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A Differential Medium for the Isolation and Cultivation of Sphaerophorus Necrophorus from Bovine Liver Abscesses

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**A DIFFERENTIAL MEDIUM FOR THE ISOLATION AND CULTIVATION OF
SPHAEROPHORUS NECROPHORUS FROM BOVINE LIVER ABSCESSSES**

BY

CHARLES LESLIE LAMKE

**A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Department of
Bacteriology, South Dakota State
College of Agriculture
and Mechanic Arts**

December, 1960

A DIFFERENTIAL MEDIUM FOR THE ISOLATION AND CULTIVATION OF
SPHAEROPHORUS NECROPHORUS FROM BOVINE LIVER ABSCESSSES

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor

Head of the Major Department

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INTRODUCTION

Sphaerophorus necrophorus is of considerable economic importance since it is the cause of many pathological conditions in a wide variety of animals. One such condition is liver abscess of cattle. This one condition costs the packing industry several million dollars a year.

The investigators have had to alter previous isolation techniques in order to isolate the organism successfully and this has led to a number of techniques. Grant (15) suggested that a selective medium be devised for the isolation and cultivation of S. necrophorus. Beerens (2) has used a veal infusion medium containing brilliant green for the isolation of the organism. This medium does not inhibit the group D streptococcus and great care has to be used in making the medium.

The difficulty of maintaining cell viability has been shared by many investigators. Orcutt (26) and Tunnicliff (32) maintained the organism on their media for several years. Some investigators have had to transfer their cultures once or twice a week to maintain cell viability, and even by doing this, the cultures may be lost in a few months.

The variety of isolation techniques have brought about the suggestion of many media that can be used to cultivate S. necrophorus. Almost all of these media are chemically undefined.

A selective medium utilizing dyes and Difco thioglycollate

medium used is not a chemically defined medium but is less complex than most suggested media.

LITERATURE REVIEW

Sphaerophorus necrophorus has long been considered the organism causing beef liver abscess. This was clearly established in 1949 by Madin (23). The organism has had many different names. Bergy (5) names the organism Sphaerophorus necrophorus (Flügge 1886) Prevot, 1938. Bergy (5) also lists ten other synonyms. Wilson and Miles (33) refer to the organism as Schmorl's bacillus, Bang's necrosis bacillus, and Fusiformis necrophorus. Dack and co-workers (8) list Bacterium necrophorum and Bacterium fundiliformis as additional synonyms.

The organism was first classified by Flügge in 1886, as Bacillus diphtheriae vitulorum according to Kennedy (19). In 1938 Prevot (27) classified the organism as Spherophorus necrophorus. Breed (5) changed the spelling of the genus name in 1955 to Sphaerophorus and the name and spelling is now accepted by Bergy (5).

The following classification appears in Bergy's Manual of Determinative Bacteriology 1957 (5):

Division I Protophyta.

Class II Schizomycetes von Naegeli, 1857.

Order IV Eubacteriales Buchanan, 1917, order of true bacteria.

Family VI Bacteroidaceae Breed, Murry, and Smith, fam. nov., pleomorphic rods.

Genus IV Sphaerophorus Prevot, 1938, 18 species, pleomorphic strict anaerobes, produces spheroids and filaments.

Genus I Bacteroides Castellani and Chalmers, 1919, rounded ends, simple, strict anaerobes.

Genus II Fusobacterium Knorr, 1922, pointed ends, simple, strict anaerobes.

Genus III Dialister Bergy et al., 1923, minute filterable, rod shaped cells, with pointed ends, parasitic, anaerobic.

Genus V Streptobacillus Levaditi, Nicolau and Poincloux, 1925, facultative anaerobes, pleomorphic, L-type growth.

There is more agreement on the morphology of the organism.

Most stains exhibit filamentous forms up to 100-200 microns in length. Bacillary forms and coccoidal cells measuring 0.5 microns have been reported. Bergy states that some authors have reported that branched forms exist. This is not confirmed by Wilson and Miles (33). Smith (30) observed that Sphaerophorus necrophorus forms irregular swellings in the center of the filaments. Kleinsberger-Nobel (20) considers these swellings or round bodies probably equivalent to a sexual process in higher organisms.

Cunningham (7) gave the following as a summation of the earliest investigations concerned with S. necrophorus:

The early work of isolation and identifying Actinomyces necrophorus and correlating the lesions produced by it attracted the attention of some notable bacteriologists. Robert Koch, Loeffler, Theobald Smith, Bang and many others investigated the organism. Dammann probably just observed the first clinical manifestations of Act. necrophorus in his investigation of diphtheritic infections in calves in 1886. He thought, however, that the disease was caused by Bacillus Diphtheriae. Loeffler (1884) disproved this by showing that the predominating organism in the diphtheritic lesions was a gram negative, thin rod with filamentous forms which he found on the border between the sound and the necrotic tissue. Koch had previously found an organism of similar morphology in the sweat glands of sheep having "sheeppox". Schultz (1888) found similar filiform bacilli in the livers of cattle, and succeeded in transferring them into rabbits and mice. Theobald Smith (1889) saw long, gram-negative bacilli in the intestinal lesions of hog cholera. In 1890, Bang identified the organism in hog cholera, and gave it the name "necrosebasillus". Schmorl (1891) isolated and cultured, for the first time, the same organism from necrotic lesions of rabbits during a virulent epizootic. Numerous other workers have subsequently confirmed these results and have investigated the morphologic, cultural and pathologic characteristics of Act. necrophorus in animals.

In 1904 Mohler and Morse (24) made a study of Sphaerophorus necrophorus. They reported that the organism was essentially a pleomorphic organism which varied in size from coccoid forms to filaments over 100 microns in length and 0.75 to 1.5 microns in width. They found that the older cultures of either animal tissues or artificial media exhibit almost exclusively bacillary forms of various lengths, some so short as to be easily mistaken for cocci.

According to these authors, the usual laboratory media are not satisfactory for the cultivation of S. necrophorus, but media employed successfully included: agar-bouillon, agar-gelatin, serum-agar, and serum-agar-gelatin. They experienced difficulty in growing the organism in petri dishes. Neither the formation of a vacuum nor displacement of the air with hydrogen would satisfy the anaerobic conditions required by the organism. Anaerobiosis was obtained by the alkaline pyrogallol method. The colonies that formed on the plates were described as follows: "...small, pinhead-sized, dirty-white, opaque, round colonies, possessing no distinctive features.... Under slight magnification, ...these colonies were seen to possess a yellowish-brown center surrounded by a thin, light, almost translucent border which under the microscope, appeared floccose." Mohler and Morse (24) were also able to isolate the organism by the use of agar-bouillon shake cultures. The production of gas was so great that the shake-cultures were often ruptured.

Holechek (25), in 1919, found that Bacillus necrophorus, as he called the organism, was an absolute anaerobe and that it grew best in

agar stab culture. The organism started to grow at the bottom of the stab near the end of the second day, and would continue to increase upward to within 1-1.5 cm of the top of the stab canal.

Fitch (12) reported in 1919 that serum-agar was the most suitable medium for the growth of Sphaerophorus necrophorus. His main problem was in maintaining viability. To maintain cultures he found it necessary to inoculate a rabbit and recover the organism from the lesions produced. This procedure was carried out every two weeks, and it was not stated in the report how he re-isolated the organism from the rabbit.

In 1923 and 1924, Hagan (16) carried out experiments to test for the production of hydrogen peroxide in cultures of S. necrophorus. He found that brief exposure to air injures vegetative forms and delays or prevents growth of the organism. The injurious substance was found to be hydrogen peroxide. Young, vigorous cultures exposed to air in shallow layers produced sufficient peroxide to give a relatively strong test with benzidine and fresh potato. Hagan found that hydrogen peroxide accumulates for four to six hours and then its production diminishes or ceases. During this time the concentration of peroxide can reach 1:10,000.

