A comparative study of enterotoxic and non-enterotoxic micrococci
by David R Morledge

A THESIS Submitted to the Graduate Committee in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University

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The results of these experiments lead one to the same conclusion reached by most of the other workers on food-poisoning micrococci, namely, that at present there is no physiological characteristic successful in separating enterotoxic from non-enterotoxic micrococci.

A COMPARATIVE STUDY OF ENTEROTOXIC AND NON-ENTEROTOXIC MICROCOCCI

by

David R. Morledge

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Submitted to the Graduate Committee

in

partial fulfillment of the requirements

for the degree of

Master of Science in Bacteriology

at

Montana State College

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Bozeman, Montana
June 1949

ACKNOWLEDGMENT

The author wishes to acknowledge the fine personal interest and supervision of Dean F. B. Cotner and Mr. W. G. Walter and the encouragement of Dr. B. L. Johnson throughout this study.

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ABSTRACT

Eighteen strains of micrococci, ten of which had been isolated from cases of food-poisoning, were studied as to their physiological reactions and the ability of their boiled filtrates to invoke enterotoxicity in cats upon intravenous injection.

All but one of the strains gave the same physiological reactions and morphological characteristics listed for Micrococcus pyogenes var. aureus in the sixth edition of Bergey's Manual, except that none of them was observed to produce hydrogen sulfide.

Incubation at 30° C brought about a more rapid liquefaction of gelatin by the strains than incubation at 37° C. The length of time the tubes were cooled after each incubation influenced the observable liquefaction in some of the tubes.

Three of the strains were found enterotoxic to cats. In addition to having all the physiological characteristics of Micrococcus pyogenes var aureus, these three strains were coagulytic, hemolytic, fibrinolytic, and gave a positive Stone reaction in 48 hours. Four strains not enterotoxic to cats gave these same reactions. No visible serological reaction was observed between homologous enterotoxin and rabbit serum.

The results of these experiments lead one to the same conclusion reached by most of the other workers on food-poisoning micrococci, namely, that at present there is no physiological characteristic successful in separating enterotoxic from non-enterotoxic micrococci.

INTRODUCTION

Eighteen strains of micrococci, some of which had been isolated from cases of food-poisoning, were studied. An attempt was made to determine whether or not those micrococci found to elaborate enterotoxin could be differentiated from ones found to be non-enterotoxigenic by their serological and physiological properties and thus be placed in a category by themselves.

HISTORICAL BACKGROUND

Food poisoning by micrococci has come to be recognized and commonly accepted as such only within the last twenty years. Micrococcus food poisoning is produced by an enterotoxin formed by organisms which were formerly classified as Staphylococcus aureus and S. albus (Dolman and Wilson, 1938) but are now classified as Micrococcus pyogenes var. aureus and M. pyogenes var. albus in the sixth edition of "Bergey's Manual of Determinative Bacteriology" (1948). Since its common recognition, a large number of outbreaks of micrococcus food poisoning have been reported in current scientific literature. According to Dack (1943), it is probably the most common of all types of food poisoning, and the number of cases cited is but a small proportion of the attacks occurring. This is probably because it follows a rapid and non-fatal course, usually among susceptible members of a single family, and is passed off as a "slight intestinal disturbance." As this sickness is of short duration, is non-fatal, and affects most individuals at various times during their lives, it is not given attention and is soon forgotten. It is when large

outbreaks occur in a community that the causative agent is tracked down and the case reported.

The symptoms of micrococcus food poisoning generally appear in two to four hours after the ingestion of food containing a large number of enterotoxin-producing micrococci (Jordan and Burroughs, 1945). Usually, cream-filled bakery goods high in starch content are the responsible items, although many other foods such as cheese, milk, chicken gravy, ham, tongue, chipped beef, fish sausage, and liver sausage have been found to have carried the contaminating organisms in the reported cases. In several instances, milk from cows suffering, or having suffered, with mastitis has been proved to be the food involved (Shaughnessy and Grubb, 1936; Minett, 1938). However, Dack (1943) points out that the most common mode of infection has resulted from the handling of the foods by individuals infected with micrococci and the subsequent storage of these foods under conditions favorable for the growth of the enterotoxin-producing organisms. The time interval between the ingestion of the contaminated food and the appearance of the first symptom is influenced by the amount of the enterotoxin consumed and the susceptibility of the individual. The first symptom is that of salivation, and is followed by nausea, retching, abdominal cramping of varying severity, and diarrhea. Some of these symptoms may not be present in milder cases. In severe cases blood and mucus may be observed in the stools and the vomitus. In the latter cases the temperature of the individual may be higher or lower than the norm, and his blood pressure

may likewise vary. Recovery usually takes place in from one to three days, although sometimes it is as long as one or two weeks until total recovery is obtained. This varies with the susceptibility and physical fitness of the person attacked, and the amount of enterotoxin ingested (Dack, 1943).

The causative agent of micrococcus food poisoning was "discovered" four times before it was commonly recognized as such. These four discoveries were by Denys in 1894, by R. W. G. Owen in 1906, by M. A. Barber in 1914, and by Dack, Carry, Woolpert, and Wiggers in 1930. These resulted in outbreaks occurring in Belgium; Kalamazoo, Michigan; the Philippine Islands; and Chicago, respectively. The contaminated food was found to be fresh beef, dried beef, milk from a cow which had mastitis, and a three-layer sponge cake. Since 1930 a large number of similar outbreaks have been reported in the literature.

