of enterotoxin have received considerable attention and many important observations have been made. The question of heat stability has been extensively studied, since its ability to survive heat treatment during cooking of food and prior to performing certain biological tests is of considerable importance. Many workers have demonstrated its heat resistance (32, 44, 50, 58, 65, 87, 92), but most agree that its potency is somewhat diminished by boiling for 30 minutes. Casman (15) has recently described a heat-labile type of enterotoxin which failed to produce emesis in cats after the hemolysins in the filtrate were inactivated by heating, but which caused emesis in monkeys when unheated filtrates were administered orally. Davison and Dack (27) have shown that autoclaving at 120 C for 20 minutes practically inactivates enterotoxin, and the use of autoclaved filtrates as negative controls has been reported (46). Stability of enterotoxin under refrigeration was noted by Jordan and Burrows (51), who found that sterile filtrates retained their potency for at least 67 days when stored in a refrigerator. Jones and Lochhead (49) found that filtrates did not lose their toxic property in 60 days at low temperatures. A formalized

filtrate from one strain was potent after 14 month's storage in a refrigerator (32).

Dolman and Wilson (32) noted that the addition of formaldehyde to filtrates in 0.3 percent concentration with subsequent incubation at 37 C did not destroy enterotoxin, but did destroy alpha and beta hemotoxins. This observation was confirmed by Minett (59) who also observed that enterotoxin was not affected by rennet. Woolpert and Dack (99) reported that small amounts of phenol apparently weakens the toxic action, and Segalove (71) has demonstrated that penicillin does not inactivate preformed enterotoxin. Its emesis-producing ability was not inactivated at pH 4.5 or at pH 8.0-8.2 after incubation at 37 C for 24 hours (44). Resistance of enterotoxin to small amounts of ascorbic acid was reported by Thatcher and Matheson (92), and Minett (59) found it resistant to acid at pH 5. Enterotoxin was reported to be resistant in crude filtrates to both trypsin and pepsin (44), while Minett (59) found that a formalized filtrate was inactivated by trypsin in four hours at 37 C. Bergdoll et al. (8) report that purified enterotoxin is resistant to trypsin.

Enterotoxin was found to be insoluble in alcohol (44, 51), and to be precipitated from solution by ethanol, methanol, and hydrochloric acid, and by saturation with ammonium sulfate (7). Hammon (44) concluded that the enterotoxic substance was a large complex carbohydrate molecule, but recent work

(45) indicates that it is a water-soluble protein with an average molecular weight of 24,000 plus or minus 3,000. The iso-electric point of purified enterotoxin was found to be pH 8.6, and analysis indicated the presence of 18 amino acids (45). Jordan and Burrows (51) found that enterotoxin would not distill, that it was resistant to a rather strong solution of chlorine, and that it was completely removed from aqueous solution by extraction with ether or chloroform. Hammon (44) was unable to find active enterotoxin in ether extracts, but found it instead in the residual extracted culture filtrate.

The ability of enterotoxin to readily dialyze was noted by Hammon (44), while Jordan and Burrows (51) found that it was not readily dialyzable. Minett (59) noted that it diffuses readily through culture medium but not through collodion. Bergdoll et al. (7) found that enterotoxin was non-dialyzable and concentrated it by this method, together with precipitation.

Reports of the antigenicity of enterotoxin have been made by many investigators, among whom are Barber (4), Woolpert and Dack (99), Minett (59), Dolman and Wilson (32, 33), Fulton (42), Davison et al. (28), Surgalla et al. (85), Casman (15), and Matheson and Thatcher (58). Hammon (44) reported that it was non-antigenic but observed a tolerance which developed in test animals. The difficulty in obtaining

enterotoxin in purified form greatly hampered the study of its antigenic, immunological, and chemical properties. Since its purification and concentration, Bergdoll et al. (8) have produced in rabbits a monovalent antiserum which is capable of producing a single band of precipitate with its antigen (enterotoxin) as shown by the gel-diffusion technique. Using the same technique, Casman (14) has presented evidence of at least two serological types of enterotoxin which he designated S-6 and 196-E. Casman states that neither type of enterotoxin was formed from 36 cultures which were non-enterotoxigenic by the cat test, nor by eight strains reported to be non-enterotoxigenic when tested by the monkey-feeding procedure. In later work (15) Casman recommended that the two heat resistant antigens (enterotoxin) be designated as type E and type F. He found that strains of staphylococci isolated from cases of enteritis produced both types E and F, but strains isolated from foods implicated in food poisoning incidents produced only type F.

Sugiyama et al. (81) verified Casman's observation of the two serological types by use of a modification of the agar diffusion technique. They demonstrated that colonies vary in their capacity to produce enterotoxin and that some colonies either did not produce it or produced it in amounts which were not detectable.

Production of Enterotoxin by Staphylococci

The conditions necessary for the production of staphylococcal enterotoxin under natural conditions have been reconstructed in detail from outbreaks of food poisoning. These conditions are contamination with sufficient numbers of a food poisoning strain of staphylococci, a suitable medium for development of the organisms, and sufficient time in the proper temperature range for growth of the organisms and development of enterotoxin.

An accurate knowledge of the percentage of strains which are capable of producing enterotoxin is still to be ascertained. Minett (59) tested 38 strains from the bovine udder; 16 of these were shown to produce enterotoxin. Slanetz et al. (76) could find no evidence of enterotoxin production in 10 strains isolated from cow's udders, while Bell and Velez (6) report 25 enterotoxigenic strains out of 37 isolated from similar sources. Staphylococci isolated from cases of enteritis following antibiotic therapy were found to produce enterotoxin in 30 out of 32 strains (86). Enterotoxin production by four out of five coagulase-positive strains of staphylococci isolated from wholesome frozen foods was noted by Evans et al. (38). Dolman and Wilson (32) found three out of four strains isolated from frozen fruits and vegetables to be enterotoxigenic. A wide variation in the estimation of the percentage of enterotoxigenic strains therefore exists

among different investigations.

The number of organisms required to produce enterotoxin in sufficient quantities to be detected is undoubtedly large. Allison (1) reports finding staphylococci in numbers ranging from 500,000 to 2 billion per gram in food samples from outbreaks. Dolman and Wilson (33) found staphylococci in suspected foods in numbers of over 1 million per gram. Production of enterotoxin was found to be associated with good growth of organisms by Segalove and Dack (72).

Dack (24) has reviewed the list of foods from which outbreaks of food poisoning have occurred. These foods include, but are not limited to, the following: custard filled bakery foods, pies, ham and ham products, tongue sandwiches, custard filled doughnuts, milk, cheese, hollandaise sauce, pressed pickled beef, chicken salad, and others. Under experimental conditions, Segalove et al. (73) found that high acid foods were incapable of supporting growth of staphylococci, while growth occurred best in low-acid foods. A selective action for staphylococci by foods containing a high salt content has been noted (61). Davison and Dack (27) noted that anaerobic conditions may decrease but not prevent enterotoxin production.

The time and temperature requirements for production of enterotoxin under laboratory conditions in commercially prepared foods were studied by Segalove and Dack (72) who found

that enterotoxin was produced at 18 C in three days and in 12 hours at 37 C. It was not produced in shorter periods at these temperatures. Production of enterotoxin was not detected in foods incubated at 9 C for 7 days or at 15 C in three days. Incubation at 4-6.7 C did not support enterotoxin production in four weeks. Jensen (48) states in order to avoid hazard from growth of these bacteria, it is well to provide a safety margin by considering the incubation zone to be from 50 F to 120 F.

Laboratory production of enterotoxin is designed to yield a product rich in the emetic principle. The purposes of its production may be to prove a particular strain capable of producing an outbreak, or for research applications. Many varied methods and media have been developed for the laboratory production of enterotoxin.

The first medium used for this purpose was milk (4); sufficient enterotoxin was developed to demonstrate its presence in human volunteers but not in test animals. Dack et al. (25) used veal infusion broth which had been inoculated with the suspected strain of staphylococcus and incubated aerobically for 40 hours. Sterile filtrates from these broth cultures were given orally to human volunteers or parenterally to test animals. The production of enterotoxin in this type of medium was undoubtedly low, although toxicity was demonstrated by use of human volunteers (25, 53) and in South

American monkeys (53). Woolpert and Dack (99), using the observations of Burnet (11), were able to produce filtrates of much higher potency by employing a 1 percent veal infusion agar adjusted to pH 6. The medium was placed in Kolle flasks, the carbon dioxide tension adjusted to 20-25 percent, and the inoculated flasks incubated for 48-72 hours. This method yielded higher hemolysin, dermotoxin, lethal toxin, and enterotoxin production than had aerobic cultures of liquid medium. Similar observations on exotoxin production were reported by Parker et al. (64).

Dolman and Wilson (32) introduced a medium which has been extensively used in the laboratory production of enterotoxin and is composed of proteose-peptone and various salts in semi-solid agar. This medium is placed in petri dishes, inoculated with the suspected strain, the atmosphere adjusted to 30 percent carbon dioxide and 70 percent oxygen, and incubated for 40 hours. After incubation, the semi-solid agar is strained through cheesecloth, the resulting liquid filtered, centrifuged, and passed through Seitz filters to accomplish sterilization. These authors described three techniques of inactivation of alpha and beta hemolysins prior to submitting the filtrate to the kitten test. These techniques consist of (a) neutralizing with specific antiserum, (b) incubating at 37 C with 0.3 percent formalin until hemolysins are no longer detected, or (c) heating the filtrate in a boiling

water bath for 20-30 minutes. Of these methods, the latter has been most often used.

Favorite and Hammon (40) recommended a casein hydrolysate medium for the production of alpha hemolysin. The mixture was placed in pyrex nursing bottles, the organisms introduced into the mixture, and the air replaced by a carbon dioxide-oxygen mixture. They concluded that this medium is capable of supporting the production of enterotoxin similar to its ability to produce hemolysins. Of interest in their results is the fact that no hemolysins were produced in this medium or in infusion broth when incubated on a shaking apparatus.

A method of producing exotoxins by bubbling a carbon dioxide-oxygen mixture through a liquid medium was described by Casman (12). He later described a simply prepared and dialyzable fluid medium which consistently supports the production of enterotoxin and is composed of commercially prepared acid hydrolysate of casein, plus vitamins and salts. A special atmosphere is not required when this medium is used.

The production of enterotoxin in chemically defined media was reported by Surgalla (82). He detected enterotoxin in the supernatants of cultures produced in media containing from 2 to 16 amino acids, and with concentrations of glucose ranging from 0.2 to 20 percent. Growth of the organisms and toxin production were correlated with the amount of available

nitrogen in the medium. Similar observations concerning the available nitrogen were made by Surgalla and Hite (88) in a study of synthetic media. Other methods of enterotoxin production, including shallow culture bottles, deep cultures in turning bottles, and deep aerated cultures were reported by Surgalla et al. (89). North (61) described a medium using ham as the basic ingredient and found that enterotoxin production was supported by this medium. An artificial carbon dioxide atmosphere was found to be unnecessary for enterotoxin production, and it was noted that hemolysin production was stimulated by the presence of proteose-peptone. North observed that there was no correlation between hemolysin production and the ability of a strain to produce enterotoxin.

The use of an enzyme hydrolyzed casein medium for the production of enterotoxin is recommended by Bergdoll.* The inoculated medium is incubated at 37 C on a shaking apparatus operating at 200-250 oscillations per minute, and does not require an artificial carbon dioxide atmosphere. This medium yields a filtrate low in hemolysins and apparently high in enterotoxin after incubation for 18-24 hours. The medium can be used in a shallow layer technique with incubation at 37 C for 72 hours under an atmosphere of 20 percent carbon dioxide, or in a deep culture aeration method for 16-18 hours. Two

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*M. S. Bergdoll, Chicago, Ill. Enterotoxin production methods. Private communication. 1960.

liters of air per minute must be bubbled through each liter of medium, and an antifoam is necessary in this method.

Cultural Methods for the Detection of Enterotoxic Strains of Staphylococci

The desire for a simple laboratory method for the detection of strains of staphylococci which are capable of producing enterotoxin has prompted various investigators to develop media which they claimed would accomplish this purpose. Liquefaction of gelatin in a beef extract gelatin medium was recommended by Stone (72) as proof of an enterotoxin-producing strain. The liquefaction was due to production of gelatinase by the organisms, and a strain which produced this enzyme was said to be "Stone positive". Chinn (20) was unable to differentiate staphylococci incriminated in food poisoning outbreaks from those isolated from infections by the use of Stone's medium. Husseman and Tanner (47) found that Stone's medium was not wholly successful in differentiating enterotoxin-forming staphylococci from non-enterotoxigenic strains. Observations by Burke and Kaplan (10) indicated that the Stone test gave a 65 percent correlation for enterotoxin production with the kitten test. Chapman et al. (19) stated that food poisoning staphylococci could be isolated by plating the suspected material on Stone's gelatin extract agar, rabbit blood agar, phenol red mannitol agar, and brom-thymol blue agar. A typical food poisoning strain would produce yellow or orange

pigment, hemolyze rabbit blood agar, produce orange or deep violet growth on crystal violet agar, grow luxuriantly on bromthymol blue agar, and ferment mannitol. In addition, a typical food poisoning staphylococcus would coagulate human and rabbit plasma.

A modification of Stone's gelatin agar for the detection of food poisoning staphylococci was introduced by Chapman (18). Strains which produce yellow or orange colonies surrounded by a clear zone (indicating gelatinase production), which ferment mannitol, and were coagulase-positive were likely to be food poisoning strains, according to Chapman. Mannitol fermentation was detected by removing pigmented colonies from the surface of the medium and adding a drop of bromcresol purple solution to several areas. A change in color of the indicator in the area from which the colonies were taken indicates mannitol fermentation. This medium is similar to Staphylococcus Medium 110 (17) except for a reduced sodium chloride content and inclusion of a developer in the medium for the detection of gelatinase production. Staphylococcus Medium 110 has been extensively used for the primary isolation of pathogenic staphylococci.