Hagan considers cooked meat medium the most favorable substrate for the cultivation of S. necrophorus. He felt that the iron residue of degenerating hemoglobin might be the component of the meat medium which caused the destruction of the peroxide, therefore allowing the organism to grow most rapidly. He also concluded that the reason

plate cultures of Sphaerophorus necrophorus were not successful was because the inoculated medium was exposed to the air from the time the plates were prepared until anaerobic conditions became established in the jar.

S. necrophorus is said to cause many different pathological conditions in a wide variety of animals. Boyd (4), in 1929 found an organism "suggestive of Bacillus necrophorus" occurring among snakes and tortoises. Details of the exact method of isolation were not given, but in the authors words: "After several passages through rabbits we were able to isolate from the liver the S. necrophorus in pure culture".

Orcutt (26) in 1930, made a study of ten strains of S. necrophorus which were isolated from cows. He stated the following about the organism:

Bacillus necrophorus is an important organism in animal disease since it produces severe septic processes in a number of domestic animals. The organism is widely distributed and is responsible for considerable losses. It may invade almost any tissue and is associated with various necrotic foci, as in calf diphtheria, necrotic ulcers of the intestines of hog cholera, foot rot of sheep and cattle, grease heel or necrotic scratches of horses, metastatic necrosis of liver and lungs of cattle and swine, necrotic stomatitis of calves, lambs and pigs.

A few human cases of infection with a similar organism have been reported. These are referred to by Harris and Brown (1927) who isolated from cases of puerperal infection in women cultures closely resembling B. necrophorus and designated by them Act. Pseudo necrophorus.

Orcutt used two methods for the isolation of S. necrophorus. He either made the isolation directly from the abscesses into agar shake cultures, or by passage through a rabbit followed by serum-agar

cultures. After colonies appeared in the serum-agar cultures the agar was removed and placed in a sterile petri dish and a colony was chosen and reinoculated in another serum-agar tube. This was carried out to assure a pure culture of Sphaerophorus necrophorus. The colonies were described by the author as "round or biconvex, smooth, even and compact". He noted that his description did not agree with those of previous investigators.

Oreutt (26) carried out an extensive study on the effect of oxygen on the cultures of S. necrophorus. He exposed the cultures to air by placing a small amount of the bouillon culture in petri dishes so as to have a very shallow layer of medium. At specific intervals of time a small amount of this material was reinoculated into fresh bouillon medium and incubated at 37 degrees Centigrade. Although his results were variable he concluded that the longer the cultures were exposed to air the longer it took for the subcultures to grow. Cultures exposed to air for two hours required one to two days for growth, whereas those exposed for eleven hours required six to seven days to show growth.

In his pathogenicity studies he found the organism to be pathogenic to several laboratory animals. He found that injection of 0.1 ml to 0.2 ml of a bouillon culture into mice produced a spreading necrosis causing the death of the mice in eleven to twenty-five days. He also injected culture filtrates and these caused illness or death within a few hours.

Elder, Lee, and Scrivner (10), in 1931, investigated the

disease known as "calf diphtheria". These workers isolated the organism by two or three passages through rabbits, followed by inoculation of the lesion material into serum-agar shake-cultures. They were not able to obtain growth on plate isolations. "Plates and surface inoculations grown in the Novy jar have not been as satisfactory in our hands."

In 1933, Shaw (29) reviewed the literature on human infections with Sphaerophorus necrophorus. Shaw stated that isolation can be effective with the use of plating or of rabbit inoculation followed by plating. He recommended a medium consisting of dextrose, peptone, veal infusion, and cystine for cultivating the organism. Serum was added to this medium for primary isolation. Shaw was able to obtain streak plates by incubating them in an atmosphere of nitrogen. He described the colonies as "...small, pinhead-sized, dirty-white, opaque, round colonies."

Beveridge (3), in 1934, was able to obtain growth on streak plates and in broth media under anaerobic conditions. He found that he could expose these cultures to air for fourteen days and the cells would still be viable. This finding is not in accord with Orcutt's (26) work on the oxygen tolerance of the organism. Beveridge reported that Sphaerophorus necrophorus is much less susceptible to anaerobic conditions when it is associated with either Staphylococcus aureus or Escherichia coli.

An investigation by Scrivner and Lee (28) in 1934 was carried out to obtain a method for the isolation of S. necrophorus. These

authors had difficulty in isolating the organism in pure culture. They noted that previous workers also had this difficulty. They quoted from Mattam and Carmichel: "Many attempts to isolate B. necrophorus in pure culture proved abortive. This failure is in accord with experience of many other authors who have worked with this organism."

After several attempts to isolate Sphaerophorus necrophorus from calf diphtheria lesions had been tried unsuccessfully by Scrivner and Lee, they finally developed a method which was considered successful. Among the methods tried was the one described by Orcutt (26).

The following steps were included in Scrivner and Lee's scheme:

1. Material from a lesion was injected subcutaneously into a rabbit.
2. The rabbit was autopsied after an average of eight days, and two milliliters of heart blood was inoculated into cooked meat medium.
3. Serum-agar shake cultures were made from the growth obtained in the cooked meat medium.
4. After three days incubation, colonies were picked from the shake-cultures and inoculated into cooked meat medium containing ten per cent serum.
5. Serum-agar shake cultures were again made following growth in the above medium.
6. Colonies picked from the above shake cultures were

inoculated into cooked meat medium enriched with serum, and this culture was considered pure.

The authors could not obtain good growth on ordinary laboratory media. As a standard medium, they recommended a cooked meat medium similar to Hagan's (16).

Scrivner and Lee also reported that the virulence of liver isolants was increased when they were injected into a rabbit in mixed culture.

Feldman et al. (11), in 1936, on the other hand, found it relatively easy to obtain pure cultures of Sphaerophorus necrophorus. Their method was to inoculate the original pus material into Rosensu's dextrose-brain broth, and examine the cultures microscopically after two to five days incubation. These workers stated, "It was not uncommon to obtain the organism in pure culture. Before the culture was considered pure, however, a shake culture was made and then a colony was secured and transferred to dextrose-brain broth."

Dack et al. (8), in 1937, were able to make routine isolations of S. necrophorus from cases of ulcerative colitis in humans. They made their isolations on streak plates of ten per cent blood agar. The plates were incubated anaerobically in Pyrex desiccators which were first evacuated, then filled with carbon dioxide and again evacuated. The remaining oxygen was absorbed by alkaline pyrogallol. They described their colonies as "...greyish, raised and smooth."

Tennicliff (32) in 1938 devised a medium for the continued cultivation of S. necrophorus. His medium was a modification of

Haslam's liver brain medium, which included gelatin, buffer salts, and ferric ammonium citrate. He reported that nine strains were viable after 460 days of storage at room temperature in this medium.

Orcutt (26) maintained a viable culture for three and one-half years but he had to transfer his cultures many times during this period.

Frederick (13), in 1943, reported on the economic and nutritional importance of bovine hepatic disturbances. He included telangiectasis, saw-dust condition, abscess, and distomiasis. The one condition, abscessed livers, was the third highest in the economic loss of the liver. In a seven year period 3,674,514 beef livers were ~~condemned~~ and at an average weight, according to Frederick, of ten pounds, that would be 36,745,140 pounds of liver. He reported that as of August 11, 1942 the average wholesale price was 28 cents a pound, so that a total of \$10,288,639.20 was lost.