The diagnosis of micrococcus food poisoning is usually reached by first feeding the suspected food to human volunteers to test its toxicity, by examining it for large numbers of micrococci, and by culturing the predominant organisms and then feeding the culture filtrates to human volunteers. The appearance of clinical symptoms indicative of micrococcus food poisoning is carefully observed. The use of monkeys (Dack et al, 1930) and kittens (Dolman and Wilson, 1938) in place of human volunteers has been given a great deal of attention. Both have their disadvantages: the monkeys are expensive to keep and use, and the kittens are difficult to obtain the year around. Hammon (1941) injected both kittens and cats intravenously with enterotoxin and found that by

his method less amounts were needed. As cats were found as susceptible as kittens and are easily obtained, the problem of procuring and keeping necessary animals was considerably lessened. However, as Hammon pointed out, kittens and cats lose their susceptibility to the enterotoxin after having had four or five attacks of the disease. Other experimental animals, including the dog (Jordan and Burroughs, 1933; Minett, 1938; Rigdon, 1938) and rodents (Jordan and McBroom, 1931; Corpening and Foxhall, 1935) have been tried, but without a comparable amount of success. Hopkins and Poland (Dack, 1943) reported that young pigs are good test animals, but other workers have not used them in order to confirm this.

There have been numerous attempts to effect a laboratory diagnosis by finding some consistent characteristic setting these organisms apart from the rest of the micrococci group. Stone (1935) developed a cultural method for the classification of food-poisoning micrococci by their production of liquefaction in his medium containing gelatin when incubated at 37° C for 24 hours. Any degree of liquefaction was considered as indicating a food-poisoning strain; the converse being true if no liquefaction occurred. Chin (1936) and Kupchik (1937) could not correlate their findings with those of Stone; whereas Chapman et al (1937) found that the Stone reaction (gelatin liquefaction) was positive in 75.5 per cent of typical food poisoning micrococci and positive in 27.6 per cent of strains with similar properties but isolated from non-food-poisoning sources.

Chapman et al (1934) found that a high correlation exists between the micrococci's ability to produce coagulase and hemolysin and their pathogenicity. Cruickshank (1937) supported the relationship between pathogenicity and production of coagulase, whereas Straiter and Jordan (1935) found that food-poisoning strains did not constitute a clearly marked division with any homogeneity in biochemical, hemolytic, or agglutinative characters. Kupchik (1937) went a step farther and found in his experiments that there was no distinctive reaction which would separate enterotoxic from non-enterotoxic-producing strains by pigment production, action on milk, liquefaction of nutrient gelatin, as well as the biochemical, agglutinative, and coagulative characters studied by Cruickshank, and Straiter and Jordan. Dolman and Wilson (1938), Haynes and Hucker (1946) and Husseman and Tanner (1949) were also of the opinion that any cultural characteristic is unsatisfactory for diagnosis. Thygeson (1938) believed that the mannitol fermentation test is a reliable indicator of the toxigenic powers of micrococci. Toxigenic ones are positive for the most part; non-toxigenic ones are negative. Chapman (1938) has remained confident throughout his studies that the food-poisoning micrococci can be separated as a distinct group by differential characteristics. Thus, according to him, they possess all or a majority of the following characteristics: (1) pigmentation, (2) coagulytic, (3) gelatinolytic, (4) fibrinolytic, (5) lactose fermentative, (6) mannitol fermentative, and (7) hemolytic powers. Through these, he believes that the organism can be thoroughly diagnosed

culturally and has designed several culture media for the differentiation of the food poisoning micrococci.

Dolman and Wilson (1938) found that enterotoxin is a specific antigen. Kittens given a series of injections of filtrates containing enterotoxin acquire active immunity. Immune rabbit and horse serum confer passive protection against the enterotoxin upon normal kittens. By a reciprocal absorption method, involving toxic filtrates prepared from two selected strains of micrococci (only one of them enterotoxigenic), and their respective antisera, a specific floculation reaction was demonstrated between the enterotoxin and its corresponding antibody. However, no other worker in this field has been able to obtain the same results.

PHYSIOLOGICAL STUDY

Materials and methods

The eighteen strains of micrococci used in this experiment are listed in Table I. To assure the purity of the cultures used in this experiment, these strains were streaked on plates containing the Chapman-Stone medium, which Chapman (1948) had devised to promote the more outstanding qualities of strains believed to produce enterotoxin. This medium has the following composition:

Water	1,000.0 ml
d-Mannitol (Difco)	10.0 g
Bacto tryptone	10.0 g
Bacto agar	15.0 g
Sodium chloride, Merck reagent	55.0 g
Bacto yeast extract	2.5 g
K_2HPO_4, anhydrous, Merck reagent	5.0 g
Bacto gelatin	30.0 g
Ammonium sulfate, Baker analysed, A.C.S. pyridine-free	75.0 g
Sodium hydroxide, 10 per cent	6.0 ml

TABLE I.