The relationship of the coagulase test to staphylococci associated with food poisoning was studied by Evans et al. (38). They reported that a strain of coagulase-positive staphylococci which failed to produce pigment on Staphylo-

coccus Medium 110 produced particularly severe symptoms when filtrates were fed to monkeys. They observed that 24 coagulase-negative strains isolated from foods were incapable of producing enterotoxin according to their methods. No difference could be detected in the physiology of enterotoxigenic staphylococci and those coagulase-positive cultures which failed to produce sickness in monkeys. Smith (78) found that the coagulase test was the only single reliable criterion for determining the pathogenicity of staphylococci. The use of the coagulase test in determining the pathogenicity of staphylococci was recommended by Christie and Keogh (21) and by Fairbrother (39).

Clark (22) describes a modified potassium tellurite agar which was developed to enumerate coagulase-positive staphylococci in milk. A suitable dilution of milk is smeared on the surface of the agar with a bent glass rod, employing a rotating platform and using a 45 second smearing time. Coagulase-positive, potentially enterotoxigenic staphylococci produce distinct black colonies. Finegold and Sweeney (41) describe a selective medium for the isolation of coagulase-positive staphylococci. This medium uses polymyxin B as an inhibiting agent, and rapid pigmentation and typical colony morphology are features of the medium.

Stritar and Jordan (80) concluded that the food poisoning strains do not constitute a clearly marked group, as there was

no evidence of homogeneity in biochemical, hemolytic, or agglutinative characteristics. They found that the power to provoke food poisoning is not limited to any recognizable variety of staphylococci.

The single criterion that most investigators are agreed upon is that the food poisoning staphylococci are contained within the coagulase-positive or pathogenic group, and that no single cultural method of dividing this group into a sub-group of food poisoning strains has yet been devised (36).

Other Methods Which Have Been Used in Attempts to Detect Enterotoxin or Enterotoxigenic Strains

Bacteriophage typing

The use of bacteriophage typing in tracing the source of strains suspected of causing staphylococcal food poisoning has shown definite promise in recent years. Allison (1) typed 47 strains of staphylococci isolated from food poisoning outbreaks. He found that 64 percent belonged to 6/47 III c, 17.2 percent to type 42 D, and that 18.8 percent could not be typed. He noted that type 6/47 is the most common type found in the nose of man, but that another type, designated 3A, is common in the nose and has not been found to be the cause of staphylococcus food poisoning. Williams et al. (95) found that Group III 6/47 is the most common type found in the feces of normal persons. In a classification system, Williams et

al. (95) designated an outbreak as "Grade A" when the same phage type was isolated from the suspected food and from a victim of the outbreak. An outbreak was designated as "Grade B" when staphylococci were isolated from one or the other but not from both. They found that strains of staphylococci from 87.5 percent of 40 well documented cases of food poisoning fell into phage Group III, and that 78 percent of 41 other cases which were not as well substantiated fell into the same group. Williams et al. (95) emphasize that it is important to remember that there is as yet no evidence of the proportion of Group III which produce enterotoxin, and a strain therefore cannot be assumed to be the cause of food poisoning merely because it belongs to Group III.

Enterotoxic strains involved in food poisoning outbreaks have been successfully traced to their source by Saint-Martin et al. (70), Gillespie (43), Wilson and Atkinson (97), Murphy and Edward (60), Williams et al. (94), and MacDonald (56), among others. The value of tracing a strain to its source is emphasized by Wilson (98), who observed that an enterotoxigenic strain persisted in the nose of a normal person for three years without loss of its toxin producing ability. An excellent example of using phage typing to trace a strain to its source, and demonstrating its enterotoxigenicity by biological means, is reported by Prince and Crowell (66). The value of the combination of these two methods is evident.

Gel-diffusion technique

A modification of the gel-diffusion technique of Oudin (62) was utilized by Surgalla et al. (83) in studies of purified enterotoxin. These workers used the rooster, monkey, and rabbit for preparation of antiserum, and studied enterotoxin in various phases of its purification. They were able to demonstrate the presence of several bands of precipitate in crude filtrates produced in a pancreatic digest medium, but were unable to determine which band represented the enterotoxic substance. A monovalent antiserum was produced in rabbits by Bergdoll et al. (8) which gave a single band of precipitate with its antigen. Casman (14) presented evidence of at least two serological types of enterotoxin by the use of the gel-diffusion technique. Verification of Casman's observation was made by Sugiyama et al. (81), who used an agar plate modification of the gel-diffusion technique. The basic agar-diffusion technique, with its various modifications, is of value in the serological study of enterotoxin, but is limited in its application because of the varied antigenic properties of enterotoxin produced by different strains of staphylococci.

Chick embryos

Placement of enterotoxin onto the surface of the chorioallantoic membrane and injection directly into the yolk sac

produced no apparent action on chick embryos (44).

Infrared spectrophotometry

Detection of enterotoxin in boiled or lypholized preparations was reported by Levi et al. (55) with the use of infrared spectrophotography. This observation has not been confirmed.

Agglutination by dilute horse serum

A fundamental difference between enterotoxigenic and non-enterotoxigenic strains of staphylococci was described by Slocum and Linden (77). Suspensions of living organisms were mixed with varying dilutions of normal horse serum and incubated at 37 C for 24 hours. Non-enterotoxic strains were agglutinated by the horse serum in very high dilutions (1-160 to 1-320) while enterotoxigenic strains were only partially agglutinated by dilutions of 1-80 and 1-160.

Action of enterotoxin on isolated rabbit small intestine

A specific action by enterotoxic filtrates on isolated rabbit small intestine was described by Anderson (2). Segments of intestine were suspended in a mixture of Ringer's solution and the filtrate being tested. A total volume of 60 ml of the mixture was maintained in the container. A characteristic increase in the tone of the intestine was noted when

not less than 4 ml of an enterotoxic filtrate was added, while no response was obtained from non-toxic filtrates or from control media. These findings were not substantiated by Anderson et al. (3), who found that the action reported was due to alpha hemolysin and was not specific for filtrates from food poisoning strains. Further confirmation of the observation that the action was due to alpha hemolysin rather than enterotoxin was made by Kelsey and Hobbs (54).

Direct testing of foods for coagulase

A suggestion for the direct determination of coagulase in foods was offered by Chapman (16) for use in those cases when no viable staphylococci are present at the time of examination of a suspected food. A loop-full or more of the food to be tested is emulsified in 0.5 ml of Bacto tryptose phosphate broth, and 0.5 ml of citrated or oxalated rabbit plasma added. Oxalated or citrated human whole blood may be used, and incubation is recommended up to seven hours. This test is not designed to detect enterotoxin, but to determine if coagulase-positive staphylococci have been present in the food.

Flocculation reaction

A flocculation reaction between enterotoxin and antiserum obtained from a horse immunized with pooled filtrates from

various strains of staphylococci was described by Dolman and Wilson (32). Absorption of this antiserum by filtrates from strains of staphylococci known to be non-enterotoxigenic enabled these investigators to demonstrate a specific flocculation zone which they attributed to enterotoxin and its antibody. A specific flocculation zone was also identified for beta hemolysin and its antibody.

Biological Methods for the Detection of Enterotoxin

Use of human volunteers

The availability of a reliable and satisfactory method of detecting enterotoxin has posed a major problem since the first report of staphylococcal food poisoning (4). Barber resorted to using human volunteers, including himself, to demonstrate the presence of a toxic substance in milk drawn from a cow suspected of being the cause of numerous cases of acute gastroenteritis. He was successful in his attempts to show the presence of a toxic agent by this method, and observed a quantitative variation in the reaction of the volunteers who consumed the suspected milk. An apparent acquired resistance to this substance was observed, since the families who regularly consumed the milk were not affected while visitors suffered attacks of gastroenteritis.

Dack *et al.* (25) utilized human volunteers to demonstrate the presence of a toxic substance in a food item being in-

vestigated (a Christmas sponge cake), and in filtrates produced from strains of Staphylococcus aureus isolated from the cake. Use of human volunteers in studies of enterotoxin has been made by many investigators, among whom are Dolman (29, 31, 34), Jordan and McBroom (53), Fulton (42), Shaugnessey and Grubb (74), Minett (59), Davison and Dack (27), and Jordan and Burrows (52).

The human volunteer is of course the most reliable means of detecting enterotoxin, but obtaining sufficient numbers of volunteers to conduct research on a large scale is almost an impossibility. Efforts have therefore been made to find suitable test animals to facilitate detection of toxic filtrates and toxin producing strains.

Use of monkeys as test animals

The first report of attempts to demonstrate enterotoxin by using monkeys yielded negative results. Barber (4) administered large quantities of milk cultures of the suspected organism, which produced little or no symptoms. Dack et al. (25) obtained negative results when monkeys were fed saline suspensions of a cake, samples of which subsequently caused food poisoning symptoms in human volunteers. Monkeys showed no ill effects when fed infected milk. Jordan and McBroom (53), using filtrates which were toxic to human volunteers, demonstrated that South American monkeys were susceptible to

the action of enterotoxin. They administered 5-20 ml of a filtrate from broth cultures of staphylococci to juvenile red spider, black spider, black howler, and white faced monkeys, and symptoms similar to those in human volunteers were observed in five out of 13 monkeys. Marked diarrhea and loss of appetite developed, and vomitus was found on the floor of the cage in one instance.

With the use of an improved method of experimentally producing enterotoxin, Woolpert and Dack (99) demonstrated that Macaca mulatta are susceptible to the action of this substance. They described the symptoms as follows:

> The symptoms are characteristic. Usually about an hour and a half after feeding, the animal becomes somewhat pale; increased salivation and swallowing motions are noted; the animal may bend over in the corner of its cage with its forearms folded across the abdomen. The pallor and salivation increase, there is usually a short premonitory period marked by regurgitation. At about the beginning of the third hour, vomiting sets in. This may be mild, but is often profuse and projectile. Paroxysms of vomiting are apt to recur over a period of an hour or so. During this time the animal is very pallid and abject. Diarrhea may be a marked feature; in two cases in which large amounts of toxic material were fed, there was profuse diarrhea without vomiting. Similar results have been noted in man. Recovery sets in rather abruptly after several hours, and on the following day the monkey may appear normal except for loss of weight. (99, pp. 11-12)

A wide variation in susceptibility of 1000 Macaca mulatta monkeys injected intra-gastrically with staphylococcal enterotoxin was noted by Surgalla et al. (84). They recommended that in testing a strain for enterotoxin production, emesis

be observed in at least two different animals before considering the strain as positive. In the interest of economy, monkeys which have previously been used should be tried first. If these should react positively with emesis, then the strain can be considered positive, but if they react negatively, the sample should be administered to unused animals before calling the strain negative. Surgalla and Dack (86) regard two out of four animals exhibiting emesis after the consumption of a filtrate as a positive reaction.

Jordan and Burrows (51) injected 5 ml of a saline solution of acid-ether extract of a potent filtrate intravenously in monkeys, and the usual signs of acute distress of the gastro-intestinal tract were noted. Davison et al. (28) concluded that the intravenous injection of a filtrate appears to offer certain advantages and is a more sensitive test. Elek (36) questioned this approach in view of the complexity of the media in which the organisms are grown.

Use of small laboratory animals

Attempts to use small laboratory animals to detect enterotoxin have been made by several investigators. Barber (4) injected living staphylococci subcutaneously into guinea pigs, producing abscesses in some cases. Dack et al. (25) injected rabbits intravenously with sterile filtrates, causing a profuse watery diarrhea and death of the rabbit in 12 hours

in one instance. Jordan and Burrows (51) failed to produce reactions in guinea pigs when a saline solution of an acid-ether extract was given intravenously. Borthwick (9) conducted tests on rabbits and guinea pigs in attempts to demonstrate staphylococcal toxins, but did not state if the strains used in his experiments were known enterotoxin-producing organisms. He adjusted the acidity of the animals' stomachs to pH 7.3 with 5 percent $NaHCO_3$, or 0.1 N HCl, and found that the majority of them were not affected, but that certain of them succumbed rapidly after administration of staphylococcal toxin. One guinea pig died within an hour after administration of the toxin. Control experiments were performed on guinea pigs by adjusting the gastric contents of one animal to pH 7.8, and the other to pH 6.8, followed by administration of staphylococcal toxin. The health of these animals was not impaired. Guinea pigs were found to be susceptible to the introduction of filtrates if the rectum was first neutralized to pH 7.3, while negative results were obtained if neutralization was not done.

The prominent post-mortem features in the animals which died were intense congestion of the mucosa of the stomach and duodenum, associated with small hemorrhages in the tissue, effusion of blood into the lumen, and congestion of various internal organs with hemorrhage. The results of Borthwick's observations were not confirmed by Dolman et al. (32), who

observed that guinea pigs and rabbits gave no detectable symptoms when injected intra-abdominally or intravenously with ten minimal kitten reacting doses. Mice did not react to more than twice the minimal dose required to evoke violent vomiting in a 500 gram kitten. Vomiting was not evoked when enormous doses of apomorphine, histamine, mineral salts, and castor oil were given to guinea pigs. Corpening and Foxhall (23) fed guinea pigs portions of a cake suspected of having caused staphylococcal food poisoning and no ill effects resulted.

Jones and Lochhead (49) report the use of guinea pigs in attempts to demonstrate enterotoxin, but did not publish the results of their experiments. Casman (13) reports that rabbits exhibit a marked, though variable, reaction to purified enterotoxin. When injections of this substance are made intravenously with one or two times the amount required to cause emesis in cats, some rabbits die within 24 hours. Rabbits, guinea pigs, and mice are used to demonstrate the lethal and dermonecrotic properties of staphylococcal filtrates (64, 72, 92).