This author reported that the invasion route was through the intestine to the portal system to the liver. He stated that no mucous membrane lesion is necessary as a predisposing factor.

Jensen (18) et al., in 1947, made a study as to when the liver abscess formed and their relationship to telangiectasis and saw-dust liver conditions. They concluded that although telangiectasis predisposes liver tissue to abscesses, this predisposing condition is not absolutely necessary for the formation of liver abscesses.

According to these workers, the Union Stock Yards of Chicago, Illinois ~~in~~ reported that in the United States 5% of the condemned livers

are condemned because of abscesses. The monetary loss was estimated to be \$2,000,000 in 1944.

Madin (23) in 1949, as stated previously in this report, clearly established that the organism now known as Sphaerophorus necrophorus is the causative organism of bovine liver abscesses. His study was carried out to determine the bacteriological flora of 55 abscessed bovine livers. He found S. necrophorus in 89.2% of the abscesses tested. He also stated this organism is the "...bacterial agent responsible for pathology seen in bovine liver abscesses."

Grant (15), in 1953, in his summary of the literature, stated that: "In general, the isolation of Sphaerophorus necrophorus is difficult to accomplish. Some workers consider animal inoculation a necessary part of isolation techniques." Grant made his isolation by searing the abscesses with a heated spatula, making an incision with a red hot scalpel and withdrawing the pus with a sterile inoculating loop. Isolation attempts were made using both the original pus material and growth which occurred in the fluid thioglycollate medium (Bacto), Brewer's medium, and Rosenau's medium.

He used shake cultures exclusively at first but later tried using the streak plate method because of its convenience. He made the shake cultures by placing a loopful of pus in a broth made from beef extract and water and inoculating varying amounts of this suspension, 0.2 ml to 1.0 ml, into the shake-cultures. After a few attempts using the standard 1.5 per cent agar he changed to a 1.0 per cent agar to allow greater ease of recovering the agar column from

his shake tubes.

The media he used for these isolation attempts included "...nutrient agar; nutrient agar enriched with sera; double strength nutrient agar, both with and without sera; fluid thioglycollate medium, both with and without dextrose, and solidified with agar; and Formulas 7 and 9, solidified with agar."

Grant found that Sphaerophorus necrophorus could compete favorably with the contaminating bacteria in the pus when thioglycollate medium was used. He used this medium in making shake-cultures and also for the streak plates. He used a 250 mm desiccator for the plates. Two methods for anaerobiosis were used, the first ending in failure because the carbon dioxide tension produced was not satisfactory since the carbon dioxide was taken up by alkaline pyrogallol. Grant developed a method by which the oxygen was absorbed by the alkaline pyrogallol preceding the release of the carbon dioxide. To allow this delayed release of carbon dioxide he placed sodium carbonate in a gelatin capsule which was then placed in a Koplín jar of concentrated hydrochloric acid.

Grant stated: "More than 200 shake-cultures utilizing the media listed in Chapter III, and involving not less than 50 strains of Sphaerophorus necrophorus, were prepared. In general, shake cultures have yielded erratic results." The shake cultures which contained glucose were usually shattered by gas before the colonies were large enough to be transferred. Omission of glucose rendered the media unsuitable for S. necrophorus. The addition of sera produced cultures

with colonies too numerous to allow selecting any one single colony. When Grant used smaller inocula for the serum shake cultures he found no growth of Sphaerophorus necrophorus.

By using his modified method for the production of carbon dioxide he found that he could get colony production on streak plates. When the colonies were transferred to thioglycollate medium they often failed to grow.

It was also stated by Grant in summarizing the isolation attempts that shake-culture colonies transferred to liquid media often developed mixed cultures.

Three other authors (6) (19) (21) later used thioglycollate medium for the isolation of S. necrophorus. Their methods employed fluid thioglycollate. Canada (6) found it difficult to subcultivate the organism after the initial growth from the pus had occurred. The other two (19) (21) were not confronted with this problem as often as was Canada.

The three authors used approximately the same method for the primary isolation attempt. They swabbed the abscess with alcohol or tincture of iodine and removed a small portion of the pus by the use of a two cubic centimeter syringe and an eighteen gauge needle. This material was inoculated into the thioglycollate broth, and subsequently incubated at 37 degrees Centigrade. Although none of the three authors used media specifically selective for S. necrophorus they were able to obtain pure cultures. Kennedy (19) stated: "...forty-six attempts to isolate Sphaerophorus necrophorus were made. Of

these forty-six attempts, 30 percent failed to show growth, 20 percent showed contaminating growth present, and 50 percent gave characteristic isolants showing good growth and apparently an absence of contaminating organisms."

Canada (6) found that injecting mice with Sphaerophorus necrophorus intraperitoneally produced death within 24 hours. The size of dosage was not given by the author. Law (21) was able to inoculate 0.1 to 0.01 ml of a 24 hour culture intraperitoneally into mice. He found that some mice survived for only a few hours after inoculation, whereas others survived for several days. After an incubation time of five to seven days the mice were autopsied and it was observed that the mice had abscessed areas internally. These abscess areas were at the site of inoculation and/or the liver, lungs, and kidneys.

Gottasacker (14) investigated the action of malachite green and its effect upon Escherichia coli and Salmonella typhosa. He was trying to find a method to inhibit E. coli and allow S. typhosa to grow. He found that in the colorless state the dye is ineffective in inhibiting either organism. If the dye is added to a medium and it is inoculated immediately with E. coli, this organism will be inhibited. He stated, "Fresh dye inhibits the growth of coli and allows the Salmonellas to grow, whereas in aged media, the coli will overgrow the Salmonellas."

Ingraham (17) made a study of the bacteriostatic action of gentian violet in relationship to the oxidation-reduction potential. This author concluded that when gentian violet is in a colorless state

it loses its inhibitory power.

Beerens (1) investigated the possibility of the differentiation of anaerobic bacteria by the use of gentian violet. He also used basic fuchsine and thionine but found only gentian violet suitable to differentiate the Ristella, Sphaerophorus, and Fusiformis groups. In this work he found that Sphaerophorus grew in concentrations of 1:20,000.

Beerens (2) in later work also used brilliant green for routine isolation of Sphaerophorus necrophorus.

PROCEDURES

Six separate attempts to isolate Sphaerophorus necrophorus were made utilizing abscessed portions of bovine liver. The livers were made available through the cooperation of John Morrell and Company of Sioux Falls, South Dakota and the Federal Meat Inspectors of the packing plant. The abscessed portions of the bovine livers were cut from the extirpated livers by the federal meat inspectors, placed in plastic bags and stored in an insulated cooler containing ice. The abscesses ranged in size from one centimeter to five centimeters in diameter.

Methods of Isolation

Three methods of isolating Sphaerophorus necrophorus from the abscessed portions were used.

1. The abscessed portions were swabbed with a tincture of iodine solution. Utilizing a two milliliter syringe and 18 gauge needle, one milliliter of sterile fluid thioglycollate broth was drawn into the syringe. One half of this was inoculated into a 22 mm X 175 mm test tube containing 15 ml of sterile fluid thioglycollate broth. This was done to insure sterility of the syringe and needle. The remaining 0.5 ml was injected into the abscess. Using the same needle and syringe, abscess material was withdrawn and this material was placed in a 22 mm X 175 mm test tube containing 15 ml of fluid thioglycollate broth. The tubes

were then incubated at 37 degrees Centigrade. The components of Difco NIH thioglycollate (9) are listed in Table 1. This medium was used as the base medium for all the experiments in this study.