SOURCE AND HISTORY OF MICROCOCCUS STRAINS

Strain No.	Source of Isolation and History
172[a]	Chipped beef, 1938. Toxic to monkeys, 1938.
178[a]	Ham, 1939. Toxic to monkeys, 1939, 1944.
196[a]	Ham, 1948. Toxic to monkeys on numerous tests.
S-16[b]	Ham, 1934. Coagulase +, Stone reaction -, 1948.
S-31B[b]	Eclair, 1935. Coagulase +, Stone reaction +, 1948.
S-290[b]	Turkey, 1942. Coagulase +, Stone reaction +, 1948.
S-295[b]	Ham, 1945. Coagulase +, Stone reaction +, 1948.
S-296[b]	Pie, 1945. Coagulase +, Stone reaction +, 1948.
S-325[b]	Lamb, 1948. Coagulase +, Stone reaction +, 1948.
9664	Case of food-poisoning, American Type Culture Collection
M-1	Stock culture, Department of Botany and Bacteriology, Montana State College
M-2	"
M-3	"
M-4	"
M-5	Urine of adult with prostatic infection, 1943. Montana State College.
2220-18[c]	Milk, 1943.
2822-2[c]	Milk of mastitic cow, 1946. Hemolytic, 1946.
2908[c]	Combined milk of mastitic herd of cows, 1947.

Cultures obtained from
a) Dr. G. M. Dack, Dept. of Bacteriology, University of Chicago.
b) Dr. R. V. Stone, Director of Public Health Laboratories, Los Angeles Co., Calif.
c) Dr. E. A. Tunnicliff, Dept. of Veterinary Science, Montana State College.

An isolated colony was removed from each of the first set of plates and streaked on a second set. From the second set of plates a colony was removed from each and streaked on a third set. All plates were incubated for 42 hours at 30° C before colonies were picked. An isolated colony from each of the third set of plates was picked into nutrient broth. All media used in testing the physiological reactions of these eighteen strains were inoculated from 24 hour nutrient broth cultures and were incubated at 30° C for a period of one week, unless otherwise specified.

The Stone Reaction and pigmentation were observed on plates containing the Chapman-Stone medium, after 42 hours incubation. The Stone Reaction is essentially that of liquefaction of gelatin within the medium and is determined by the cleared areas immediately surrounding the colonies. The pigment color of the strains was compared with the colors given on Plate IV of "Color Standards and Nomenclature" by Robert Ridgway (1912).

Fermentation studies were carried out on the following carbohydrates: glucose, fructose, xylose, galactose, l-arabinose, d-arabinose, sucrose, trehalose, lactose, maltose, cellobiose, melibiose, raffinose, glycerol, mannitol, dulcitol, inositol, and salicin. Each carbohydrate was made up in five per cent aqueous solution, sterilized by filtration through pyrex brand fritted discs of ultra-fine porosity, and stored at 10° C until used. Five days before use, one ml of each carbohydrate was transferred aseptically to test tubes which contained Durham fermentation tubes and four ml of nutrient broth with brom cresol purple as an

indicator. The tubes were subsequently held at room temperature to check for any contamination. They were then inoculated and observed at various intervals for acid and gas production.

Nitrate reduction, production of indol, hydrogen sulfide, ammonia from peptone, motility, starch hydrolysis, gelatin liquefaction, growth on $NH_4H_2PO_4$, and reactions on litmus milk and brom cresol purple milk were studied on standard media and tested according to the methods presented in the "Manual of Methods for Pure Culture Study of Bacteria" (1948-1949).

Hemolysis of sheep erythrocytes was tested on plates using bacto blood agar base and five per cent sterile, defibrinated blood.

The coagulase test was run according to Chapman's procedure (1946), viz.: "Mix a loopful of culture, grown exactly 42 hours on the special medium (Chapman-Stone agar) with 0.1 ml of bacto brain-heart infusion; shake violently at frequent intervals for exactly 15 minutes and add 0.2 ml of tested blood (previously oxalated). Incubate the mixture, preferably in a water bath, at 37° C and inspect in exactly one hour." Coagulation of the blood is indicative of a positive test.

The test for fibrinolysin production was run according to the technic of Tillett and Garner (1933). The plasma used for the determination was prepared by mixing 20 ml of human blood with one ml of a two per cent potassium oxalate solution. The blood was shaken thoroughly and centrifuged. One-half of the supernatant of each cultured strain, grown in nutrient broth for twenty-four hours, was mixed with one ml

of 1:5 dilution of plasma; then 0.25 ml of an 0.25 per cent calcium chloride solution in normal saline was added; the tubes were shaken thoroughly and kept at 37° C to be observed at the end of 24 hours for lysis of the plasma clot.

Results

Fermentative abilities of the eighteen strains of micrococci are given in Table II. With the exception of strain M-1, all organisms produced acid without gas from glucose, fructose, galactose, sucrose, trehalose, lactose, maltose, glycerol, and mannitol, whereas they produced neither acid nor gas from xylose, l-arabinose, d-arabinose, cellobiose, melibiose, raffinose, dulcitol, inositol, and salicin. Strain M-1 differed from the rest in not fermenting sucrose, galactose, lactose, or mannitol. These fermentations were, for the most part, complete at the end of a 48-hour incubation. However, the tubes were retained for three weeks and observations were made periodically. These results are in agreement with those obtained by Chapman (1940) and others in their studies on the carbohydrate fermentation reactions of staphylococci.