Use of the frog (Rana pipiens)

The use of the frog to detect enterotoxin was reported by Robinton (68), who prepared filtrates by Dolman's method from known positive and negative enterotoxin-producing strains

of staphylococci. Filtrates were dropped directly into the frog's stomach with an eye-dropper, and a positive test was considered to be a spasm with reverse peristalsis of the stomach. The frog was reported to give swallowing motions, to have changes in the tone of the skin color, and to assume a characteristic sitting position, expelling mucus from the mouth. A frog could be used for only one test and actual vomition was not a symptom. In a continuation of her previous work, Robinton (69) described a decerebration technique, with subsequent testing of the frog with staphylococcal filtrates. She stated that anti-peristalsis of the stomach in decerebrated frogs occurred only when emetic or enterotoxic substances were administered, and that this reaction occurred with each frog tested. She found that this reaction occurred within 30 minutes after giving the filtrate, and that the use of concentrated filtrates could shorten the time to one minute.

The non-specificity of the frog test was attacked by Eddy (35), who used non-toxic material and noted actual vomition. Her conclusions were that the occurrence of a spasm in frogs fed staphylococcal culture filtrate products is either a non-specific response to various substances of an especially viscid nature which is more readily elicited at certain seasons, or is a presponse at a particular season to some stimulus not yet determined. Other workers (24, 58, 84, 91) have demonstrated that the use of frogs is not a satisfactory

method of detecting enterotoxin.

Use of young pigs

The use of young pigs as test animals for the detection of enterotoxin was introduced in 1943 by Hopkins and Poland (46). These investigators found that oral administration of active filtrates was unsatisfactory and adopted intraperitoneal injection as the most satisfactory approach. Filtrates in the amounts of 3 to 4 ml per kilogram of body weight were administered, and it was noted that discomfort resulted soon after the injection. Periods of restlessness alternated with drowsiness, and vomition was preceded by convulsive movements of the abdominal wall. Only vomition was considered to be a positive reaction. Vomiting resulted in most cases in 30-90 minutes after injection, but was sometimes delayed to nearly 3 hours. In a comparative test, pigs were found to react to a filtrate while monkeys did not respond. The strains of staphylococci used in the study included a known enterotoxin producer and other suspected strains of various origin. Control preparations were autoclaved toxic material, uninoculated filtered medium, and filtrates from non-toxic strains. All control preparations gave negative reactions.

Use of chimpanzees

A comparison of the susceptibility of monkeys and chimpanzees to oral administration of partially purified entero-

toxin was reported by Wilson (96). Toxic levels were determined in 30 Macaca mulatta monkeys, and were compared with the results obtained from feeding the toxic substance to five chimpanzees. Wilson found that expressing the respective toxin doses as milligrams per kilogram, the chimpanzee appears approximately 10 times as susceptible to the action of enterotoxin as is Macaca mulatta.

Use of cats and kittens

The use of kittens was reported by Barber (4), who fed them large quantities of milk cultures of organisms with few or no symptoms resulting. Dack et al. (25) fed infected milk to kittens with no ill effects. Tanner and Ramsey (90) fed milk cultures of living staphylococci to kittens and caused definite symptoms in two out of four animals in less than a week. One had severe diarrhea with bloody stools, while the other passed considerable mucus. The two remaining kittens had persistent diarrheal stools until the inoculated milk was removed from the diet. They then used three week old kittens which had received only their mother's milk, and fed them 10 ml of a sterilized 24 hour milk culture of staphylococci for four days. After the fourth feeding, all had watery stools, one vomited, and another had mucus in its stool, while the control was normal. Jordan and Burrows (51) found that kittens showed no ill effects when injected intravenously with

saline solutions of acid-ether extracts of a toxic filtrate.

Dolman et al. (34) found that adult cats reacted with projectile vomiting 2 1/2 hours after they consumed 50 ml of a toxic filtrate. The cats passed several loose stools a few hours after consumption of the filtrate. Similar results were obtained with 10 ml of a filtrate which had been concentrated by evaporation under low pressure. Jones and Lochhead (49) used a pipette feeding method utilizing both kittens and adult cats and produced vomiting and diarrhea with active filtrates. The typical symptoms observed in kittens to which toxic filtrates were fed was at times so slight that they might easily have passed unnoticed. In many instances the kittens were observed to regurgitate, but did not actually vomit. Positive reactions generally occurred 2 to 5 hours after feeding toxic filtrates, while the greatest number of positive reactions were observed between 3 and 4 hours after consumption of the filtrate. Adult cats were fed up to 25 ml of an active filtrate, and reacted in many instances in a manner similar to that of kittens. Minett (59) administered toxic filtrates to monkeys and adult cats in a parallel feeding test, and noted that the susceptibility of the two species was of much the same order. Matheson and Thatcher (58) found that adult cats did not react to oral administration of 150 times the amount of enterotoxin which caused vomition by the parenteral route.

A parenteral method of detecting enterotoxin in active filtrates was reported by Dolman et al. (34), who used kittens 6 weeks to 3 months old, and administered filtrates intraperitoneally. The hemotoxins present in the filtrates had been inactivated by neutralization with specific antisera, formalization, or by heating in a water bath at boiling temperature for 20 to 30 minutes. These authors described the symptoms resulting from the injection of an active filtrate as follows:

> In a positive reaction, the kitten displays lassitude, weakness and unsteadiness, occasionally interspersed with bouts of restlessness, commencing a few minutes after injection. Strong peristaltic movements may be noted which culminate, in 15 minutes to 1 1/2 hours, in the first of a series of attacks of retching and vomiting, which may recur over a period of an hour or more. Several loose stools may meanwhile be passed, and occasionally the diarrhea is a more conspicuous feature than the vomiting. Passage of a single small stool shortly after the injections may be disregarded. Control kittens should remain unaffected. (33, p. 70)

Davison et al. (28) injected kittens intravenously and intracardially with toxic filtrates in a study of specific antiserum prepared against enterotoxin. Emesis was not prevented when the antiserum and enterotoxin were mixed and injected intracardially, but was prevented when the mixture was injected intraperitoneally.

The mechanism of the vomiting reaction in cats was thoroughly documented by Bayliss (5), who administered 1.0 ml per kilogram of boiled filtrate intraperitoneally or intravenously

and described the symptoms in cats from the time of injection to recovery of the animal. He noted that an excessive dose may produce progressive symptoms ending in death, but that in most cases the animal appeared normal in all respects the following day. In those animals which died from toxin or from operative procedures, the post mortem examination showed an excessive amount of mucus in the entire gastro-intestinal tract, a contracted urinary bladder, and a distended gall bladder. He found that enterotoxin has no direct action on isolated strips of cat intestine, and that emesis never occurred after surgical destruction of the vomiting center. Emesis resulted from intravenous or intraperitoneal injections, but failed to appear following oral, subcutaneous, or intramuscular administration of a toxic filtrate. His conclusions were that the action of staphylococcal enterotoxin on peripheral sensory structures is of greater importance in the initiation of emesis than the direct action of this substance on the vomiting center. Bayliss used Dolman's technique of producing the filtrates, and inactivated the alpha and beta hemolysins by heating the filtrates in a boiling water bath for 20 minutes. Dack (24) states that although Bayliss' experiments were controlled, the amount of surgery and trauma involved in them makes further work desirable to establish the mode of action of enterotoxin. Other investigators have noted that excessive amounts of enterotoxin may result in the

death of the test cat or kitten (15, 32, 59, 72), while Matheson and Thatcher (58) state that in their hands, death of a cat has never resulted from a heated preparation.

A modification of the kitten test was introduced in 1940 by Phatak and Pentler (65), which consisted of anesthetizing the animals with sodium pentobarbital and injecting them intraperitoneally with the heated filtrates. The kittens had been fed a small meal about 30 minutes prior to administration of the anesthetic. Only definite vomiting with expulsion of vomitus between 20 and 90 minutes after injection of the filtrate was considered positive. They found that a large number of animals could be handled in this manner, but that it was necessary to keep the kittens warm and to turn them over every hour to avoid hypostatic congestion. These investigators claimed reduced sensitivity but increased specificity with this method, and found that it was important to use 50 percent larger doses of filtrate than when working with conscious kittens.

An improved cat test was introduced by Hammon (44). The improvements over the kitten test consisted of using the intravenous injection of filtrates rather than the intraperitoneal route, and the use of mature or adult cats instead of young kittens. He found adult cats easier to procure, maintain, and handle than were young kittens, and that they were more susceptible to intravenous administration of an active

filtrate than were young kittens injected intraperitoneally. A moderate sized meal eaten shortly before the inoculation was found to increase the effectiveness of the vomiting stimulus. Non-specific vomiting occurred less frequently than with kittens, and adult cats would always vomit on the first injection of a reasonable amount of an enterotoxic filtrate. He found that the train of symptoms differed little from those described by Dolman et al. (32) following intra-abdominal inoculation of kittens with a toxic filtrate. The usual dosage of 2 ml of filtrate for the first injection produced a severe reaction, the same amount for the second injection normally produced a less severe reaction, and an increase of 50 to 100 percent for the third inoculation gave a moderate or severe reaction. An animal was seldom used the fourth time and the results of a negative test was not accepted as final unless repeated on at least two previously unused animals in a minimal dose of 3 ml. Dolman (30) verified Hammon's contention in respect to the greater sensitivity of the intravenous route of administration.

Observations by Rigdon (67) revealed that kittens which were injected intra-abdominally with uninoculated control medium vomited within a period of 30 minutes. The control medium and toxin preparation used were prepared by a commercial laboratory without the source of the organisms being stated. Rigdon heated the toxin for 2 hours at 100 C, and

found that none of the kittens injected with this preparation vomited. He then injected the unheated toxin, and all were dead within 4 hours. Singer and Hagen (75) found that the hemolytic exotoxins present in staphylococcal filtrates could be demonstrated after boiling for more than 6 hours. They found that the uninoculated medium caused vomiting in about 25 percent of the cases, and that filtrates presumed to contain enterotoxin failed in nearly half the cases to induce emesis. They concluded, as did Rigdon (67), that the kitten test was unreliable for detection of enterotoxin. Slanetz et al. (76) found that beta toxin was not inactivated by boiling for 30 minutes, and considered that beta hemotoxin and enterotoxin might be the same entity.

The validity of the kitten test was questioned by Fulton (42) who reported that a kitten-positive extract was non-toxic by mouth to a susceptible human volunteer, and that a kitten-negative extract was toxic to the same human volunteer. He concluded that the kitten test was not specific for staphylococcal enterotoxin but was rather an index of peritoneal irritation. Fulton found that boiling filtrates for 20 minutes destroyed most of the alpha lysin, leaving insufficient amounts to cause vomiting, but that there was sufficient beta toxin remaining to induce this effect. He concluded that beta lysin or some toxin closely associated with it is the responsible factor in the kitten test.

Surgalla and Hite (87) reported that vomiting reactions were not obtained following injections of monkeys and kittens with boiled filtrates from beta hemolytic strains of staphylococci, and that unheated preparations of these strains were non-enterotoxigenic when fed to monkeys. They concluded that staphylococcal enterotoxin and hemolysins are separate and distinct entities.

Matheson and Thatcher (58) undertook a reappraisal of the validity of the kitten test, using mature or adult cats and administering filtrates by the intraperitoneal route. They found that not a single "false positive" reaction occurred following the injection of four different types of control medium. In an exhaustive study, they concluded that the presence of an emetic principle in a staphylococcal filtrate can readily be determined by the intraperitoneal injection of cats, thereby confirming the work of Dolman et al. (32).

In a second report (92) Thatcher and Matheson investigated the abilities of specific lysin (alpha, beta, and delta) to induce vomiting in cats. They reported that there is no evidence that alpha lysin survives boiling to any degree to cause confusion with any other emetic principle, but that relatively large amounts of beta lysin may behave similarly to alpha lysin to the extent of causing death. Beta lysin did not occur in filtrates subjected to boiling for 30 minutes in amounts sufficient to cause vomiting in cats, although they

found that smaller amounts (16 units per kilogram or greater) in filtrates heated to less than boiling may cause emesis in the presence of subemetic amounts of enterotoxin. Delta lysin was found to be incapable of causing emesis. They specifically endorsed the Dolman kitten test, and substantiated reports of many investigators that enterotoxin is a specific substance distinct from known lysins.

Use of dogs

A few reports on the use of dogs to detect enterotoxin have appeared in the literature. Barber (4) administered milk cultures of living staphylococci to puppies with no ill effects. Dack et al. (25) fed infected milk to puppies and no adverse symptoms were noted. Jordan and Burrows (51) administered a saline solution of an acid-ether extract of enterotoxin intravenously in dogs and observed no ill effects. Dolman et al. (34) observed that 3 to 5 ml of an active filtrate injected intra-abdominally in a litter of collie puppies evoked a response similar to that shown by kittens. Minett (59) orally administered 20-40 ml of an active filtrate mixed with an equal volume of pasteurized milk to dogs 6 to 12 months old, and only one out of five vomited when treated by this method. In another experiment, Minett used a filtrate which had caused toxic symptoms to appear in a human volunteer when 4 ml were consumed. He gave 6 ml of this filtrate orally

to a dog and observed no symptoms on the day of feeding, but in 24 hours signs of a slight intestinal disturbance appeared, with the stools appearing dull green and exceedingly moist. During the next 6 hours the diarrhea increased and the stools were very watery. Four days later the same animal was given 20 ml of another filtrate, and symptoms of intestinal disturbances were again observed in 24 hours. Minett concluded that the feeding of filtrates to dogs was not a useful means of detecting enterotoxin.

The intra-abdominal injection of puppies with a staphylococcal toxin prepared by a commercial laboratory was reported by Rigdon (67) who heated the toxin at 100 C for 2 hours. The puppies were drowsy for approximately 4 hours after the injection and none receiving the heated toxin vomited, while all receiving the uninoculated control medium vomited within 30 minutes after the injection. The animals were injected the following day with the unheated toxin and all were dead after 4 hours. The pathological changes were similar to those occurring from the intravenous injection of staphylococcal exotoxins.