2. The abscessed portions were treated exactly as they were in the first method. After incubation for 24 hours at 37 degrees Centigrade four tenths of a milliliter of the broth containing growth was transferred to a tube containing 15 ml of fluid thioglycollate plus a measured amount of dye solution and these tubes were incubated at 37 degrees Centigrade. The dye solutions were each prepared by adding one gram of dye to 100 ml of distilled water. The dyes included eosin, brilliant green, methylene blue, crystal violet, basic fuchsin, and thionin. One milliliter of dye solution was added to 100 ml of fluid thioglycollate broth. Fifteen milliliters of this medium were placed in 22 mm X 175 mm test tubes and sterilized at 121 degrees Centigrade and 15 pounds pressure for 15 minutes.
3. Five more dye solutions were added (malachite green, ~~hematoxylin~~, congo red, erythrosin, and acid green) to those used in method two. These five dyes were prepared using the same method used for the original five dyes. These five solutions and the preceding six dye solutions constituted the stock solutions of dyes used throughout this study. To the fluid thioglycollate, 1.1 per cent agar was

TABLE I. COMPOSITION OF DIFCO FLUID THIOGLYCOLLATE MEDIUM

Components	Amount per liter
Casitone	15.0 grams
Yeast extract	5.0 grams
Glucose	5.0 grams
Sodium chloride	2.5 grams
l-Cystine	0.5 grams
Thioglycollic acid or Sodium thioglycollate	0.3 milliliter 0.5 grams
Agar	0.75 grams
Resazurin	0.001 grams
Distilled water	1000.0 milliliters

added to make a semi-soft agar. Two tenths, four tenths, and one milliliter of each dye solution were added to 100 ml of the thioglycollate agar. The same method of isolation of the organisms present in the abscess used in method one was used up to the incubation period. After placing the abscessed material from the syringe into the thioglycollate broth, two tenths milliliter of the resulting dilution was placed in a petri dish by using a one milliliter sterile pipette. Two tenths of a milliliter of the same material was also transferred into another 15 ml of fluid thioglycollate. To each petri dish approximately 40 ml of the thioglycollate-dye medium were added. The plates were then rotated slowly to assure mixing of the added organisms and medium. Control plates were made by adding plain thioglycollate agar to plates similarly inoculated. All plates were incubated at 37 degrees Centigrade and observed periodically.

Continued Preservation

The method for continued preservation to maintain cell viability was the same when methods 1 and 2 were used to isolate Sphaerophorus necrophorus from bovine livers. This method included the transfer of the broth cultures to freshly prepared thioglycollate broth every two days for one week. Following this the cultures were transferred every four days and incubated at 37 degrees Centigrade.

Periodically during the cultivation of Sphaerophorus necrophorus a check for virulence was made. This was accomplished by inoculating mice intraperitoneally with 0.1 ml and 0.01 ml of the broth culture suspension.

The mice were observed for symptoms of the disease. The mice that remained alive after seven days were autopsied. Those that succumbed to the S. necrophorus before the end of seven days were autopsied immediately after death.

When the third method of isolation was used the continued preservation was simplified. The plates were sealed to prevent the evaporation of water from the medium and placed in a large desiccator jar which was then evacuated. The desiccator containing the petri dishes was then incubated at 37 degrees Centigrade.

Every week for a period of twelve weeks a colony was taken from each petri dish and inoculated into 25 ml of thioglycollate broth and incubated at 37 degrees Centigrade. The cultures obtained in this manner were serially transferred every three days until cell viability was lost.

Culture Contaminant Determination

Culture contamination was determined by making gram stains of all cultures and by streaking a sterile nutrient agar slant with a loopful of a 24 hour culture. This was a double check for contamination. Considering the pleomorphism of S. necrophorus it is difficult to determine that only S. necrophorus is present in a culture. S.

necrophorus is unable to grow on nutrient agar slants and the contaminants normally found existing in most abscesses are able to grow on the slants.

Morphology

Many shapes and forms of Sphaerophorus necrophorus have been observed from the cultures obtained during the course of this study. S. necrophorus was noted to be a very pleomorphic organism. Changes in environment or media usually changed the morphology of the organism. It was observed that even in the same culture the organism could range from filamentous 40 to 100 microns long to coccoid cells .5 to 1.5 microns in length. The Gram's method of staining was used on cultures in this study. All forms of S. necrophorus were Gram negative.

Broth Cultures

Initial growth of broth cultures arising from the abscess material usually had the long filament form predominating, although small rod shapes were also present. This is shown in Plate I. After several transfers the filamentous form was absent and the rod and coccoid shapes predominated in the cultures (Plate II). When S. necrophorus was transferred from a broth culture, which contains the rod shape of the organism, to the media containing the dyes, the filamentous form predominated. After several transfers in dye media the organism reverted to the rod shaped forms.

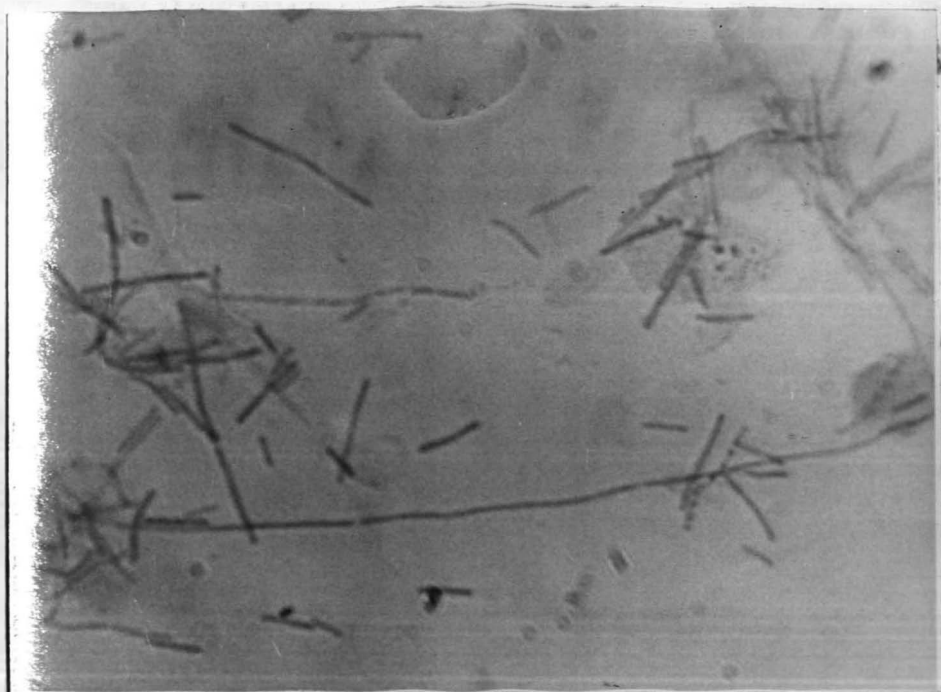


Plate I. Initial growth in broth culture after 24 hours incubation. 2000X. Gram's stain.