Thygeson (1938) found that mannitol fermentation is a reliable indicator of the toxigenic powers of staphylococci. Straiter (1935) and Chapman (1940) believed the fermentation of lactose and mannitol has some differential value in setting apart enterotoxin-producing micrococci. Strain 9664 failed to ferment mannitol within 24 hours (the other strains, with the exception of strain M-1, did). Strain S-16 produced much less acid in lactose within 24 hours than did the rest of the strains.

TABLE II.

FERMENTATIVE REACTIONS OF MICROCOCCI

Strain No.	glucose	fructose	xylose	galactose	l-arabinose	d-arabinose	sucrose	trehalose	lactose	maltose	cellobiose	melibiose	raffinose	glycerol	mannitol	dulcitol	inositol	salicin
172	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
178	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
196	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
S-16	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
S-31B	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
S-290	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
S-295	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
S-296	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
S-325	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
9664	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
M-1	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-
M-2	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
M-3	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
M-4	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
M-5	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
2220-18	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
2882-2	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
2908	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	-	-	-
*M. pyogenes	+						+		+				-	+	+			-

*Fermentative characteristics for Micrococcus pyogenes var. aureus as given in the sixth edition of Bergey's Manual.

Other physiological reactions, nitrate reduction, indol production, hydrogen sulfide production, production of ammonia from peptone, motility, hydrolysis of starch, liquefaction of gelatin, growth on $NH_4H_2PO_4$, reactions on litmus milk and brom cresol purple milk, pigmentation, liquefaction of gelatin and the Stone reaction are given in Table III.

It was observed after one week's incubation that all strains reduced nitrate to nitrite, produced ammonia from peptone, produced acid in litmus and brom cresol purple milk (with the exception of strain M-1), reduced litmus milk, and produced pigments--from light yellow to deep orange. All strains gave negative results when tested for hydrogen sulfide, indol, starch hydrolysis, growth on ammonium acid phosphate agar, and motility. These are, with one exception, the characteristics listed for Micrococcus pyogenes var. aureus as given in the sixth edition of Bergey's Manual, in which group enterotoxin-producing micrococci are included. The one general exception is that none of the eighteen strains produced hydrogen sulfide on the medium containing peptone, meat extract, sodium sulfite, ferrous sulfate, and agar, whereas Bergey's Manual lists a slight amount of hydrogen sulfide as a characteristic.

The liquefaction of gelatin and the Stone reaction, as observed after various lengths of incubation, are given in Table IV. Twelve of the eighteen strains gave a Stone reaction within a 48-hour incubation period. Three strains (S-31B, 9664, and 2809) later showed the Stone reaction at 96 hours, and one strain (M-1) was considered positive at the end of nine days. Two strains (S-16, M-2) failed to give any

TABLE III.

PHYSIOLOGICAL REACTIONS OF MICROCOCCI ON STANDARD MEDIA

Strain No.	gelatin liquefaction	Stone reaction	NO_3 reduction	H_2S production	NH_3 production	indol production	starch hydrolysis	motility	growth on $NH_4H_2PO_4$	litmus milk	brom cresol purple milk	Pigment
172	+	+	+	-	+	-	-	-	-	AR	A	light cadmium
178	+	+	+	-	+	-	-	-	-	AR	A	aniline yellow
196	+	+	+	-	+	-	-	-	-	ARC	AC	aniline yellow
S-16	-	-	+	-	+	-	-	-	-	AR	A	light cadmium
S-31B	-	-	+	-	+	-	-	-	-	AR	A	maize yellow
S-290	+	+	+	-	+	-	-	-	-	AR	A	apricot yellow
S-295	+	+	+	-	+	-	-	-	-	ARC	AC	Martus yellow
S-296	+	+	+	-	+	-	-	-	-	AR	A	aniline yellow
S-325	+	+	+	-	+	-	-	-	-	ARC	AC	Martus yellow
9664	+	+	+	-	+	-	-	-	-	ARC	AC	lemon yellow
M-1	+	+	+	-	+	-	-	-	-	BCW	B	maize yellow
M-2	+	-	+	-	+	-	-	-	-	ARC	AC	aniline yellow
M-3	+	+	+	-	+	-	-	-	-	AR	A	pinnard yellow
M-4	+	+	+	-	+	-	-	-	-	AR	A	apricot yellow
M-5	+	+	+	-	+	-	-	-	-	ARCW	ACW	Martus yellow
2220-18	+	+	+	-	+	-	-	-	-	AR	A	apricot yellow
2882-2	+	+	+	-	+	-	-	-	-	ARC	AC	light cadmium
2908	+	+	+	-	+	-	-	-	-	ARC	AC	maize yellow
*M. pyogenes	+		+	+	+	-	-	-	-	AC	AC	yellow to orange

*Physiological characteristics for Micrococcus pyogenes var. aureus as given in the sixth edition of Bergey's Manual.

A, acidic; B, basic; C, curd; R, reduction; W, whey.

TABLE IV.

LIQUEFACTION OF GELATIN AND THE STONE REACTION

Strain No.	Gelatin Liquefaction Days			The Stone Reaction Days		
	3	4	5	2	4	9
172	+	+	+	+	+	+
178	+	+	+	+	+	+
196	+	+	+	+	+	+
S-16	-	-	-	-	-	-
S-31B	-	-	-	-	-	-
S-290	-	+	+	+	+	+
S-295	+	+	+	+	+	+
S-296	+	+	+	+	+	+
S-325	+	+	+	+	+	+
9664	-	+	+	-	+	+
M-1	+	+	+	-	-	+
M-2	-	+	+	-	-	-
M-3	-	+	+	+	+	+
M-4	+	+	+	+	+	+
M-5	+	+	+	+	+	+
2220-18	+	+	+	+	+	+
2908	-	+	+	-	+	+
2882-2	+	+	+	+	+	+

indication of the Stone reaction. The Stone reaction was very difficult to ascertain for the most part. Many of the strains which were considered positive merely showed a very narrow zone of decreased opacity adjacent to growth on the plates.