The intravenous administration of heated filtrates in dogs was reported by Dekse (91), who found that as much as 6 ml of a non-toxic filtrate did not produce vomition in healthy young animals. On the other hand, intravenous injections of heated filtrates prepared from known enterotoxi-

genic strains always caused vomiting at varying intervals, and symptoms other than depression were absent. Bowel movements were usually noted shortly after injection but these were normal in consistency. Vomiting was observed in from 5 to 40 minutes, while no reactions were observed following administration of filtrates from non-toxic strains or from uninoculated control medium. The filtrates were prepared by Dolman's method, and the hemolysins inactivated by heating the filtrates in a boiling water bath for 20-30 minutes. Tekse concluded that the use of dogs as test animals offered more promise than formerly presumed, and recommended that further work be accomplished on this subject.

MATERIALS AND METHODS

Source of Strains of Organisms

The strains of Staphylococcus aureus used in this study were obtained in pure culture, and were of three types: known enterotoxigenic, known non-enterotoxigenic, and strains unknown as to their enterotoxigenic ability. The strains of staphylococci used in the first phase of the study were numbers 137, 161, 184, 269, 273, and 305. These cultures were received from Dr. M. S. Bergdoll, and the only information received with the cultures was that there were three known enterotoxigenic and three known non-enterotoxigenic strains in the group. The purpose of this procedure was to determine the value of the dog as a test animal under conditions similar to those which would exist in testing a strain of staphylococcus isolated from a food suspected of having caused food poisoning. Appendix A gives a complete listing of all the strains used in the study.

Structure of the Study

This study was divided into a preliminary phase and a final phase. In the preliminary study, the initial problem to be resolved was to determine if dogs would react to enterotoxin in filtrates which had been boiled for 30 minutes to inactivate the hemolysins. The coded cultures referred to

above were used in the first part of this phase. After a sufficient number of tests had been made with these strains to determine that dogs did react to enterotoxin in the filtrates, a number of strains of unknown enterotoxigenic ability were tested in an attempt to find one which produced enterotoxin. During the preliminary phase, cats were used to make comparisons with the results obtained in dogs. In most instances two or more cats were used per test, but in some cases only one cat was employed. At least three dogs were used for each test in the preliminary study.

The final phase was devoted to a further study of the reactions of dogs to filtrates from both enterotoxigenic and non-enterotoxigenic strains of staphylococci. It was deemed desirable to determine the minimum amount of enterotoxic filtrate required to cause emesis in dogs as well as to determine the most effective level which would cause this effect. The filtrates from non-enterotoxigenic strains were used to determine the amount of filtrate, within practical limits, which could be tolerated without exhibition of symptoms by the animals. To accomplish this purpose, 10 known enterotoxigenic and nine known non-enterotoxigenic cultures of Staphylococcus aureus were obtained. Filtrates were produced and prepared from these 19 strains and used for test purposes.

Preliminary work had tentatively established the level at which emesis occurred following the injection of an entero-

toxic filtrate at .125 ml per pound of body weight. Filtrates from enterotoxigenic strains were injected slightly above, at, and below this level. Filtrates from non-enterotoxigenic strains were injected on and well above this level. Five dogs were used for testing each strain, and the body weight of each dog was determined and recorded.

Cultural Characteristics of Strains

Coagulase production of the strains was determined by inoculating a loop-full of a broth culture of the organisms into 0.5 ml fresh rabbit plasma which had been diluted 1-5 in sterile physiological saline. Horse plasma was used in the same manner. The tubes were incubated at 37 C and read for coagulation at 2 hours, 4 hours, and after overnight in the incubator. The final results were recorded as positive or negative.

Pigment production of the strains was determined by streaking a loop-full of a 24 hour broth culture of the respective strains onto the surface of Staphylococcus Medium 110. These plates were incubated aerobically at 37 C for 24 hours, and then at room temperature for another 24 hours. Color of the colonies was recorded as white, yellow, or golden.

Hemolytic patterns of the strains of staphylococci used were determined by the lysis of erythrocytes from different

species of animals, using 5 percent blood agar plates. Whole citrated sheep, horse, and rabbit blood were washed three times in sterile physiological saline to remove any antitoxins which might have been present in the blood.* Blood from each species was added to cooled Bacto tryptose blood agar base which had been prepared in accordance with the manufacturer's directions. The plates were poured with approximately 15 ml of the medium and allowed to harden. Rabbit and sheep blood agar plates streaked with a loop-full of the respective strains were incubated aerobically, inoculated sheep and horse blood agar plates were incubated under 10 percent CO_2, and inoculated sheep blood agar plates were incubated anaerobically. The temperature of incubation for all plates was 37 C.

The appearance of a large zone of complete lysis with hazy margins on rabbit and sheep blood agar plates which had been incubated aerobically was interpreted to be due to alpha hemotoxin. The appearance of a large sharply defined zone of incomplete lysis on sheep blood agar plates incubated aerobically was due to beta hemotoxin. Beta hemotoxin also exerted an action on sheep blood agar plates incubated under 10 percent CO_2 and under anaerobic conditions. A small zone of complete lysis with sharply defined margins on horse blood

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*The author is indebted to Dr. R. A. Packer for the gift of sheep blood and to Dr. W. W. Kirkham for the gift of horse and sheep blood used in this phase of the study.

agar plates incubated under 10 percent CO_2 was due to delta hemotoxin. A large zone of complete lysis produced by coagulase-negative strains on rabbit or sheep blood agar plates was interpreted to be due to epsilon hemotoxin. The interpretation of hemolytic patterns was based on the work of Elek and Levy (37) and Marks and Vaughn (57).

Production of Enterotoxin

The medium used for enterotoxin production was prepared as follows:

Amigen powder*	20 grams
Niacin	10 milligrams
Thiamin	0.5 milligram
Distilled water	1 liter

The medium was adjusted to pH 7.7 with NaOH and distributed in 90 ml amounts in 500 ml Erlenmeyer flasks, and in 10 ml amounts in test tubes. Flasks and tubes were plugged with cotton and sterilized in the autoclave at 121 C for 15 minutes.

Preparation of Filtrates

The strain of staphylococcus being tested was inoculated into tubes containing 10 ml of the Amigen medium and incubated at 37 C for 24 hours. After two or more such passages the

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*Amigen is a trade name for casein protein hydrolysate powder manufactured by Mead Johnson and Company, Evansville, Indiana. The author is indebted to Dr. R. C. Ellingson for the supply of powder used in the study.

entire contents of a tube were used to inoculate the flask containing 90 ml of Amigen medium, effecting a 10 percent inoculation by volume. The flasks were then placed on an Eberbach shaking apparatus (Model 55) and the speed adjusted to approximately 250 excursions per minute. Incubation was at 37 C for 24 hours.

After the incubation period, the resulting culture was centrifuged at 2000 RPM for 30 minutes to sediment the organisms. The supernatant fluid was filtered through an EK Seitz filter pad, using negative pressure to accomplish filtration. The resulting filtrate was stored in a sterile 4-ounce medicine bottle placed in the refrigerator at approximately 20 C. Filtrates were normally used within one week. The first filtrate produced from a strain was designated as filtrate A, while subsequent filtrates were designated B, C, D, etc.

Inactivation of Hemolysins

Five ml of the untreated filtrate were removed from the medicine bottle and reserved for titration of hemolysins. The remainder was adjusted to pH 7 with 5 percent acetic acid and dispensed in 5 ml amounts in sterile screw-cap test tubes. These tubes were placed in a water bath and the temperature brought up to boiling. A thermometer was placed in a tube of filtrate (without cap) in order to determine when the filtrate reached 100 C. A cover was placed over the water bath to allow the temperature to reach this point and the filtrates

were maintained at 100 C for exactly 30 minutes. The tubes were then removed from the water bath, allowed to cool, the caps tightened, and stored in the refrigerator. The filtrate in the tube which held the thermometer was discarded.

Titration of Hemolysins

Sheep erythrocytes were washed three times in sterile physiological saline, and a 2 percent suspension prepared. The third centrifugation was timed for exactly 8 minutes in order to obtain uniform density of cell suspension with each preparation. A 2 percent suspension of rabbit red blood cells was prepared in a similar manner.

A series of doubling dilutions of the unheated filtrate was made using 2.5 ml of physiological saline in each tube. Two and one-half ml of the unheated toxin were placed in the first tube, mixed well, and half the volume transferred to the second tube. This procedure was carried out until a dilution of 1-128 was reached. One-half ml of each dilution was transferred to a clean test tube; two such series of tubes were prepared. One-half ml of the 2 percent rabbit red blood cell suspension was added to one series of tubes, and 0.5 ml of the 2 percent sheep red blood cell suspension was added to the other series. A final concentration of 1 percent red blood cells was present in the tubes. A control tube for each type of cells was prepared by adjusting the concentration to 1 per-

cent with saline.

Hemolysin titration of the heated filtrates was conducted in exactly the same manner as for the untreated filtrates. The filtrate remaining in the tube from which the sample was taken was discarded.

The tubes containing the hemolysin tests were incubated in a water bath at 37 C for one hour, and the initial hemolytic titer determined. The end-point was taken as the last tube showing hemolysis of approximately 50 percent of the cells. Alpha hemotoxin titers were determined by lysis of rabbit red blood cells, and were recorded after the one-hour incubation period. Beta hemotoxin titers were determined by lysis of sheep red blood cells after an additional hour in the refrigerator.

Performance of the Test in Dogs

No attempt was made to select any particular size or type of dog as a test animal, although most animals used in the first phase of the study were in the 20-30 pound range. In the final phase of the study dogs which varied in weight from 7 to 44 pounds were used. Only dogs in obviously good health were used. Dogs selected to receive control preparations or test filtrates were given a small meal 30 minutes prior to injection. Animals which refused to eat and old dogs were not used for test purposes. Three dogs per trial were usually

used in the preliminary study, and five dogs per trial were used in the final study. Dogs were not usually used more than once in the preliminary study, but were used as many as three times in the final study.

The findings of Tekse (91) were used as a basis for establishing an initial dosage of filtrate. Five ml were first administered intravenously to dogs weighing 20-30 pounds and emesis was found to result in 40 to 120 minutes with an enterotoxic filtrate. The reactions caused by this amount were quite severe and the dosage was accordingly adjusted downward. It was found that 2.5-3.5 ml of an enterotoxic filtrate administered to a 20-30 pound dog caused emesis, while no reactions were observed with a non-enterotoxic filtrate.

The technique of injection consisted of properly restraining the dog, clipping the hair over the cephalic vein, disinfecting the area with alcohol, and injecting the control preparation or test filtrate very slowly intravenously. The preparations were first heated to body temperature in warm water, since they had been stored in the refrigerator. The test animals were observed continously for a 2 1/2-3 hour period and at intervals thereafter. An attempt was made to observe each animal 5 hours after the injection but this was not possible in some cases. They were always observed the following morning.

The criterion established for a positive reaction was

emesis. Symptoms such as bowel evacuation, urination, nervousness, depression, and exhibition of anxiety were noted but were not considered conclusive proof of reaction to enterotoxin. For the purpose of this study, a positive reaction entails the observation of emesis, while a negative reaction is one in which this action did not occur.

Performance of the Cat Test

The cat test was used for comparison purposes and was performed essentially in accordance with Hammon's technique (44) except that smaller amounts of the boiled filtrate were administered. In addition, the filtrates were injected in the cephalic vein as described by Tekse (91). Both adult cats and kittens were utilized and in a few instances when the animals proved extremely irretractable, intraperitoneal rather than intravenous injections were made. As with dogs, a small meal was given 30 minutes prior to injection, and cats were observed for the same period described for dogs. Cats were normally used four times for test purposes; it was felt that results might not be valid if this number was exceeded.

EXPERIMENTAL RESULTS

Cultural Characteristics

The results of studying the cultural characteristics of the strains of staphylococci used are presented in Table 1. It is significant to note that all enterotoxigenic cultures produced coagulase and alpha hemotoxin. Coagulase-negative cultures produced a hemolysin similar to that described by Elek and Levy (37) which they designated as epsilon. It is also interesting to note that some enterotoxigenic strains produced white pigment on Staphylococcus Medium 110. One culture (number 137) which produced severe reactions in dogs and cats produced white pigment. No other outstanding features were noted in the study of cultural characteristics.

Hemolysin Production and Inactivation

Table 2 shows the results of titrating the untreated and the boiled filtrates used in this investigation. Since these filtrates were produced from cultures incubated aerobically on a shaking apparatus, hemolysin production was found to be extremely low. It is fortunate that this was the case, for no difficulty was encountered in inactivating the small amounts of hemolysins present. Differences were observed in hemolysin titers between production lots of the same strain but these differences were not greater than one dilution.

Table 1. Cultural characteristics of strains of staphylococci used in the study

Strain	Hemolytic pattern	Pigment color	Coagulase	Enterotoxin production
137	alpha, beta, delta	white	positive	positive
161	alpha, beta, delta	golden	positive	positive
184	alpha, beta	golden	positive	negative
269	alpha, beta, delta	white	positive	negative
273	alpha, beta, delta	golden	positive	positive
305	alpha, beta, delta	golden	positive	negative
8	alpha, delta	golden	positive	negative
DFI 12	alpha, beta, delta	golden	positive	negative
DFI 1310	alpha, beta, delta	golden	positive	negative
Heidi	alpha, beta, delta	golden	positive	negative
Wood 46	alpha, beta, delta	white	positive	negative
Slayton	alpha, beta, delta	white	positive	negative
Baird	alpha, beta, delta	golden	positive	negative
Davis 3	alpha, beta, delta	golden	positive	negative
307	alpha, delta	golden	positive	positive
C 246-3A	alpha, beta, delta	golden	positive	positive
87	alpha, beta, delta	golden	positive	negative
216	epsilon	white	negative	negative
251	epsilon	white	negative	negative
280	epsilon	white	negative	negative
227	epsilon	yellow	negative	negative

Table 1. (Continued)

Strain	Hemolytic pattern	Pigment color	Coagulase	Enterotoxin production
204	epsilon	white	negative	negative
230	alpha	golden	positive	positive
238	alpha, beta, delta	golden	positive	positive
246-3A	alpha, beta, delta	golden	positive	positive
243	alpha, beta, delta	golden	positive	positive
248	alpha, beta, delta	white	positive	negative
249	alpha, beta, delta	white	positive	positive
Wood 46	alpha, beta, delta	white	positive	negative
Staph no. 1	alpha	golden	positive	negative
FDA 209	alpha	golden	positive	negative
239	alpha, beta, delta	golden	positive	positive
338	alpha, delta	golden	positive	positive
C 242	alpha, beta, delta	golden	positive	positive

Filtrates were titrated for residual hemolysins before injection into test animals.