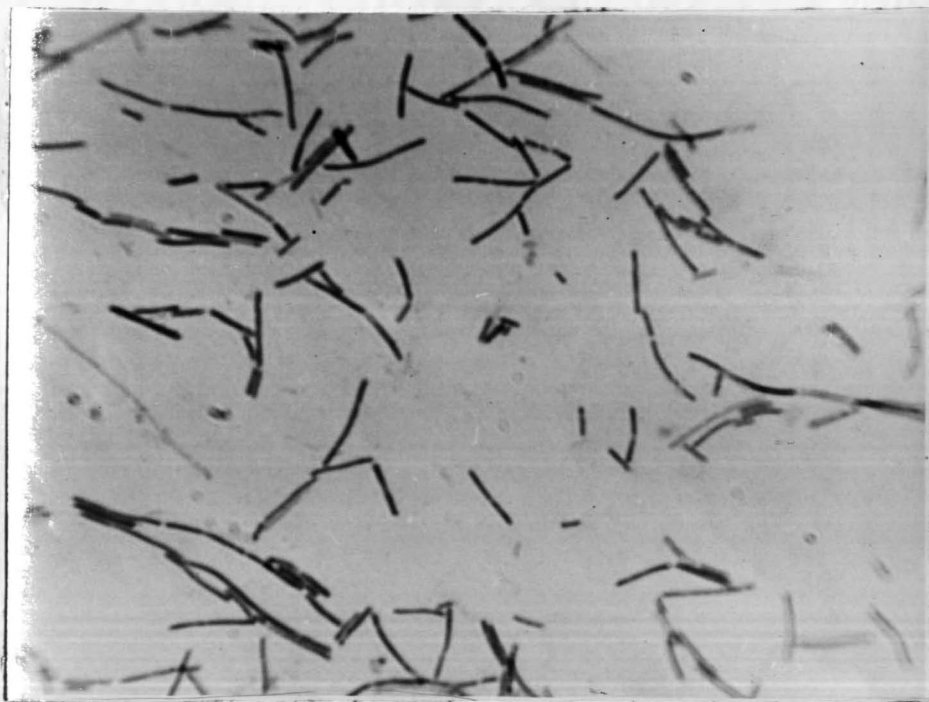


Plate II. Rod and coccoid forms after eight transfers in thioglycollate. 2000X, Gram's stain.

Agar Cultures

Colony growth on the agar plates supported the filaments as the predominant form of the organism. There was less morphological change of the organisms when they were grown in the petri dishes. This was true regardless of the age of the colony. Plate III is of a colony taken from a plate which had been incubated at 37 degrees Centigrade for six weeks. The colonies were taken from the plates with the use of an inoculating loop. The loop was sterilized and then the agar just above the colony was removed. The loop was again sterilized and a circle was cut in the agar around the colony. This agar circle which contained the colony was then placed in the desired medium and macerated. When crystal violet was used as the dye the medium surrounding the colony was decolorized and the colony became more densely colored. When malachite green and basic fuchsin were used the colony became denser, and a precipitate formed around the colony. These observations are in accord with those of Beerens (1).

RESULTS AND DISCUSSION

The first isolation attempt was made using the first method discussed in the procedure. Seventeen abscessed portions were procured for this attempt to isolate Sphaerophorus necrophorus. Of the seventeen cultures obtained, one failed to grow, four were pure cultures of S. necrophorus and the remaining twelve were contaminated with gram positive cocci and bacilli. No attempts to classify the

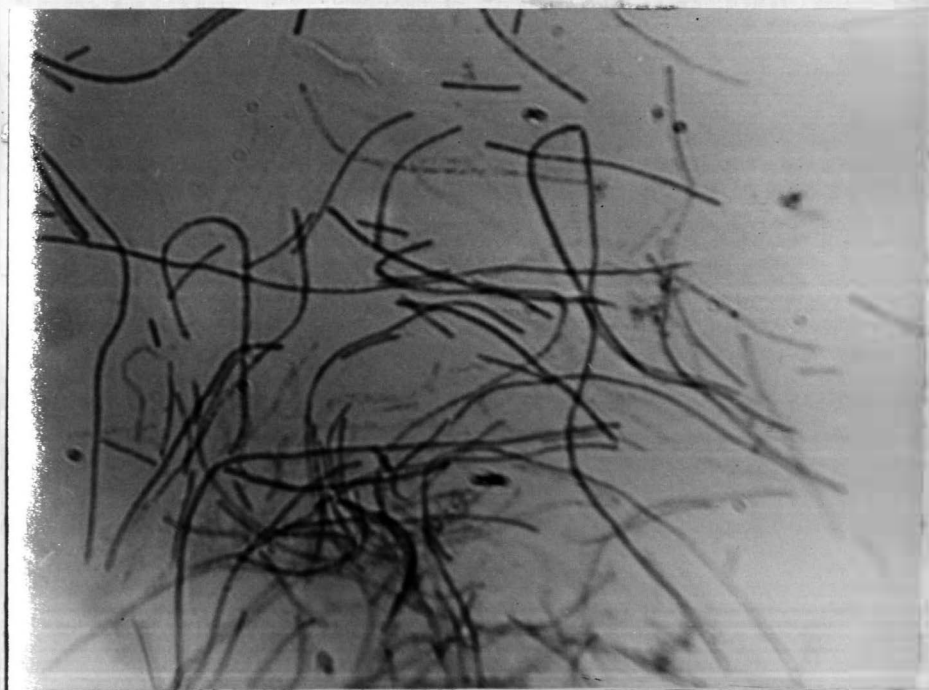


Plate III. Morphology of Sphaerophorus necrophorus grown in agar medium for six weeks. 2000X. Gram's stain.

contaminants were made during the course of study. Beveridge reported that Staphylococcus aureus is found in conjunction with Sphaerophorus necrophorus in many liver abscesses (3).

After four days of cultivation four cultures were chosen to be used for mouse inoculation. Two of these cultures were contaminated with gram positive cocci and bacilli, and two were pure cultures of S. necrophorus. Four mice were used for each culture, of these four mice two were small (approximately 15 grams) and two were large (approximately 35 grams). The mice were inoculated with 0.10 ml and 0.01 ml of the broth culture. One small and one large mouse was inoculated with each of the dosages.

After twelve hours the mice inoculated with viable cultures all showed ruffled fur and were less active than those mice inoculated with sterile fluid thioglycollate medium. At the end of thirty hours two small mice inoculated with a 0.10 ml viable culture succumbed. The autopsies revealed an inflamed area around the site of inoculation. After 48 hours two more small mice and one large one had died. The autopsy revealing an abscessed area at the site of inoculation but no other abscesses could be found in the mice. On the seventh day after inoculation there were eleven mice still alive. At this time the mice were sacrificed and autopsied. Each of the mice had an abscess at the site of inoculation and small abscessed areas on the liver. As these abscesses develop either the internal organs are destroyed or the skin is eroded and the viscera are exposed. The abscesses do not regress. Four mice also had abscessed areas in the

lungs and kidneys. It should be noted that all of the mice that were sacrificed would have succumbed to the infection. No correlation between the pure cultures and those cultures that were contaminated could be noted as to the time required for the mice to succumb to the dosage administered.

The autopsies of the mice used in this study were done aseptically in order that the small abscessed areas in the mice could be removed and cultivated in fluid thioglycollate medium. The abscesses used were removed and placed in tubes containing 15 ml of sterile fluid thioglycollate. These test tubes were subsequently incubated at 37 degrees Centigrade. The sites from which abscesses were procured and the isolant designation are listed in Table 2.

Twenty cultures were made from the mouse abscesses. Of these twenty cultures, eight failed to grow, six were contaminated, and six were pure cultures of Sphaerophorus necrophorus. Three of the pure cultures were from mice that had been inoculated with the contaminated cultures. Three of the contaminated cultures were from mice that had been inoculated with a contaminated culture.

Media containing dyes were introduced into the study at this point. Beerens reported that the bacteriostatic action of dyes has been used by a number of investigators (Rettger, Pratt, Spaulding and Berry) as a selective agent for the isolation, in the presence of pathological materials, of the gram negative anaerobes (1). Beerens has successfully used brilliant green in a veal infusion medium to isolate S. necrophorus in pure culture.