Two strains (S-16, S-31B) did not liquefy gelatin after a five-day incubation period at 30° C. Most of the strains liquefied gelatin within a 72-hour incubation; however, liquefaction became obvious with five of them (M-2, M-3, 9664, 2908, and S-290) only after a five-day incubation. It was found that incubation at 30° C brought about a more rapid liquefaction of gelatin by the organisms than incubation at 37° C. The length of time the tubes were held in the refrigerator after each incubation (in order to determine which strains actually liquefied the gelatin) influenced the observable liquefaction in some of the tubes. When held at 8° C for a period of two hours, eight of the eighteen tubes became solid and any possible liquefaction in them was undiscernible. When held at 8° C for a period of 45 minutes, only two of the tubes showed no liquefaction. The uninoculated control tubes were solid after each of the different cooling periods.

Table V gives the results of the coagulytic, fibrinolytic, and hemolytic abilities of the cultures. All but four strains (M-3, M-4, 2882-2, S-16) showed some fibrinolytic action on coagulated human blood plasma, five strains (M-1, M-4, 2908, S-16, S-31B) did not hemolyze sheep erythrocytes, and all eighteen strains coagulated defibrinated rabbit whole blood to a certain extent. Chapman et al (1934) found that a high correlation exists between the micrococci's ability to produce coagulase

TABLE V.

MICROCOCCUS REACTIONS WITH BLOOD

Strain No.	Hemolytic	Coagulytic	Fibrinolytic
172	4+	3+	3+
178	1+	3+	3+
196	4+	2+	2+
S-16	-	1+	-
S-31B	-	4+	2+
S-290	2+	3+	3+
S-295	2+	3+	-
S-296	3+	4+	4+
S-325	3+	4+	4+
9664	1+	4+	1+
M-1	-	3+	4+
M-2	3+	3+	3+
M-3	2+	3+	-
M-4	-	3+	-
M-5	4+	3+	4+
2220-18	4+	3+	3+
2882-2	3+	4+	-
2908	-	2+	3+

1+ slight
to
4+ complete

- no reaction

and hemolysin and their pathogenicity. Cruickshank (1937) supported the relationship between pathogenicity and production of coagulase, whereas Jordan and Burrows (1935), Kupchik (1937), Dolman and Wilson (1939), and Hussemann and Tanner (1949) could not find the coagulase test or any other physiological characteristic wholly successful in separating the enterotoxin-forming from the non-enterotoxin-forming micrococci.

BIOLOGICAL STUDY

The biological study of the eighteen strains of micrococci consisted of testing culture filtrates for enterotoxin through cat inoculation.

Methods and materials

Favorite and Hammon (1941) devised the following medium for enterotoxin production from micrococcus food-poisoning strains:

Casein hydrolysate	15 g
Distilled water	900 ml
Phenol red (0.2%)	4 ml
Thiamin chloride	5 gamma
Nicotinic acid	1200 gamma

The pH of this medium was adjusted to 7.6 with 40 per cent NaOH and the volume brought to 990 ml. Sixty ml portions of this medium were placed in Kolle flasks and sterilized. The ratio of surface area in contact with atmosphere to the volume of culture medium is high in Kolle flasks; thus, more growth and greater amounts of toxic substances in a given amount of medium are obtained. After autoclaving, one ml of a 25 per cent aqueous solution of sterile glucose was added to the medium of each

flask. They were then inoculated with one ml of 24-hour broth cultures and incubated at 30° C for 72 hours in a pyrex brand 5000 ml vacuum dessicator jar under a tension of 30 per cent carbon dioxide and 70 per cent oxygen. At the end of this period the cultures were filtered through pyrex brand fritted discs of ultrafine porosity and the filtrates transferred to vaccine bottles. The bottles were then immersed in boiling water for 30 minutes to destroy alpha and beta hemolysins and any other thermolabile toxins elaborated by the organisms.

It was impossible to obtain a sufficient number of kittens upon which to test the filtrates, as this part of the experiment was carried out before the kittening season occurring in late spring and early summer. Therefore, it was necessary to test the enterotoxicity of the cultures by cat inoculation. Nine cats, and two kittens of one of the cats, were used to test the presence of enterotoxin in the culture filtrates. Each cat was injected with four different filtrates, three days to a week allowed between injections. Three ml of each filtrate were first injected intramuscularly behind the shoulder blades of the cats in order to see whether injections using this route would elicit food-poisoning symptoms. As no enterotoxic symptoms were observed upon intramuscular injections of the filtrates, intravenous injections, in accordance with the Hammon improved cat test (1941) were used. The inner surfaces of the cat's thighs were clipped of hair. Then pressure was applied in the inguinal region to make the veins "stand out" and the needle inserted into the saphenous vein. This vein, although small, is the easiest vein to work with in the cat. It was found necessary to have the help of another person in order to hold

the cat perfectly still while inserting the needle properly into the vein to prevent its destruction through sudden movement. The two kittens were each given three ml of a filtrate previously showing positive reactions on a cat. This was in accordance with the Dolman kitten test (1938). Actual vomiting was considered a positive reaction.