Effect of Control Preparations in Dogs

In order to determine the effect of uninoculated medium in dogs, control preparations were injected intravenously in either 5 or 10 ml amounts. Since Amigen powder is intended

Table 2. Results of hemolysin titrations of filtrates used in the study

Strain	Filtrate	Alpha hemotoxin		Beta hemotoxin	
		Before boiling	After boiling	Before boiling	After boiling
137	A	1-32	0	1-16	0
161	A	1-16	0	1-4	0
184	A	1-16	0	1-16	0
269	A	1-16	0	1-0	0
273	A	1-16	1-1	1-8	0
269	B	1-8	0	1-0	0
273	B	1-8	0	1-0	0
305	A	1-16	0	1-16	0
204	A	1-4	0	1-0	0
269	C	1-8	0	1-0	0
273	C	1-16	0	1-4	0
137	B	1-16	0	1-16	0
161	B	1-16	0	1-8	0
184	B	1-8	0	1-16	0
269	D	1-16	0	1-0	0
273	D	1-8	0	1-8	0
305	B	1-16	0	1-16	0
8	A	1-32	0	1-0	0
DFI 12	A	1-8	0	1-32	0
Wood 46	A	1-8	0	1-4	0
DFI 1310	A	1-4	0	1-0	0

Table 2. (Continued)

Strain	Filtrate	Alpha hemotoxin		Beta hemotoxin	
		Before boiling	After boiling	Before boiling	After boiling
Heidi	A	1-4	0	1-8	0
Davis 3	A	1-16	0	1-4	0
Slayton	A	1-4	0	1-8	0
Baird	A	1-4	0	1-4	0
C 242	A	1-8	0	1-4	0
216	A	1-2	0	1-0	0
87	A	1-16	0	1-8	0
C246-3A	A	1-8	0	1-16	0
307	A	1-4	0	1-0	0
248	A	1-16	0	1-4	0
249	A	1-4	0	1-0	0
280	A	1-1	0	1-0	0
338	A	1-2	0	1-0	0
251	A	1-4	0	1-0	0
246-3A	A	1-16	0	1-8	0
W-46	A	1-16	0	1-8	0
Staph no. 1	A	1-8	0	1-0	0
239	A	1-8	1-1	1-2	0
238	A	1-16	0	1-4	0
209	A	1-8	0	1-4	0

Table 2. (Continued)

Strain	Filtrate	Alpha hemotoxin		Beta hemotoxin	
		Before boiling	After boiling	Before boiling	After boiling
227	A	1-4	0	1-2	0
243	A	1-8	0	1-4	0
230	A	1-4	0	1-0	0

for use in preparing liquids for parenteral injections in human beings, it was thought that no adverse symptoms should result from its intravenous use in liquid form in dogs. Sterile uninoculated Amigen medium was injected in 5 or 10 ml amounts and the animals were observed continuously for 2 1/2 hours following administration. Once the dogs had recovered from the indignity of being restrained and subjected to an intravenous injection, no reactions of any type were observed which could be attributed to administration of the uninoculated medium. Since other investigators have stated that dogs vomit with little provocation, two other enzyme hydrolyzed protein preparations were also tested for reactions in dogs. These were 2 percent solutions of N-Z-Case powder* and

*N-Z-Case is a pancreatic digest of casein produced by the Sheffield Chemical Company, a Division of National Dairy Products Corp., Norwich, N.Y. The author is indebted to Dr. P. A. Hartman for the supply of product used in this study.

Trypticase powder* which had been prepared in the same manner as Amigen culture medium. No reactions followed the intravenous administration of 10 ml of either preparation. These latter two solutions were not used to produce filtrates for test purposes. The results of injecting control preparations are summarized in Table 7.

Results of Injections of Filtrates into Test Animals

The first trials in dogs were conducted with several unknown factors existing. The reaction of dogs to the injection of enterotoxic and non-enterotoxic filtrates was not known with certainty, and the strains of organisms used were the six coded cultures. The enterotoxigenic ability of these cultures was unknown to the author. There were no reports of the reaction of dogs to the medium which was used for enterotoxin production or to filtrates produced by the method used in this study. No information was available as to the dosage of these filtrates required to cause emesis in dogs. The results of the preliminary study were on a trial and error basis, and the information obtained was used as a guide for further study of the problem.

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*Trypticase is a pancreatic digest of casein produced by Baltimore Biological Laboratory, Baltimore, Maryland.

Strains 137 and 161 were used to produce the first filtrates, and the filtrate from strain 137 was selected for the first trials. Average sized dogs (20-30 pounds) were used and were fed a small meal as previously described. Two animals were injected with 5 ml each of the filtrate, two with 4 ml, and one received 1.75 ml. All acted normally for 5 to 10 minutes, and then went into a state of depression. In approximately 30 minutes, the four receiving the larger amounts of the filtrate began to show symptoms of restlessness and anxiety. They showed signs of apprehension and one began to whine. Emesis occurred in these dogs between 40 and 90 minutes and was quite severe in character. Vomiting occurred several times, and retching occurred after their stomachs were empty. The dog receiving 1.75 ml of the filtrate exhibited depression during the 2 1/2 hour observation period, but emesis did not occur. Since emesis had been established as the single criterion for a positive reaction, the results were tabulated as four positive and one negative.

Filtrates from the other strains were subjected to testing in the same manner except that no more than 3.5 ml of filtrate were administered. The results of injecting these filtrates, including the dosage range, are given in Table 3. The reactions obtained with four strains, numbers 137, 161, 184, and 305 were clear-cut. However, the results obtained from two strains, numbers 269 and 273, were not so definite,

Table 3. Summary of results obtained in the preliminary studies of coded cultures of Staphylococcus aureus

Strain	Filtrate	Number of dogs	Dosage range	Number positive	Number negative	Provisional designation
137	A	5	1.75-5 ml	4	1[a]	enterotoxic
161	A	8	1.75-5 ml	6	2[b]	enterotoxic
184	A	12	2-3.5 ml	0	12	non-enterotoxic
269	A	9	2.5-3.5 ml	6	3	?
273	A	8	2.5-3.5 ml	2[c]	6	?
305	A	5	3.5 ml	0	5	non-enterotoxic
269	B	6	2.5-3.5 ml	0	6	non-enterotoxic
273	B	6	2.5-3.5 ml	2[c]	4	?

[a]This dog received 1.75 ml of filtrate.

[b]These dogs received 1.75 ml of filtrate.

[c]These dogs received 3.5 ml of filtrate.

therefore a second filtrate (filtrate B) was produced from each of these strains. This time negative results were obtained from strain 269, while positive reactions were again obtained from strain 273. The percentage of positive reactions was much lower, however, than with other strains. Emesis occurred only when the amount of filtrate injected was more than 3 ml. It appeared that there was considerable variation in the amount of enterotoxin produced by the different strains.

It will be noted that emesis did not occur in any animal which received less than 2 ml of filtrate. The amount required to produce emesis with strain 273 appeared to be 3-3.5 ml, while emesis occurred with other strains from lesser amounts.

At this time, the information pertaining to the enterotoxigenic abilities of the six coded strains was requested and received.* Strain numbers 137, 161, and 273 are enterotoxin-producing, while strain numbers 184, 269, and 305 are non-enterotoxigenic. The results reported in Table 3 for strain numbers 137, 161, 184, 273, and 305 are in agreement with those supplied by Dr. M. S. Bergdoll. Because of the differences in results obtained with strain 269, another culture of this strain was requested and received. Due to

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*M. S. Bergdoll, Chicago, Ill. Identification of staphylococcal strains. Private communication. 1961.

the low percentage of reactions obtained with strain 273, a transfer from the original culture was made. Filtrates were produced from these new cultures and submitted to retesting in dogs. The results of these retests are presented in Table 4.

Since the enterotoxigenic capabilities of the six strains were now known to the author, it was decided to repeat the experiment. Filtrates were produced in the manner previously described and were injected into dogs. Injection of a minimum of 3 ml were made with the exception of two injections of the filtrate from strain 273. In these instances 2 ml were administered to two dogs. The results obtained in retesting these strains are given in Table 5. Included in this table are the reactions to these strains shown by other animals and methods of testing conducted by other investigators. Credit is due to Dr. M. S. Bergdoll for so generously supplying this information.

During many stages of the preliminary study, comparisons between dogs and cats as test animals were conducted. It was significant to note that the response to identical filtrates by these two species was the same in every instance. One trial involving five cats injected with filtrate C, strain 273, is of special interest. In this trial all five cats used gave a positive reaction, while eight dogs out of 10 reacted with emesis when injected with the same filtrate. The two dogs which gave negative responses received 2 ml, while the other

Table 4. Retest of filtrates from strain numbers 269 and 273

Strain	Filtrate	Number of dogs	Dosage range	Number positive	Number negative	Designation
269	C	10	3-3.5 ml	0	10	non-enterotoxic
273	C	10	2-3.5 ml	8	2[a]	enterotoxic

[a]These dogs received 2 ml of filtrate.

Table 5. Results of repeat testing of six strains of Staphylococcus aureus and results obtained by other investigators

Strain	Filtrate	Number of dogs	Positive	Negative	Results of other investigators
137	B	3	3	0	Enterotoxigenic in monkeys, cats, and one human volunteer
161	B	3	3	0	Enterotoxigenic in monkeys
184	B	3	0	3	Non-enterotoxigenic in monkeys
269	D	8	0	8	Non-enterotoxigenic in monkeys and cats
273	D	8	6	2[a]	Enterotoxigenic in monkeys and cats, reacts positively to the gel-diffusion technique for detection of enterotoxin
305	B	3	0	3	Non-enterotoxigenic in monkeys and cats

[a]These two animals received a dosage of only 2 ml.

eight received 3 ml or more. The details of this series of injections in cats are presented in Table 6. All comparisons of the two species as test animals are listed in Table 7.

In an attempt to find an enterotoxigenic strain among unknown cultures, seven strains of staphylococci of miscellaneous origin were obtained and filtrates produced. These filtrates were injected intravenously in dogs and cats, and not a single positive reaction was observed in either species following the injections. In all instances the dosage administered to dogs was 2.5 ml or greater, while the amount administered to cats was 2.5 ml except for one instance in which 1 ml was injected into a kitten. A coagulase-negative strain, number 204, and a known non-enterotoxigenic strain, W-46, were also tested.

All injections made in the preliminary study are summarized in Table 7. It will be noted that in those instances in which an identical filtrate was given, the results of cat injections and dog injections appear on the same line.

Final Phase of the Study

In the final phase of this study, an attempt was made to establish, on a body weight basis, the amount of enterotoxic filtrate required to produce emesis in dogs. The three known enterotoxic strains previously used had shown variation in the amount of filtrate required to consistently produce emesis.

Table 6. Results of injecting an enterotoxic filtrate into cats

Animal	Amount of filtrate	Route	Results
Small kitten	.75 ml[a]	i/p[a]	Uneasiness in 30 minutes, salivation in 35 minutes. Vomiting at 1 hour, repeated at 1 3/4 hours. Ate at 14 hours, dead in 24 hours.
Small kitten	1 ml	i/p	Uneasiness in 15 minutes. Vomiting at 30 minutes. Diarrhea in 1 hour. Normal in 24 hours.
Medium sized cat	1.75 ml	i/v[a]	Uneasiness in 15 minutes. Vomiting at 25 minutes, at 45 minutes, and at 2 hours. Diarrhea occurred at 1 hour. Normal in 24 hours.
Large cat	2 ml	i/v	Uneasiness in 15 minutes, vomiting at 30 minutes and 45 minutes. Diarrhea at 1 hour. Retching at 1 hour and 15 minutes. Urination and straining at 1 1/2 hours. Normal in 24 hours.
Large cat	2.5 ml	i/v	Depressed at 30 minutes, at 1 hour, and at 1 1/2 hours. Vomited at 2 hours. Normal in 24 hours.

[a]Legend: ml - milliliters; i/p - intraperitoneally; i/v - intravenously.

Table 7. Summary of results obtained in the preliminary study of reactions in dogs and cats following the injection of filtrates produced from strains of staphylococci

Strain	Filtrate	Cats			Dogs		
		No. animals	Positive	Negative	No. animals	Positive	Negative
137	A	1	1	0	5	4	1
137	B	2	2[a]	0	3	3	0
161	A	1	1	0	8	6	2
184	A	2	0	2	12	0	12[b]
269	A	2	2	0	9	6	3
269	B	2	0	2	6	0	6
273	A	2	2	0	8	2	6
273	B	2	2	0	6	2	4
269	C	4	0	4	10	0	10
273	C	5	5[c]	0	10	8	2

[a]One cat receiving an injection of this filtrate died within 24 hours.

[b]One dog receiving an injection of this filtrate died within 24 hours. One other dog went into shock, but recovered within 30 minutes and exhibited no further symptoms.

[c]One kitten receiving an injection of this filtrate died within 24 hours.