TABLE II. AUTOPSY FINDINGS IN MOUSE INOCULATIONS

Inoculants	Size of Mouse	Dose (ml)	Site of Infection	Resulting Cultures
Ko	Large	0.10	Right kidney	+
Kn	Small	0.01	Left lobe of liver	+
Ko	Small	0.01	Right lobe of liver	o
Kp	Small	0.01	Subcutaneous at injection	■
Kn	Small	0.01	Blood from tail	o
Ke	Large	0.10	Left lobe of liver	o
Kb	Small	0.01	Spleen	+
Kx	Large	0.10	Blood from tail	0
Ip	Large	0.10	Subcutaneous at injection	o
Iv	Small	0.01	Substernal	o
Io	Small	0.01	Blood from tail	0
Ic	Large	0.01	Stomach wall	0
Ib	Large	0.01	Right kidney	0
Ia	Large	0.01	Liver	0
no	Large	0.01	Lung	0
Kp	Small	0.01	Subcutaneous at injection	+
Kx	Large	0.10	Liver	+
On	Large	0.10	Blood from tail	+
Op	Large	0.01	Right lung	0
On	Small	0.01	Right lobe of liver	0

- + = Sphaerophorus growth.
 o = Sphaerophorus growth with contaminants.
 0 = No Sphaerophorus growth.

The dyes and fluid dye media were made as described on Page 18. Of these six dyes two were eliminated immediately. Brilliant green was reduced to a colorless state by the thioglycollate medium. Methylene blue reacted with the thioglycollate in such a manner as to give a precipitate during sterilization.

The pure cultures of Sphaerophorus necrophorus and the cultures known to be contaminated were inoculated into the remaining four dye media (eosin, crystal violet, thionin, basic fuchsin) which were incorporated into the thioglycollate medium by adding 1 ml of the stock solution to 100 ml of fluid thioglycollate. These cultures were transferred every three days for nine days. A gram stain of each culture was made after each transfer and a nutrient agar slant was inoculated to check for the presence of contaminants. S. necrophorus was able to grow in the presence of each dye. Eosin and thionin had little or no effect on the contaminants present in the cultures. Basic fuchsin and crystal violet eliminated the contaminants from four of the five contaminated cultures (Table 3). The one culture that did not yield S. necrophorus in pure culture was very heavily contaminated with gram positive cocci and rods. At the end of twelve days no S. necrophorus organisms could be noted in the Gram stains made from this culture and the growth of the contaminating organisms was reduced markedly. The characteristic odor produced by S. necrophorus when grown in vitro was absent from this culture.

A check was made to ensure that S. necrophorus had not lost its virulence when subjected to the dyes. Four mice were used for each

TABLE III. RECOVERY OF SPHAEROPHORUS NECROPHORUS FROM MICE

Days	Eosin			Thionin			Crystal violet			Basic fuchsin			Thio glycollate		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Transfers															
Isolants															
L ₀	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L _u	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L _c	c	c	c	c	c	c	+	+	+	+	+	+	c	c	c
L _p	c	c	c	c	c	c	+	+	+	+	+	+	c	c	c
Kn	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
Ka	0	0	0	c	c	c	+	+	+	+	+	+	c	c	c
K _b	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+
Iv	0	0	0	c	c	c	+	+	+	+	+	+	c	c	c
I _v	c	c	c	c	c	c	c	+	+	+	+	+	c	c	c
Oc	0	-	-	-	-	-	0	-	-	+	+	+	+	+	+
Op	0	-	-	0	-	-	+	+	+	+	+	+	+	+	+
Or	0	-	-	+	+	-	+	+	+	+	+	+	+	+	+

+ = Pure culture of Sphaerophorus necrophorus.

0 = No Sphaerophorus growth.

c = Sphaerophorus growth with contaminants.

- = No growth.

culture. These included two small mice (approximately 15 grams) and two large mice (approximately 35 grams). One isolant was used for the inoculating organism. This isolant was grown in the fluid thioglycollate and the fluid dye thioglycollate media.

Control mice were injected with sterile thioglycollate medium and with the fluid dye-thioglycollate media. All of these control mice survived and were not autopsied. Cultures of Sphaerophorus necrophorus that had been grown only in thioglycollate were used as controls to compare with those cultures which had been subjected to the dyes. After twelve hours the mice inoculated with viable cultures all had ruffled fur and were less active than the control mice which received the fluid thioglycollate or the fluid dye-thioglycollate medium. At the end of 24 hours one small mouse inoculated with a fluid thioglycollate culture succumbed. The autopsy revealed an inflamed area at the site of inoculation. After 48 hours two small mice and one large mouse succumbed. All three of the mice had been inoculated with the basic fuchsin culture. The area surrounding the site of inoculation was inflamed in all three mice. The remaining twelve mice were alive after seven days. All of the mice inoculated with a viable culture were very inactive and appeared to be very ill from the S. necrophorus infection. The mice were autopsied at the end of seven days. The mice injected with 0.10 ml of the cultures were more heavily infected than those inoculated with 0.01 ml. The mice inoculated with the higher dosage had small abscessed areas on the liver, lungs, intestinal walls and the kidneys. The mice receiving the smaller dosage

had abscessed areas on the lungs and liver. Both dosages produced a large, cheesy, odiferous lesion at the site of inoculation.

The cultures grown in thionin and eosin were contaminated, but the results of the mouse inoculations were the same as those cultures used that were grown in basic fuchsin and crystal violet. The dyes had no adverse effect on the Sphaerophorus necrophorus organism. The characteristic appearance and lesions produced by the cultures exposed to the dyes compared favorably with those from the cultures grown in fluid thioglycollate. The cultures acted in much the same manner as those cultures used in the first mouse inoculation (page 28). Scribner and Lee (28) reported that S. necrophorus was more virulent when injected into rabbits if it was in a mixed flora. This does not seem to be true when mice are used as the experimental animals. The experiment described on page 28 and the one discussed above bear this out. According to Spector (31) the concentrations of dyes and sodium thioglycollate used are not toxic to mice.

The preliminary experiments using the dye-agar medium dealt with the sensitivity of S. necrophorus to the dyes in relation to the dye concentrations which were bacteriostatic for the contaminants. Both acid and basic dyes were used. Congo red, erythrosin, acid green, eosin, and thionin were all eliminated because they had no bacteriostatic action even at as high a concentration as 1:10,000. S. necrophorus was able to tolerate all of these dyes, however. Crystal violet, basic fuchsin, and malachite green seemed suitable for further study when they were incorporated in the thioglycollate agar. Crystal

violet and basic fuchsin were also shown to be effective when they were incorporated in the fluid thioglycollate.

Beerens (1) reported that eleven out of twelve strains of Sphaerophorus necrophorus were not susceptible to concentrations of crystal violet ranging from 1:80,000 to 1:120,000. Gottsacker (14) states that malachite green loses its bacteriostatic action in the reduced state (colorless). During sterilization malachite green is slightly reduced but it is thought that the high concentrations that are able to be incorporated allow enough dye to remain unreduced to maintain its bacteriostatic action.

To further establish that S. necrophorus was not affected by the three dyes (crystal violet, basic fuchsin, and malachite green) concentrations of 1:5000, 1:2000, and 1:1000 were made of each dye. Four plates were used and were incubated at 37 degrees Centigrade and observed for colony formation. The high concentrations of the dyes made it very difficult to observe colony formation; however after one week colonies could be found on all plates containing 1:5000 dyes. There were no colonies on the 1:1000 plates and the 1:2000 plates.