Results

None of the filtrates from the eighteen strains was found to invoke food-poisoning symptoms upon intramuscular injection of the cats. However, upon intravenous inoculation, cats injected with the filtrates of three strains (178, M-5, S-325) showed typical food-poisoning symptoms with vomiting. The two kittens, each about three weeks old, were inoculated intraperitoneally with three ml amounts of the enterotoxigenic strain S-325 and also showed typical food-poisoning symptoms with vomiting.

The following are descriptions of the symptoms of food-poisoning demonstrated by each cat upon intravenous inoculation with three ml of each filtrate proving enterotoxigenic.

Male cat, age one year:

4:00 p.m. Injected with filtrate from strain S-325. 4:10 appeared nauseated and dejected. 4:12 showed retching motions. 4:20 vomited. 4:20-4:30 vomited twice more. This cat refused to eat during the next two or three days and became much thinner. At the completion of the experiment some 30 days later, it had not yet fully recovered in weight or spirit.

Female cat, age two years:

4:05 p.m. Injected with filtrate from strain S-325. 4:20 showed dejection, nausea, and salivation, followed by restlessness and retching motions. 4:40 vomited. 4:41 vomited. During the next 30 minutes it did not vomit again, but exhibited dejection and lassitude. The next day it appeared to be eating normally and none the worse for having had an attack of food-poisoning.

Male cat, age three years:

3:10 p.m. Injected with filtrate from strain 178. 3:30 showed dejection and excessive salivation. 3:35 retching motions observed. 4:05, 4:08, 4:10 vomited. This was followed by dejection but no additional signs of nausea or retching. The next day it ate normally and showed no visible signs of loss of weight or weakness.

Female cat, age one year:

3:05 p.m. Injected with filtrate from strain M-5. 3:30 appeared nauseated and dejected. 3:35 showed heavy swallowing movements, licking of lips, and unrest. 3:36, 3:37, 3:45 vomited. An hour after injection of the filtrate it still appeared dejected, but the next day it ate normally.

The two kittens showed similar symptoms when injected intraperitoneally with three ml amounts of the filtrate from strain S-325, but nursed normally the next day and appeared to lose little, if any, weight.

Of the ten strains used in this experiment which were isolated from actual cases of micrococci food-poisoning, only two, strain 178 and strain S-325, proved to invoke vomiting when injected intravenously into cats. Evidently the other strains, if they were actually the source of the food-poisoning cases from which they were isolated, had lost their ability to produce enterotoxin after being cultured on artificial media (some as long as fifteen years), whereas those invoking food-poisoning symptoms in the cats had not lost this ability. It is possible that the technic followed to obtain enterotoxin filtrates was at fault, but as each filtrate was obtained in the same way, and as two of them actually produced vomiting in cats, this does not seem to be the cause.

Strain M-5, which also invoked vomiting in a cat, was not isolated from a case of food-poisoning but was obtained from the urine of an individual suffering with a prostatic infection.

The Hammon Cat Test and the Dolman Kitten Test are considered by most workers in this field as being the most reliable means of determining the enterotoxicity of a micrococcus strain.

SEROLOGICAL STUDY

Woolpert and Dack (1933) were the first investigators to demonstrate the antigenicity of enterotoxin. They accomplished active immunization in monkeys without difficulty, but were unable to immunize them passively. Although Dolman, Wilson, and Cockcroft (1936) reported that preliminary experiments indicated that enterotoxin was antigenic, sufficient confirmation of this fact was not forthcoming until Dolman and Wilson (1938) and

Minett (1938) published their observations. Dolman and Wilson demonstrated a flocculation reaction involving the enterotoxin and the specific antibody. However, no other author has been able to confirm this.

Methods and Materials

In this experiment, two rabbits were given a series of injections following a schedule suggested by Boyd (1947). One rabbit was injected with a filtrate from strain S-325 which had showed a positive cat test, the other with a filtrate from strain S-295 which had showed negative results in the cat test. Preceding each series of injections, blood was obtained from the rabbits' ears. Serological tests were run with the serum of this blood in order to determine whether there were any antibodies present directed against the enterotoxin which would give a visible serological reaction. The following immunization schedule was used for both rabbits:

Day	Bled	Dosage	Mode of Inoculation
1	5 ml	1.5 ml	Intravenous
2	-	1.5 ml	Intravenous
3	-	1.5 ml	Intravenous
7	5 ml	4.5 ml	Intraperitoneal
8	-	1.5 ml	Intravenous
9	-	1.5 ml	Intravenous
13	5 ml	4.5 ml	Intraperitoneal
14	-	1.5 ml	Intravenous
15	-	1.5 ml	Intravenous
23	5 ml	—	—

The intraperitoneal injection preceding the second and third series of intravenous inoculations prevented the possible occurrence of anaphylactic shock.