Table 7. (Continued)

Strain	Filtrate	Cats			Dogs		
		No. animals	Positive	Negative	No. animals	Positive	Negative
305	A	3	0	3	5	0	5
8	A	2	0	2	5	0	5
DFI 12	A	2	0	2	5	0	5
DFI 1310	A	2	0	2	4	0	4
Heidi	A	3	0	3	5	0	5
Davis 3	A	2	0	2	3	0	3
Baird	A	3	0	3	3	0	3
Slayton	A	3	0	3	3	0	3
204	A	-	-	-	4	0	4
Wood 46	A	-	-	-	5	0	5
161	B	-	-	-	3	3	0
184	B	-	-	-	3	0	3
269	D	-	-	-	8	0	8
273	D	-	-	-	8	6	2
305	B	-	-	-	3	0	3

Table 7. (Continued)

Strain	Filtrate	Cats			Dogs		
		No. animals	Positive	Negative	No. animals	Positive	Negative
Amigen control	-	2	0	2	18	0	18
Trypticase control	-	-	-	-	4	0	4
N-Z-Case control	-	-	-	-	4	0	4
Total injections		45			170		

It was therefore considered advisable to use several different strains to establish an effective level as well as to study further the reactions of dogs to enterotoxic and non-enterotoxic filtrates. Ten known enterotoxigenic strains were obtained for this purpose, as were nine known non-enterotoxigenic strains which were used for comparison purposes.*

The amount of enterotoxic filtrate required to produce emesis in a 20-30 pound dog appeared to be 3 ml as shown by results previously obtained. On the basis of body weight, this amount is .100-.150 ml per pound. The figure of .125 was established as a reference point, and filtrates from enterotoxigenic strains were administered to dogs in varying amounts from this level. The purpose of this procedure was to establish the most dependable dosage, and the level which could not be expected to produce vomition. Injections were made slightly above, at, and below the .125 ml per pound level in order to make these determinations.

Filtrates from known non-enterotoxigenic strains were injected in varying amounts beginning at .125 ml per pound, and increasing to a maximum of .625 ml per pound. In no instance was the amount administered less than that level which appeared to consistently produce emesis with the administration of an enterotoxic filtrate. By this procedure, a comparison of

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*These cultures were obtained through the courtesy of Doctors L. S. Bergdoll and E. P. Casman.

the level at which emesis consistently occurred with an enterotoxic filtrate could be made with the level of non-enterotoxic filtrate administered.

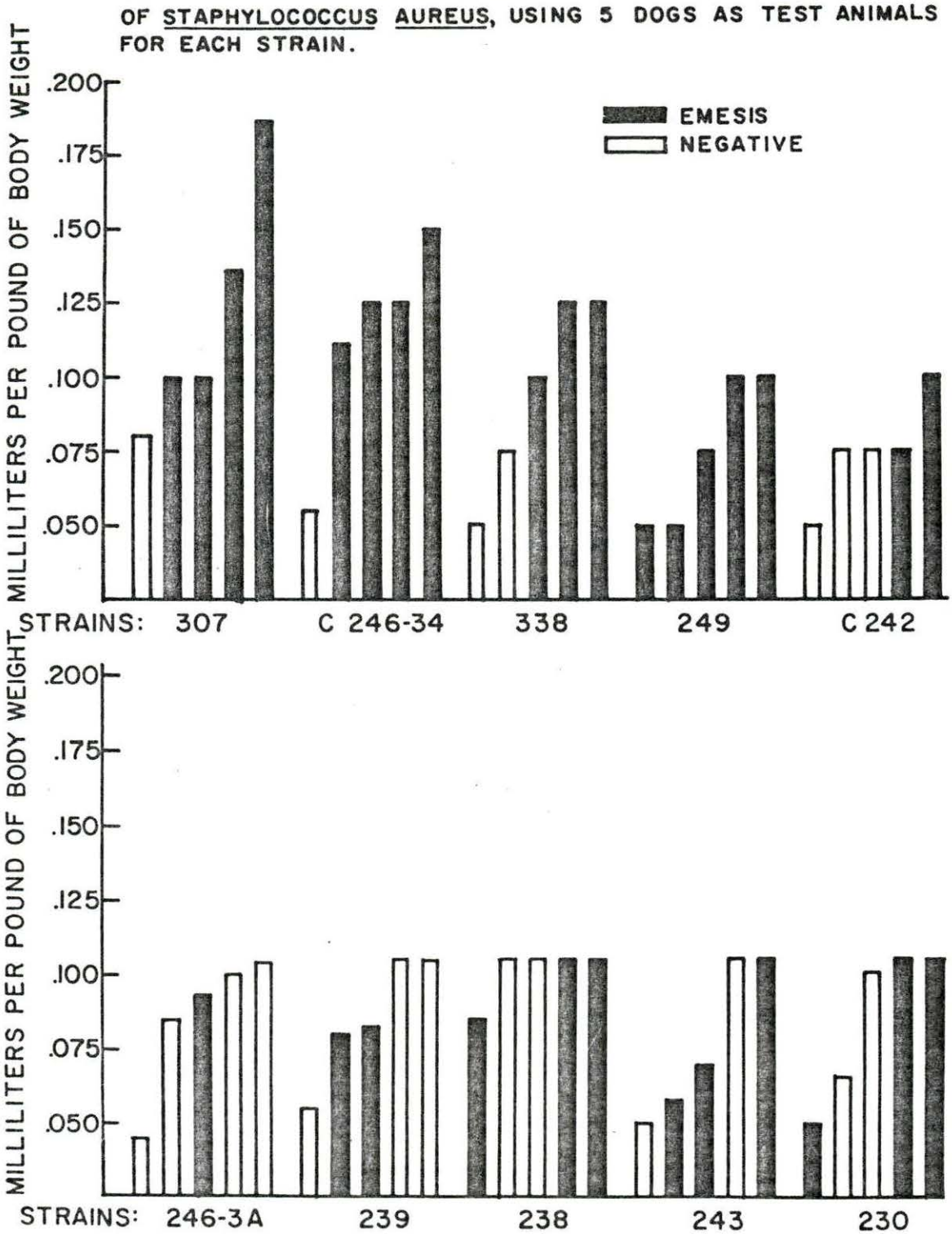
Figure 1 is a comparison of the reactions obtained by the injection of the filtrates from the 10 known enterotoxigenic strains of staphylococci. The largest amount administered was .187 ml per pound, and the smallest amount administered was .045 ml per pound. It is significant to note that at least one animal exhibited a positive reaction with each filtrate. In one instance, however, only one animal out of five suffered emesis (strain 246-3A). In contrast, all five animals used gave a positive reaction when injected with the filtrate from strain 249. Even with the conditions of decreased dosage, 60 percent of the animals injected suffered emesis.

In the course of these injections, it became increasingly apparent that considerable variation existed in the ability of different strains of staphylococci to produce enterotoxin. No claim is made for the use of dogs as a quantitative method of assay for enterotoxin, but it is obvious from the reactions obtained that strains vary in their ability to produce enterotoxin. On the basis of body weight, the reactions of the dogs injected with the filtrate from strains 249 and C 242 clearly demonstrate this variation.

The results shown in Figure 1 indicate that positive

Figure 1. Comparison of filtrates from 10 enterotoxigenic strains of Staphylococcus Aureus, using 5 dogs as test animals for each strain

COMPARISON OF FILTRATES FROM 10 ENTEROTOXIGENIC STRAINS OF STAPHYLOCOCCUS AUREUS, USING 5 DOGS AS TEST ANIMALS FOR EACH STRAIN.



reactions are more frequent as the dosage per pound is increased. There are, however, some instances which cannot be satisfactorily explained on the basis of dosage per pound of body weight. The five strains shown on the lower half of Figure 1 are examples of this. Some animals receiving low doses on this basis exhibited emesis, while others receiving larger doses did not. It appeared that some factor other than dosage per pound was influencing the number of positive reactions. An examination of the data revealed that total volume of filtrate was an important factor. The weights of animals used, dosage administered, and detailed accounts of the reactions observed are given in Appendix B.

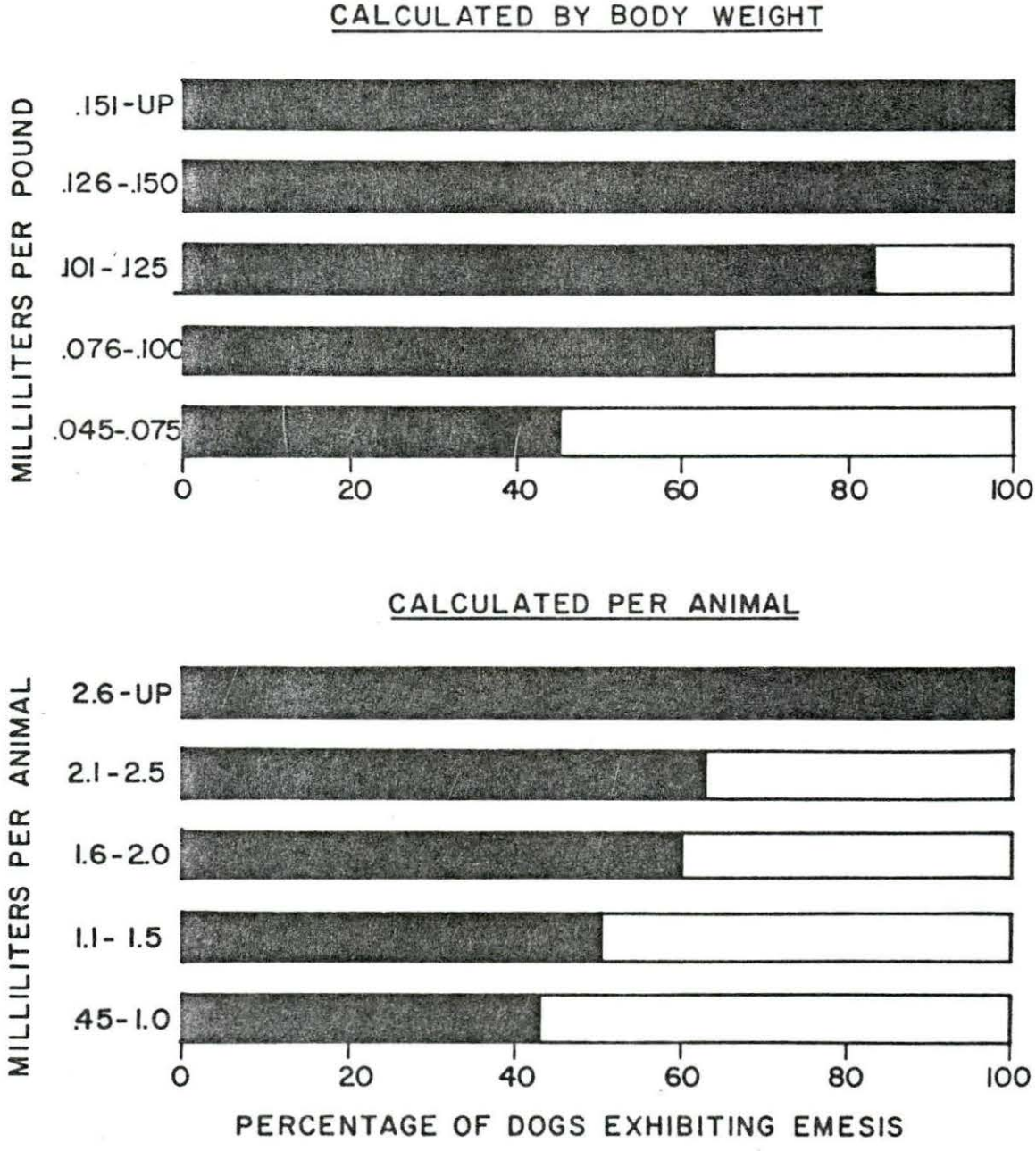
Figure 2 shows the percentages of positive reactions, calculated on a milliliter per pound basis, obtained from injection of filtrates from the 10 enterotoxigenic strains. Emesis was found to occur in every instance when the amount of filtrate exceeded .125 ml per pound, while it occurred in only 44 percent of the cases when the amount was .075 ml per pound or less.

In Figure 3 the percentage of positive reactions are calculated on a total volume per animal basis, irrespective of body weight. It is significant to note that in the range of 2.1 to 2.5 ml per animal, the positive reactions totaled only 63 percent, whereas emesis occurred in every case when the amount of filtrate administered exceeded 2.6 ml. This

Figure 2. Percentage of positive reactions in dogs following intravenous administration of enterotoxic filtrates calculated by body weight

Figure 3. Percentage of positive reactions in dogs following intravenous administration of enterotoxic filtrates calculated per animal

PERCENTAGE OF POSITIVE REACTIONS IN DOGS FOLLOWING INTRAVENOUS ADMINISTRATION OF ENTEROTOXIC FILTRATES



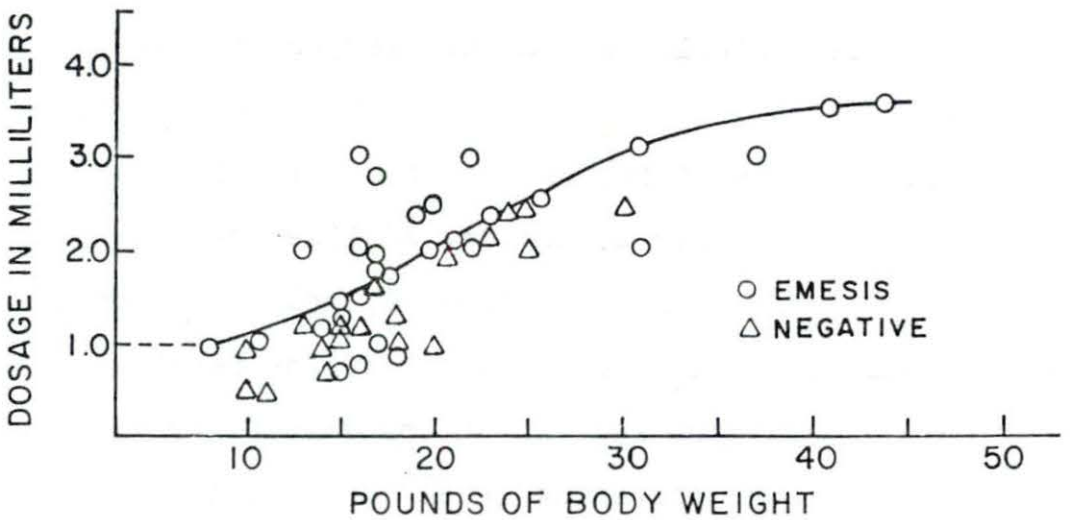
would indicate that the dosage of 3 ml per animal established in the preliminary study was well founded, and is further substantiated by the results shown in Figure 4.