These three dyes, crystal violet, basic fuchsin, and malachite green, were used in further primary isolation attempts, made as previously described on page 19. Twelve abscesses were used in the first attempt to utilize dyes. Ten of these showed contaminating organisms in the control broth tubes and petri dishes. (The concentrations of dye used in the first isolation attempt were 1:50,000, 1:25,000 and 1:10,000). S. necrophorus was obtained in pure culture from the ten

contaminated cultures from all three dyes. All concentrations of the three dyes were effective in removing the contaminating organisms from the cultures.

The petri dishes were incubated at 37 degrees Centigrade for three months. Every week during this time a colony from each plate was transferred to a tube of thioglycollate broth. Two of the colonies chosen failed to grow. The two isolates that were obtained originally in pure culture were transferred in thioglycollate broth every three days. After nine weeks these two cultures were lost. Cultures representing all twelve isolates taken from the plates, after being transferred to the broth, were serially subcultured at three day intervals. These isolates that were transferred serially remained viable for seven to fifteen weeks. The length of time they remained viable in broth was not dependent on the length of time that the isolants were left in the petri dishes. Lee (21), Canada (6) and Kennedy (19) were unable to maintain cultures in broth media longer than two to four months. The isolants they obtained were not grown in the presence of dyes or in a semi-solid medium.

The morphology of the isolants taken from these plates was similar to the morphology of isolants grown in the thioglycollate broth. There were more filaments present when the isolant was growing in the colony form on the plates. This was true of all colonies of Sphaerophorus necrophorus throughout the study.

Four more attempts were made to isolate S. necrophorus from bovine livers. There were twenty-five abscesses used in these four

different attempts. Thirteen of these yielded pure cultures of Sphaerophorus necrophorus, nine showed contaminants in the control medium, and four failed to yield viable cultures. Two of these isolation attempts will be described in this paper.

Eight abscessed portions were procured on January 17, 1960. An attempt to isolate S. necrophorus in pure culture was made using the dye-agar media. Four of the resulting cultures were contaminated and four were pure cultures when grown on the thioglycollate agar control medium. Only S. necrophorus grew on the plates containing the dyes. The 1:10,000 dilution plates were sealed and incubated at 37 degrees Centigrade for six months. At the end of each month a colony was subcultured in fluid thioglycollate. All colonies used up to five months were viable when placed in the fluid thioglycollate. Five of the colonies selected at the end of six months were viable. Tunncliff's (32) modification of Haslem's medium was said to maintain S. necrophorus in a viable state for 460 days. It should be noted that Tunncliff's medium is more complex and more undefined than the media used in this study.

After eighteen weeks of cultivation on the plates, one colony from each plate was selected to be injected intraperitoneally into a mouse. A portion of the colony was drawn into a two milliliter syringe. A 24 gauge needle was then placed on the syringe and one milliliter of sterile fluid thioglycollate was drawn into the syringe. The syringe was shaken gently to assure an even suspension of the colony in this medium. Then 0.10 ml of this suspension was given

to one mouse and 0.01 ml was given another mouse. This procedure was followed for all eight cultures. Only large mice were used in this experiment.

The over-all results of this experiment were the same as those obtained in the two previous mouse inoculations. All of the mice that succumbed to the infection after two days had a small, grayish, ediferous, and cheesy lesion at the site of inoculation. At the end of seven days only five mice remained alive. These mice were sacrificed and autopsied. These mice all had lesions in the liver and lungs.

Six abscess portions of bovine liver were procured on August 25, 1960. The isolation attempt was made using the dye-agar media. Two of these resulting cultures were contaminated when grown on the thio-glycollate agar control medium. The concentration of crystal violet, malachite green, and basic fuchsin used in this experiment was 1:25,000. This concentration was effective in removing the contaminants in the two contaminated cultures. The primary isolation techniques used in this experiment were the same as those described on page 18.

The control plates and dye-agar plates were incubated for fourteen days at 37 degrees Centigrade. At the end of this time a colony was picked from each of four plates for mouse inoculation. The procedure used to remove the colony is described on page 28. Two mice (one large and one small) were injected from each colony. The large mouse received 0.1 ml and the small mouse received 0.01 ml. Control mice were inoculated with the same dosages. One mouse

inoculated with 0.1 ml from the crystal violet colony died within forty-eight hours. The remaining mice were autopsied after seven days of incubation. The two mice injected with material from the basic fuchsin colony were not infected. The remaining mice showed the usual pathological condition previously described in this work. Plate IV is a photograph of the mouse injected with .01 ml of the crystal violet colony. The light areas represent the abscessed areas of the liver and spleen. This photograph is representative of the pathological conditions produced by Sphaerophorus necrophorus cultures in this work.

Plates V through VIII show colonies as they appear on the plates. Plate V is the colonies as they appear when grown in basic fuchsin thioglycollate agar medium; Plate VI is crystal violet; Plate VII is malachite green; and Plate VIII is plain thioglycollate agar. The hazy appearance around the basic fuchsin and malachite green colonies is thought to be a precipitation of the dye. A spreading growth which is apparent in the control plate is not uncommon if the colony arises at the bottom of the petri dish. This effect has been noted a number of times during this study in both control and dye-agar plates.



Plate IV. Visceral lesion in mouse eight days after intraperitoneal inoculation with phaerocephalus necrophorus. 2X.

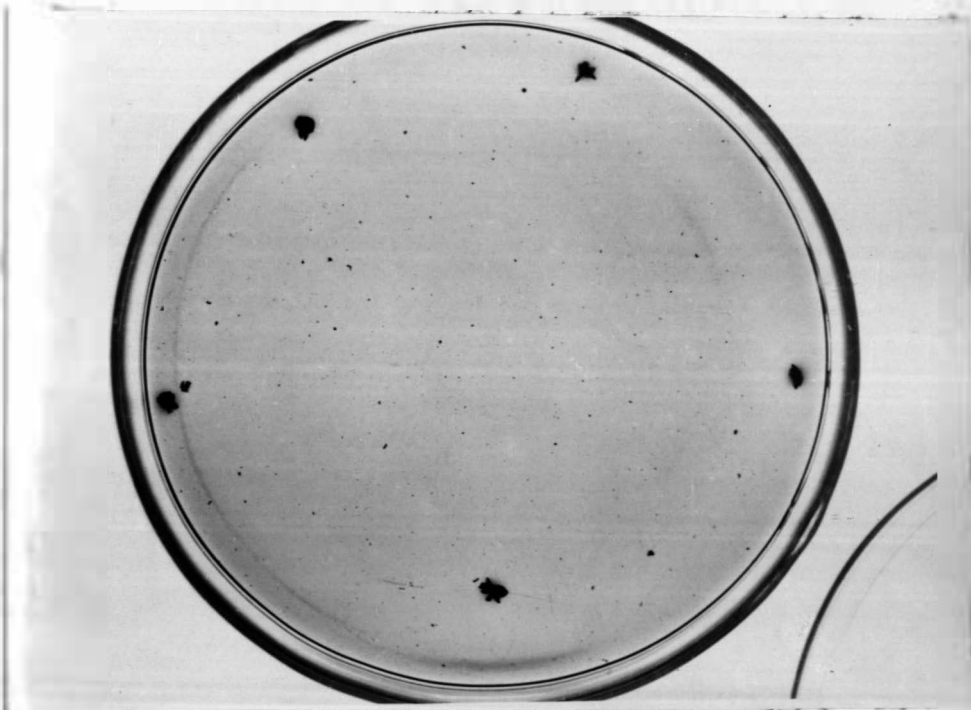


Plate V. Sphaerophorus necrophorus colonies in basic fuchsin-thioglycollate agar. Hazy appearance around colony is precipitated dye.