The serum obtained from the above rabbit bleedings was tested for the presence of precipitating antibodies directed against the filtrate according to the procedure outlined in the "Manual of Methods for Pure Culture Study of Bacteria" (1948-1949). Progressively doubled serial dilutions of antigen were prepared in saline beginning with 1:100 and extending through 1:25,000. One-tenth ml of the serum was transferred to the bottom of small tubes and an equal amount (0.1 ml) of each dilution of the antigen (filtrate) was layered onto the serum. The tubes were incubated at 37° C for two hours and observed at 30 minute intervals for precipitate at the juncture of the serum and antigen. They were then shaken and incubated overnight at 4° C. By this time the precipitate should have settled out so that it could be read by a gentle shaking of the tubes. Controls of antigen with saline and serum with saline were included.

Results

Although Dolman and Wilson (1938) demonstrated a specific floculation reaction between enterotoxin and its corresponding antibody obtained from rabbit immunization, no visible serological reaction was observed in this experiment. After a four weeks' immunization period, no reaction whatsoever was observed between various amounts of analogous enterotoxin and rabbit serum run in the precipitation test.

GENERAL DISCUSSION

All but one of the eighteen strains studied gave the same physiological reactions and morphological characteristics as those listed for Micrococcus pyogenes var. aureus in the sixth edition of Bergey's

Manual. Strain M-1 was the interesting exception to this. It gave a basic reaction in litmus and brom cresol purple milk, failed to ferment sucrose, galactose, lactose, and mannitol, and proved to have cells of twice the diameter of the other strains. No other organism listed under the genus Micrococcus in Bergey's Manual is characterized by a basic reaction in litmus milk. Even with these discrepancies, strain M-1 is closer to the species of Micrococcus pyogenes var. aureus than it is to any other organism classified in Bergey's Manual. A possible explanation is that strain M-1 is a variant of the species to which the other strains belong. Variation sometimes occurs among bacteria because of their high generation rate and the tremendously different environmental changes to which they are constantly being subjected.

It was found that incubation at 30° C brought about a more rapid liquefaction of gelatin by the organisms than incubation at 37° C. The length of time the tubes were held in the refrigerator after each incubation (in order to determine which strains actually liquefied the gelatin) influenced the observable liquefaction in some of the tubes. When held at 8° C for a period of two hours, the gelatin in eight of the eighteen tubes became solid and any possible liquefaction in them was undiscernible. When held at 8° C for a period of 45 minutes, only two of the tubes showed no liquefaction. The uninoculated control tubes were solid after each of the different cooling periods. Most of the strains liquefied gelatin within a 72-hour incubation; however, liquefaction became obvious with five of them (S-290, 9664, 2908, M-2, M-3) only after a five-day incubation. Gelatin is generally not liquefied rapidly in the group to which these

strains belong. Two strains (S-16, S-31B) failed to liquefy gelatin at the end of five days.

The Stone reaction, the liquefaction of gelatin in the Chapman-Stone medium, was positive for twelve of the eighteen strains within 48 hours. This reaction was very difficult to ascertain for the most part. Many of the strains which were considered positive merely showed a very narrow zone of decreased opacity adjacent to growth on the plates. Only two of the strains (M-5, S-290) brought about a pronounced clearing (translucency) of the medium. Three strains (9664, 2908, M-1) out of the six which showed no discernible Stone reaction within 48 hours did so when observed at 96 hours and nine days. Of the three strains which failed to give the Stone reaction, S-16, and S-31B did not liquefy gelatin, and M-2 was a slow liquefier.

All eighteen strains coagulated defibrinated rabbit whole blood to a certain extent. For the most part it can be assumed that all strains were isolated from pathological sources (strains M-1, M-2, M-3, and M-4 were obtained from stock cultures which had no history of isolation source). Chapman (1934) and Cruickshank (1937) found that a high correlation exists between the ability of micrococci to produce coagulase and their pathogenicity, but subsequent experiments carried out by other workers (Jordan and Burrows, 1935; Kupchik, 1937; Dolman and Wilson, 1939; and Russemann and Tanner, 1949) could not substantiate this.

Table VI compares the enterotoxicity on cats of the eighteen strains with the physiological reactions which various workers have thought at one time or another to be significant in regard to food-poisoning micrococci. The three strains found enterotoxic to cats (178, S-325, M-5) liquefied gelatin, fermented lactose and mannitol, were coagulytic, fibrinolytic, and hemolytic, and produced a positive Stone reaction within 48 hours.

Four strains (172, 196, S-296, 2220-18) of the remaining fifteen which were non-enterotoxigenic to cats produced the same physiological reactions. The remainder of the strains differed by being negative in one or more of the physiological reactions. If these strains had originally been able to produce these reactions, they had apparently lost this ability after being cultured on artificial media for fairly long periods of time since their original isolation. It is to be noted that the three strains which produced food-poisoning symptoms in cats had been cultured on artificial media for a shorter time than most of the rest of those isolated from food-poisoning cases.

The six strains furnished by Stone (1949) were all found to be coagulase positive when tested in his laboratory in 1948. Our results confirmed this reaction. The Stone reaction he found for the six strains corresponded with ours with one exception. He had found strain S-31B to give a positive Stone reaction whereas it gave a negative Stone reaction in this study. Dack (1948) had found his three strains (172, 178, 196) to be toxic to monkeys shortly after isolation. However, in the present experiment we found only strain 178 to be enterotoxic to cats.

TABLE VI.