Figure 4 shows the relationship of body weight to the total amount of filtrate administered. In every instance, an emetic response was obtained when the dosage was 3 ml or greater. A line has been plotted through the graph so that all negative results are on or below the line. Figure 4 also illustrates the variation in response shown by individual animals of the same weight range. Positive responses were observed in certain animals, while the same amount of filtrate administered to other animals of lesser weight gave negative reactions. It should be emphasized that all animals receiving an enterotoxic filtrate exhibited some symptoms but unless emesis was observed it is tabulated as negative. Figure 4 shows the minimum amount of filtrate which caused an emetic response. This amount is .75 ml in a 15 pound animal, and is .05 ml per pound of body weight. The maximum amount administered was 3.5 ml; this amount was administered to a 41 pound dog and a 44 pound dog and both gave positive responses. The results show that it was unnecessary to administer more than 3.5 ml to any animal used in order to obtain emesis from an enterotoxic filtrate. Because of the extremely severe reactions produced by amounts greater than 3.5 ml, larger doses should not be administered.

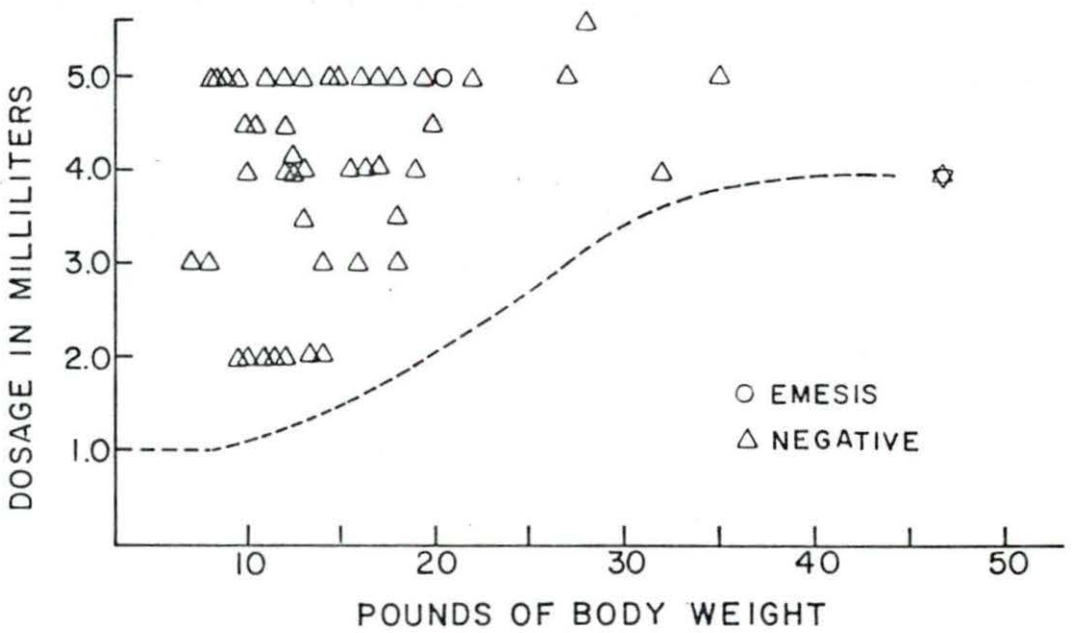
Figure 4. Reaction of dogs to intravenous administration of enterotoxic filtrates

Figure 5. Reaction of dogs to intravenous administration of non-enterotoxic filtrates

REACTION OF DOGS TO INTRAVENOUS ADMINISTRATION OF ENTEROTOXIC FILTRATES



REACTION OF DOGS TO INTRAVENOUS ADMINISTRATION OF NON-ENTEROTOXIC FILTRATES



✡ LINE WHICH WAS ESTABLISHED IN FIGURE ABOVE.

In Figure 5, the amounts of filtrate from the non-enterotoxigenic strains are shown, together with the weights of the animals. In general, non-enterotoxic filtrates were administered to smaller animals and in much larger amounts than were enterotoxic filtrates. The reason for this was to exceed the level which caused vomition when enterotoxic filtrates were administered. In Figure 5, the dotted line present is the line which was established in Figure 4, and which was transposed to demonstrate the relationship of the reactions obtained from enterotoxic and non-enterotoxic filtrates. All injections made were above the level required to produce emesis, while 73 percent were 3.5 ml or more per animal. The minimum amount administered was 2 ml, while the maximum amount was 5.5 ml. A complete record of the weights of dogs used and the amount of filtrate administered is given in Appendix C.

DISCUSSION

The results obtained in this study clearly indicate that dogs can be used as satisfactory test animals for detecting staphylococcal enterotoxin in boiled filtrates. In every instance, similar results have been obtained when identical filtrates were administered to dogs and cats (Table 7). It was shown in this study that the results of administering filtrates to dogs by the intravenous route are the same as those other investigators have reported by using human volunteers, the monkey feeding technique, the cat test, and the gel-diffusion technique (Table 5).

Some difficulty was experienced in establishing correct dosage levels in dogs. With the dosages employed, actual vomition did not occur in all animals in each trial. Variation in susceptibility of individual animals was noted in the final phase of the study, since some animals receiving low dosages vomited while others which received larger amounts did not (Figures 1 and 4). Variation in enterotoxin production among strains was observed in the preliminary phase and again in the final phase (Figure 1). As a result of trial and error in the preliminary study, it appeared that 3 ml of an enterotoxic filtrate should give consistently positive results with a 20-30 pound dog.

An attempt was made in the final phase of the study to determine the amount of enterotoxic filtrate per pound of body

weight required to consistently produce emesis in dogs. The results obtained indicated that the total volume administered as well as the milliliters per pound of body weight exerted an influence on the number of positive reactions. Under the experimental conditions of the study, consistently positive reactions occurred when the amount of filtrate administered was 2.6 ml or more. In the preliminary study, however, positive reactions were not always obtained with the administration of this amount of filtrate. On a body weight basis, more than .125 ml per pound consistently produced emesis with the intravenous administration of an enterotoxic filtrate. It appears, then, that the administration of 3 ml of an active filtrate for a 20-30 pound dog or .125 ml per pound for dogs of other sizes should give consistently positive responses.

Animals which varied considerably in weight were used in the final phase of the study and were administered varying dosages of enterotoxic filtrates. It was possible by this procedure to develop a curve established at a level at which emesis consistently occurred for dogs varying between 8 and 44 pounds in weight. The curve is of particular interest since it was developed with 10 known enterotoxigenic strains of staphylococci. It therefore compensates for the variation in enterotoxin production of strains as well as for the individual resistance of test animals to the effects of enterotoxin. Under the experimental conditions of the study, every

injection exceeding the amount indicated by the curve resulted in emesis when an enterotoxic filtrate was administered. The results obtained indicate that the curve would be accurate for any weight dog available.

Observations of the reactions of dogs to filtrates produced from non-enterotoxigenic strains of staphylococci were made in all phases of the study. Inconsistent results were obtained with both dogs and cats with one strain (filtrates A, B, C, and D, strain 269). Filtrate A was inconsistent with the other three filtrates. It would seem that the strain from which filtrate A was produced was mis-labeled or that it was possibly mixed with another culture since filtrates B, C, and D were consistent and clear-cut.

In the final phase of the study, the administration of filtrates produced from nine non-enterotoxigenic strains were compared with the level established for emesis with enterotoxic filtrates. In every instance the amount administered was .125 milliliter per pound or greater, and in some instances the amount administered was almost five times the established emetic dose. If enterotoxic rather than non-enterotoxic filtrates had been administered in these amounts, every animal would have suffered emesis according to the level established in Figure 4.

One false positive reaction occurred following the injection of a non-enterotoxic filtrate in the final phase of the

study. This animal, which weighed 20 pounds, received 5 ml of the filtrate produced from Staph No. 1, a non-enterotoxigenic strain. Vomition occurred 60 minutes after injection, however, the usual symptoms of depression following administration of an enterotoxic filtrate were not observed before or after the act of vomition. No adverse symptoms of any kind were observed in the other four animals, one of which weighed 10 pounds and received an injection of 4.5 ml. The reason for this false positive reaction is obscure, although it might well be that the animal was suffering from an undetected case of mild gastritis prior to the injection. The animal appeared normal in every respect the following day.

It appears that false positive reactions are not of major consideration when using dogs for test animals to detect staphylococcal enterotoxin. False positive reactions do occur, but not in proportions which would be misleading in interpreting the results of the test. In the final phase of the study, not a single false positive reaction occurred within the dosage range which would normally be administered in conducting the test.

A comparison of the reactions obtained in dogs and cats is of considerable interest. As previously mentioned, the reactions of the two species to the parenteral administration of enterotoxic and non-enterotoxic filtrates were the same in every instance. The symptoms exhibited by the two species,

however, differ in many respects.

It was observed that cats react sooner than dogs when injected with an enterotoxic filtrate, and appear to suffer much greater distress. It was common for cats to utter cries of pain, and the depression so commonly noted in dogs was usually absent in cats. On the contrary, cats usually exhibited symptoms of restlessness and uneasiness soon after injection. Salivation was a common symptom in cats but was seldom seen in dogs. When vomition occurred in cats it was of a more forceful nature than with dogs and seemed to be more painful to the animal. Diarrhea was common in cats following the injection of an enterotoxic filtrate but was observed only occasionally in dogs. Dogs seemed to recover from the effects of an enterotoxic filtrate more quickly than did cats, for in a few instances dogs appeared to be normal 5 hours after a positive reaction. On the other hand, some cats showed severe prostration and weakness after a siege of vomiting and did not usually appear normal in less than 24 hours. Of 45 injections made in cats, two died as a result of receiving an injection of enterotoxic filtrate. Not a single dog died as a result of receiving an enterotoxic filtrate. One dog, however, died from other causes 24 hours after receiving an injection of a non-enterotoxic filtrate (filtrate A, strain 184).

Other investigators have reported the necessity of

increasing the amount of filtrate administered when cats are used for more than one test, but this was not observed in the trials conducted with cats in this study. The method used for production of enterotoxin undoubtedly accounts for this, since the severity of reactions observed indicated that enterotoxin was present in considerable quantity in all filtrates.

It seems appropriate to point out that cats are more difficult to handle than are dogs, since at times it was necessary to make intraperitoneal instead of intravenous injections. This was necessary in the case of small kittens because of the size of the veins, and in some adult cats because of an occasional unmanageable animal.

There have been a few reports in the literature of attempts to use dogs as test animals for detecting staphylococcal enterotoxin. Some of the attempts to use dogs were feeding trials, and were uniformly unsuccessful (4, 25, 59). This phase of the reaction of dogs to enterotoxin was not in the scope of the present study, so no attempt will be made to compare the results obtained. Further work is undoubtedly desirable to determine the effect of oral administration of staphylococcal culture filtrates on dogs.

Parenteral administration of an acid-ether extract of a potent filtrate was reported by Jordan and Burrows (51). They administered this substance intravenously in dogs and

no ill effects resulted. It is significant to note that cats and rabbits exhibited no symptoms following the administration of the same solution. The reaction of cats to enterotoxin has been established (33), and Casman (13) has demonstrated that rabbits are susceptible to the intravenous administration of purified enterotoxin. If enterotoxin had been present it would appear that part, if not all, of these animals would have reacted. A possible explanation of the failure of these animals to react lies in the findings of Hammon (44). He was unable to find any trace of enterotoxin in ether extracts of enterotoxic filtrates but did find it in the residual culture extract.

Dolman et al. (34) state that dogs are more prone to vomit than are cats. This fact cannot be denied, for numerous instances of unprovoked emesis were observed in dogs during the course of this study. It is felt, however, that by the use of dogs which are obviously in good health, coupled with the rejection of animals which refuse to eat prior to injection, the tendency to vomit need not be of major concern. Tekse (91) observed no reactions in dogs following the intravenous administration of 5 ml of control medium or non-toxic filtrate. The administration of 10 ml of uninoculated control preparations did not cause vomition in the present study. Only one false positive reaction which could be attributed to spontaneous vomition was seen. It appears

that with adequate selection of test animals, the tendency to vomit does not lessen the value of the dog as a test animal.

The intra-abdominal route of administration of staphylococcal toxins to dogs was reported by Rigdon (67). On the basis of his work, it has generally been concluded that dogs could not be used as satisfactory test animals for detecting staphylococcal enterotoxin. The results of the present study are not in agreement with Rigdon's observations.

The control medium and staphylococcal toxins Rigdon used were prepared in a commercial laboratory by a method reported to be similar to Dolman's technique (32). The source of the organisms used in preparing the toxins was not reported. The method of inactivation of hemoysins was to heat the toxin preparation for 2 hours at 100 C. In the light of present day knowledge, even if enterotoxin had been present it would have been destroyed or greatly weakened by this treatment. The puppies receiving intra-abdominal injections of the control medium vomited within 30 minutes after the injection, while none of the animals receiving the heated toxin vomited. It would seem that if the control medium caused vomition the toxin preparation should also cause this effect. The toxin contained all the ingredients of the control preparation plus the by-products of metabolism from the organisms. Rigdon administered unheated toxin to two puppies and both died after 4 hours. This effect was to be expected, since the hemolysins

present in the filtrate were not inactivated in any manner.

Rigdon also used kittens for injections with the staphylococcal toxins and obtained results similar to those he reported in puppies. The kittens receiving the control preparations vomited, while those receiving the heated staphylococcal toxin did not. The results of Rigdon's studies are therefore not in accord with the observations of numerous investigators.