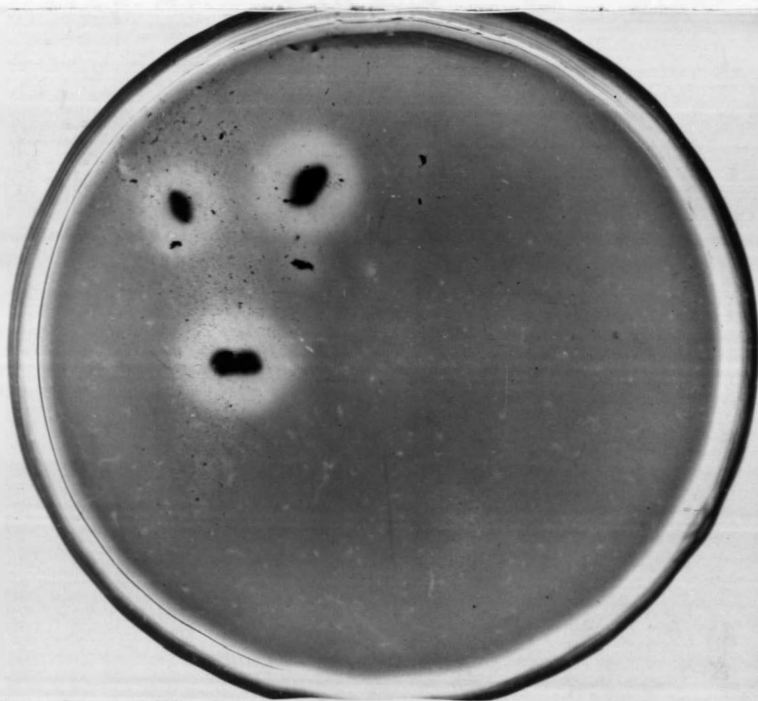


Plate VI. Sphaerophorus necrophorus colonies in
crystal violet-thioglycollate agar.
Clear zone surrounding colony is caused
by reduction of the dye by the organism.

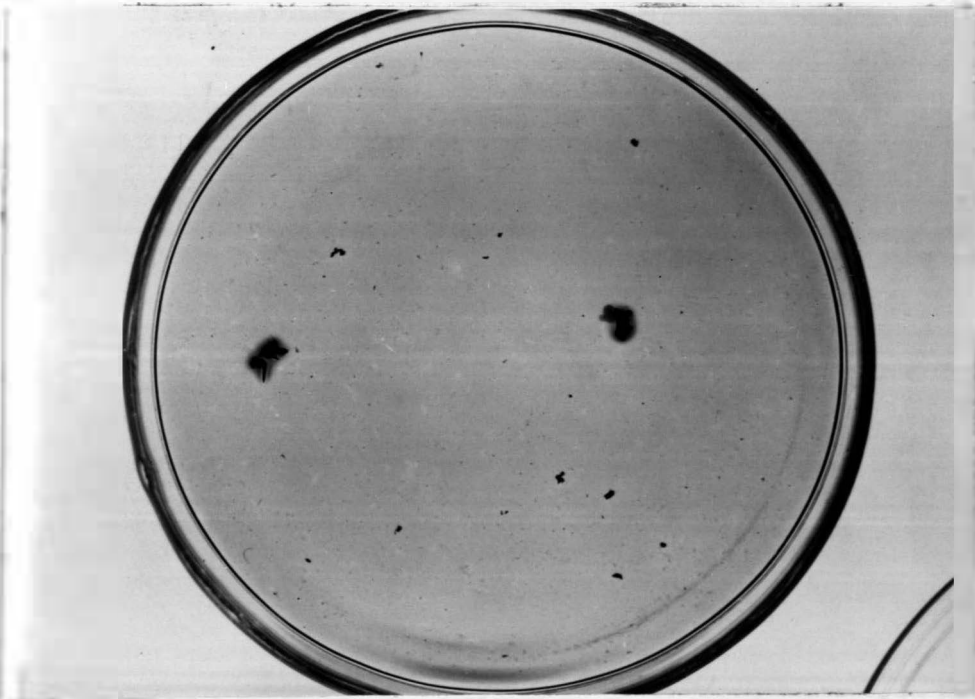


Plate VII. Sphaerophorus necrophorus colonies
in malachite green-thioglycollate
agar. Hazy appearance around
colony is precipitated dye.

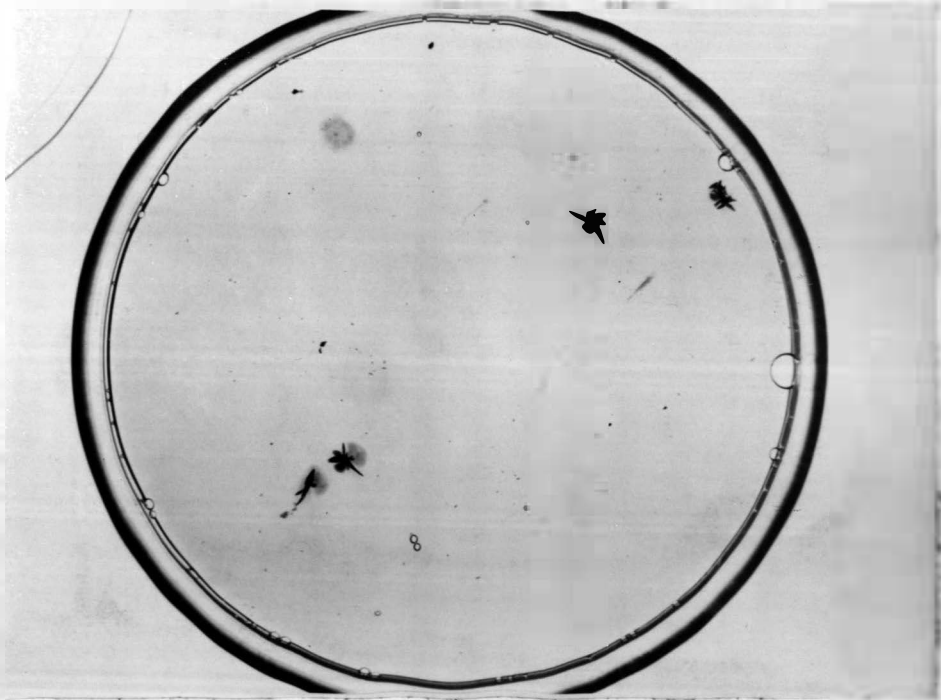


Plate VIII. Sphaerophorus necrophorus colonies in thioglycollate agar. The two lower left hand colonies show spreading type growth.

SUMMARY AND CONCLUSIONS

Culture media are described in which the presence of certain dyes permits primary isolation of Sphaerophorus necrophorus in pure culture from abscessed portions of bovine livers in which contaminating bacteria could be shown to be present. The cultures so obtained were compared with other pure cultures of this organism, and were not found to differ from them in any significant way. In no instance did these media yield contaminated cultures of S. necrophorus.

The conclusions drawn from this work are as follows:

1. Crystal violet, basic fuchsin and malachite green can be used to remove contaminants from Sphaerophorus necrophorus cultures.
2. Viability and pathogenicity are seemingly not affected by dyes.
3. S. necrophorus can withstand high concentrations of dyes.
4. Petri dishes containing thioglycollate agar are effective media for maintenance of S. necrophorus.
5. Mice can be infected by using 0.1 or 0.01 ml of a broth culture or suspension of a colony.
6. Mice surviving for more than two days after inoculation show abscessed areas at the site of inoculation, on the liver and sometimes on the spleen, kidneys and lungs.

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