COMPARISON OF PHYSIOLOGICAL REACTIONS WITH CAT ENTEROTOXICATION

Strain No.	coagulytic	gelatin liquefaction	lactose fermentation	mannitol fermentation	fibrinolytic	hemolytic	48-hour Stone reaction	enterotoxic to cats
178	+	+	+	+	+	+	+	+
S-325	+	+	+	+	+	+	+	+
M-5	+	+	+	+	+	+	+	+
172	+	+	+	+	+	+	+	-
196	+	+	+	+	+	+	+	-
S-296	+	+	+	+	+	+	+	-
2220-18	+	+	+	+	+	+	+	-
S-290	+	+	+	+	+	+	-	-
M-2	+	+	+	+	+	+	-	-
9664	+	+	+	+	+	+	-	-
M-3	+	+	+	+	-	+	-	-
2908	+	+	+	+	-	+	-	-
2882-2	+	+	+	+	+	-	-	-
S-295	+	+	+	+	-	-	+	-
M-4	+	+	+	+	-	-	-	-
M-1	+	-	+	+	-	-	-	-
S-16	+	-	+	+	-	-	-	-
S-318	+	-	+	+	+	-	-	-

Cats are presumably as sensitive to enterotoxin as are monkeys, so the enterotoxic ability of the other two strains (which produced no reaction in cats) must have been lost.

The only conclusive test for enterotoxin would have to be carried out using human volunteers. However, as Hussemann and Tanner (1949) brought out, the whole filtrates of micrococci thought to be the source of food-poisoning should be used to test their pathogenicity, as all the metabolic products of the micrococci are ingested with the contaminated food. Some micrococci produce lethal toxin, dermatoxin, and hemolysins as well as enterotoxin, and these could be fatal if ingested. Therefore, experimental animals must of necessity be used, even if the eliciting of food-poisoning symptoms within them cannot be considered as conclusive to enterotoxin. At the present time cats are the most commonly used animal to test for enterotoxin. It would be of advantage to find a more suitable animal, however, for cats are difficult to maintain and handle, and they develop an increasing tolerance to enterotoxin after several attacks of food-poisoning.

Although Dolman and Wilson (1938) demonstrated a specific flocculation reaction between enterotoxin and its corresponding antibody obtained from rabbit immunization, no visible serological reaction was observed in this experiment. After a four weeks' immunization period, no reaction whatsoever was observed between various amounts of homologous enterotoxin and rabbit serum run in the precipitation test. W. M. Hammon (1941) was also unable to obtain any visible serological reaction between homologous

enterotoxin and serum.

Workers at various times have thought that some physiological reactions characterized food-poisoning micrococci. However, subsequent experiments by other workers on these have not led to confirmation. Jordan and Burrows (1935), Chinn (1936), Kupchik (1937), Dolman and Wilson (1938), Hammon (1941), Haynes and Hucker (1946), Stone (1949), and Hussemann and Tanner (1949) could not find any physiological characteristic wholly successful in separating the enterotoxin-forming from the non-enterotoxin-forming micrococci. The results of the present experiment indicate the same conclusion.

SUMMARY

Eighteen strains of micrococci, ten of which had been isolated from cases of food-poisoning, were studied as to their physiological reactions and the ability of their boiled filtrates to invoke enterotoxicity in cats upon intravenous injection. An attempt was made to obtain a visible serological reaction between one of the strains (S-325) found to elicit vomiting in cats, and its homologous serum obtained by rabbit immunization.

All but one of the strains gave the same physiological reactions and morphological characteristics as listed for Micrococcus pyogenes var. aureus in the sixth edition of Bergey's Manual, except that none of them was observed to produce hydrogen sulfide. The strain (M-1) differing from the rest gave a basic instead of acidic reaction on litmus and brom cresol purple milk, failed to ferment sucrose, galactose, lactose, and mannitol, and proved to have cells of twice the diameter of the other

strains. Even with these discrepancies, strain M-1 is closer to the species of Micrococcus pyogenes var. aureus than to any other organism classified in Bergey's Manual.

Three of the strains (178, S-325, M-5) were found to be enterotoxic to cats. Two of these (178, S-325) were isolated from cases of food-poisoning, whereas strain M-5 was isolated from the urine of an adult with a prostatic infection. These three strains, in addition to having all the physiological characteristics listed for Micrococcus pyogenes var. aureus, were coagulytic, fibrinolytic, hemolytic, and gave a positive Stone reaction in 48 hours. Four other strains (172, 196, S-296, 2220-18) gave these same characteristics but were not enterotoxic to cats. The remaining eleven strains differed by not giving one or more of these reactions.

No visible serological reaction was observed between various amounts of enterotoxic filtrate and homologous rabbit serum run in the precipitation test.

It was found that incubation at 30° C brought about a more rapid liquefaction of gelatin by the organisms than incubation at 37° C. It was also observed that the length of time the tubes of gelatin were held in the refrigerator after each incubation influenced the observable liquefaction in some of the tubes.

The Stone reaction was very difficult to ascertain for the most part. Many of the strains which were considered positive merely showed a very narrow zone of decreased opacity adjacent to growth on the plates. only two of the strains brought about a pronounced clearing. Some of the strains which showed no discernible Stone reaction within 48 hours did so

when observed at 96 hours and nine days.

The results of these experiments lead one to the same conclusion reached by most of the other workers on food-poisoning micrococci, namely, that at present there is no physiological characteristic wholly successful in separating the enterotoxin-forming from the non-enterotoxin-forming micrococci.

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