A report by Tekse (91) indicated that the use of dogs for detecting staphylococcal enterotoxin had not been thoroughly investigated. Tekse prepared filtrates by Dolman's method from known enterotoxigenic and known non-enterotoxigenic strains of staphylococci. In his work the hemolysins were inactivated by heating the filtrates in a water bath for 20-30 minutes at 100 C. He administered the filtrates by the intravenous route and obtained vomition in dogs with the administration of 3 to 5 ml of enterotoxic filtrate. No reactions were noted following the administration of control preparations or filtrates prepared from non-enterotoxigenic strains. In a comparison with the results obtained with the use of cats, Tekse found that the two species reacted similarly to identical filtrates. It is significant to note that the results obtained from the use of two non-enterotoxigenic strains, Wood 46 and FDA 209, were the same in the present study as those observed by Tekse. The results obtained in the present study substantiate Tekse's observations as well

as those of Dolman and Wilson (32).

A search of the literature has revealed no previous work on the amount of filtrate per pound of body weight required to cause emesis in dogs.

SUMMARY

A technique has been described for the use of dogs as satisfactory test animals for detecting staphylococcal enterotoxin in boiled filtrates. Dogs selected for test purposes should be in good health and should be fed a small meal 30 minutes prior to injection of control preparations or test filtrates. It is recommended that they be in the 20-30 pound weight range, although larger or smaller animals can successfully be used. The dosage of filtrate required to consistently produce emesis has been found to be .125 ml per pound, and the total volume should not exceed 3.5 ml for dogs of any weight. In the present study, five dogs per trial appeared to be the most preferable number. These animals should be observed continuously for a minimum of 2 1/2 hours and for a longer period if possible. Observations should be made at intervals thereafter, and all animals should appear normal the following day. At least four out of five animals should react with emesis following the administration of an enterotoxic filtrate, while no symptoms should follow the administration of a non-enterotoxic filtrate.

The use of the dog as a test animal is offered as an alternate to the existing methods of detecting staphylococcal enterotoxin. It is believed that the ease of handling and maintaining dogs, together with their availability, far

outweighs any slight disadvantage which might exist as a result of their tendency to vomit occasionally without provocation.

CONCLUSIONS

1. It has been shown that dogs react with emesis when boiled filtrates produced from enterotoxigenic strains of Staphylococcus aureus are injected intravenously. The results obtained in the present study show that dogs injected intravenously with boiled filtrates produced from non-enterotoxigenic strains of Staphylococcus aureus exhibit no symptoms.
2. Under the experimental conditions used, the amount of enterotoxic filtrate required to produce emesis has been found to be .125 ml per pound of body weight, up to a maximum of 3.5 ml total volume.
3. It is concluded that dogs can be used as satisfactory test animals for detecting enterotoxin in sterile, boiled filtrates produced from enterotoxigenic strains of Staphylococcus aureus.

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APPENDIX A

Table 8. Identification of strains of staphylococci used in the study

Culture	Obtained from	Designation	Isolated from
137	Dr. M. S. Bergdoll	Known enterotoxigenic	Abscess
161	Dr. M. S. Bergdoll	Known enterotoxigenic	Food poisoning outbreak
273	Dr. M. S. Bergdoll	Known enterotoxigenic	Enteritis after antibiotic therapy
184	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Food poisoning outbreak
269	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Enteritis after antibiotic therapy
305	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Nose culture
204	Dr. R. A. Packer	Coagulase-negative	Nose culture
W-46	Dr. R. A. Packer	Known non-enterotoxigenic	Information not available
8	Dr. R. A. Packer	Unknown	Information not available
DFI 12	Dr. R. A. Packer	Unknown	Bovine milk
DFI 1310	Dr. R. A. Packer	Unknown	Cheese
Davis 3	Dr. R. A. Packer	Unknown	Bovine milk
Slayton	Dr. R. A. Packer	Unknown	Bovine milk
Baird	Dr. R. A. Packer	Unknown	Osteomyelitis
Heidi	Dr. T. M. Ford	Unknown	Upper respiratory infection
C 242	Dr. M. S. Bergdoll	Known enterotoxigenic	Information not available
C 243-3A	Dr. M. S. Bergdoll	Known enterotoxigenic	Information not available
307	Dr. M. S. Bergdoll	Known enterotoxigenic	Information not available
249	Dr. M. S. Bergdoll	Known enterotoxigenic	Information not available
338	Dr. M. S. Bergdoll	Known enterotoxigenic	Information not available
230	Dr. E. P. Casman	Known enterotoxigenic	Information not available
238	Dr. E. P. Casman	Known enterotoxigenic	Information not available
239	Dr. E. P. Casman	Known enterotoxigenic	Information not available
246-3A	Dr. E. P. Casman	Known enterotoxigenic	Information not available
243	Dr. E. P. Casman	Known enterotoxigenic	Information not available

Table 8. (Continued)

Culture	Obtained from	Designation	Isolated from
Wood 46	Dr. E. P. Casman	Known non-enterotoxigenic	Information not available
Staph No. 1	Dr. E. P. Casman	Known non-enterotoxigenic	Information not available
FDA 209	Dr. E. P. Casman	Known non-enterotoxigenic	Information not available
227	Dr. E. P. Casman	Known non-enterotoxigenic	Information not available
87	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Information not available
216	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Information not available
248	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Information not available
251	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Information not available
280	Dr. M. S. Bergdoll	Known non-enterotoxigenic	Information not available

APPENDIX B

Table 9. Reactions of dogs to injections of filtrates from 10 enterotoxigenic strains of Staphylococcus aureus

Strain	Weight of dog	Dose	Ml/lb.	Time in minutes 30	60	90	120	150	180	5 hrs.	24 hrs.	Results
307	25 lbs.	2 ml	.080	D[a]	D	D	D	D	D	N[a]	N	Negative
307	10 lbs.	1 ml	.100	Ner[a]	D	D	D	E[a]	D	D	N	Emesis
307	20 lbs.	2 ml	.100	D	U,BE[a]	D	E	D	D	N	N	Emesis
307	22 lbs.	3 ml	.136	E	E	E	E	D	D	D	N	Emesis
307	16 lbs.	3 ml	.187	E	E	E	D	D	D	D	D, ill	Emesis
C 246-3A	18 lbs.	1 ml	.055	N	N	N	N	N	N	N	N	Negative
C 246-3A	17 lbs.	2 ml	.117	N	N	N	E	E	D	D	N	Emesis
C 246-3A	8 lbs.	1 ml	.125	N	N	N	E	D	D	D	N	Emesis
C 246-3A	16 lbs.	2 ml	.125	N	N	E	E	D	D	D	D	Emesis
C 246-3A	14 lbs.	2 ml	.150	N	N	E	E	E	D	D	N	Emesis
338	17 lbs.	1.7 ml	.100	S	D	D	D	D	E	D	N	Emesis
338	14.5 lbs.	.75 ml	.051	BE	D	D	D	D	D	D	N	Negative
338	19.5 lbs.	2.4 ml	.125	N	E, Ner	D	D	D	D	D	N	Emesis
338	20 lbs.	2.5 ml	.125	D	D	E	E	D	D	D	N	Emesis
338	16 lbs.	1.2 ml	.075	N	N	D	D	D	D	D	N	Negative

[a]Legend: D is depressed; N is normal; U is urinated; Ner is nervous; BE is bowel evacuation; LS is loose stool; S is salivating; E is emesis; Tre is trembling.

Table 9. (Continued)

Strain	Weight of dog	Dose	Ml/lb.	Time in minutes 30	60	90	120	150	180	5 hrs.	24 hrs.	Results
249	16 lbs.	.8 ml	.050	D	D	D	D	E	D	D	N	Emesis
249	18 lbs.	.9 ml	.050	D	D	D	U,LS[a]	E	E	D	N	Emesis
249	16.5 lbs.	1.25 ml	.075	D	D	E	E	E	D	D	N	Emesis
249	28 lbs.	2.8 ml	.100	Ner	E	E	D	D	D	D	N	Emesis
249	23 lbs.	2.3 ml	.100	BE	D	D	E	E	Tre[a]	D	N	Emesis
C 242	20 lbs.	1 ml	.050	N	D	D	DD	D	-	N	N	Negative
C 242	16.5 lbs.	1.25 ml	.075	N	N	BE,D	D	D	-	N	N	Negative
C 242	18 lbs.	1.35 ml	.075	N	N	BE,D	DD	D	-	N	N	Negative
C 242	15 lbs.	1.15 ml	.075	N	N	D	E	E	-	D	N	Emesis
C 242	21 lbs.	2.1 ml	.100	N	E,BE	D	D	D	-	D	N	Emesis
246-3A	11 lbs.	.5 ml	.045	N	D	D	D	D	D	N	N	Negative
246-3A	30 lbs.	2.5 ml	.084	D	D	D	D	D	D	D	N	Negative
246-3A	16 lbs.	1.5 ml	.093	D	D	U,D	D	E	D	D	N	Emesis
246-3A	21 lbs.	2 ml	.095	D	D	D	D	D	D	D	N	Negative
246-3A	24 lbs.	2.5 ml	.104	D	D	D	D	U,D	D	D	N	Negative
239	14 lbs.	.8 ml	.057	N	D	D	D	D	D	D	N	Negative
239	31 lbs.	2.5 ml	.080	D	D	D	E	D	D	D	N	Emesis
239	36 lbs.	3 ml	.083	D	D	D	E	E	D	D	N	Emesis
239	10 lbs.	1 ml	.100	N	D	D	D	D	D	D	N	Negative
239	17 lbs.	1.7 ml	.100	N	D	BE,D	D	D	D	D	N	Negative
238	41 lbs.	3.5 ml	.084	U,D	D	D	D	E	D	D	N	Emesis
238	23 lbs.	2.3 ml	.100	Ner,U	LS	D	D	D	D	D	N	Negative
238	25 lbs.	2.5 ml	.100	D	D,S	D	D	D,S	D	D	N	Negative
238	22 lbs.	2.2 ml	.100	D	Ner	D	E	E	D	D	N	Emesis
238	31 lbs.	3.1 ml	.100	N	D	D	D	U,E	E	D	N	Emesis

Table 9. (Continued)

Strain	Weight of dog	Dose	Ml/lb.	Time in minutes 30	60	90	120	150	180	5 hrs.	24 hrs.	Results
♂43	10 lbs.	.5 ml	.050	N	BE	D	D	D	D	D	N	Negative
♂43	17 lbs.	1 ml	.058	E,BE	D	D	D	D	D	D	N	Emesis
♂43	44 lbs.	3.5 ml	.079	D	D,BE	D	D	E	D	D	N	Emesis
♂43	13 lbs.	1.3 ml	.100	D	U,D	D	D	D	D	D	N	Negative
♂43	15 lbs.	1.5 ml	.100	D,BE	D	D	E	D	D	D	N	Emesis
♂30	15 lbs.	.75 ml	.050	E	D	D	D	D	D	D	N	Emesis
♂30	15 lbs.	1 ml	.066	D	D	D	D	D	D	D	N	Negative
♂30	21 lbs.	2 ml	.095	D	D	D	D	D	D	D	N	Negative
♂30	17 lbs.	1.7 ml	.100	Ner	D	U,D	D	E	E	D	N	Emesis
♂30	25 lbs.	2.5 ml	.100	DD	BE,D	Ner	D	E	D	D	N	Emesis

APPENDIX C

Table 10. Administration of nine non-enterotoxic filtrates to dogs[a]

Strain	Weight of dog	Dosage	Ml/lb.
87	14 lbs.	2 ml	.142
87	12 lbs.	2 ml	.166
87	11 lbs.	2 ml	.181
87	10 lbs.	2 ml	.200
87	10 lbs.	2 ml	.200
248	14 lbs.	2 ml	.143
248	18 lbs.	3 ml	.166
248	11 lbs.	2 ml	.181
248	16 lbs.	3 ml	.187
248	17 lbs.	3.5 ml	.206
216	32 lbs.	4 ml	.125
216	22 lbs.	5 ml	.227
216	20 lbs.	5 ml	.250
216	8 lbs.	3 ml	.375
216	7 lbs.	3 ml	.427
251	15.5 lbs.	4 ml	.260
251	13 lbs.	4 ml	.310
251	12 lbs.	4 ml	.333
251	12 lbs.	4 ml	.333
251	10 lbs.	4 ml	.400
280	35 lbs.	5 ml	.142
280	20 lbs.	4.5 ml	.225
280	14 lbs.	4 ml	.284
280	10 lbs.	4.5 ml	.450
280	9 lbs.	5 ml	.555
W-46	17 lbs.	4 ml	.235
W-46	15 lbs.	5 ml	.333
W-46	12 lbs.	5 ml	.416
W-46	9 lbs.	5 ml	.555
W-46	8 lbs.	5 ml	.625

[a]All animals receiving the injections listed above acted in a normal manner throughout the observation period except for one animal receiving 5 ml of the filtrate produced from Staph no. 1. This animal reacted with emesis in 60 minutes after receiving the injection. No symptoms of emesis or discomfort were observed in the other four animals in the trial.

Table 10. (Continued)

Strain	Weight of dog	Dosage	Ml/lb.
Staph no. 1	14 lbs.	3 ml	.211
Staph no. 1	16.5 lbs.	4 ml	.242
Staph no. 1	20 lbs.	5 ml	.250
Staph no. 1	12 lbs.	3.5 ml	.291
Staph no. 1	10 lbs.	4.5 ml	.450
209	19 lbs.	4 ml	.210
209	18 lbs.	5 ml	.277
209	14.5 lbs.	5 ml	.340
209	12 lbs.	4.5 ml	.375
209	11 lbs.	5 ml	.550
227	27 lbs.	5 ml	.185
227	28 lbs.	5.5 ml	.196
227	17 lbs.	5 ml	.294
227	16 lbs.	5 ml	.312
227	13 lbs.	5 ml	.384