Cultural characteristics of certain pathogenic anaerobes isolated from sheep
by Theodore Thomas Chaddock

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

Abstract:
1. Three anaerobes isolated from sheep suspected of having died of a disease resembling "black disease" were studied© These were com-pared with a New Zealand strain 7 which had been isolated from a known ease of "black disease".

2. A brain-liver broth medium with a pH of 7.2- 7.4, consisting of minced beef brain, liver broth plus 0.1 per cent dextrose, an iron Wire, and .5 cc. laked sheep blood, was found to produce optimum growth© 3. Optimum growth conditions were determined by direct counts of bacteria in definite quantities of culture media.

4. A medium composed of a sugar free broth, laked sheep blood and an iron wire was found to favor optimum growth in carbohydrate test cultures.

5. Two strains (572 and 591) proved to be Clostridium sporogenes, while strain 590 was Clostridium oedematiens and identical with strain 7. 6. It could not be demonstrated that immunity could be produced by bacterins prepared from strain 590 of Clostridium oedemations.

CULTURAL CHARACTERISTICS OF CERTAIN PATHOGENIC ANAEROBES ISOLATED FROM SHEEP

by

Theodore T. Chaddock

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INTRODUCTION

A disease of sheep occurring in western Montana has been investigated by the Montana Veterinary Research Laboratory. This disease had the characteristics of the "black disease" of sheep described by Turner (10, 11, 12) and Albiston (1) in Australia.

The data recorded in this paper are derived from a study of the disease as it occurred in Montana and deals only with the identification and cultural characteristics of organisms which were isolated from sheep that had died of a disease which resembled "black disease".

METHODS OF PROCEDURE

Strains 572, 590, and 591 were obtained from the livers of two sheep dying of suspected black disease. Strain 7 was obtained from New Zealand and used as a control throughout the work.

Morphology and staining.

These organisms are among the larger anaerobes.

Strain 572 measured 3 to 8.2 microns in length and 1 micron in width.

Strain 591 measured 5 to 10 microns in length and 1 to 1.5 microns in width.

Strain 590 measured 4.5 to 6.7 microns in length and .8 to 1.2 microns in width.

Strain 7 measured 1.5 to 5.5 microns in length and .8 to 1 micron in width.

In young cultures, in a modified brain-liver broth medium (see page 8) the rods usually occurred singly or in filamentous chains of from 2 to 5 individuals. In a medium favoring growth the organisms occurred singly or in pairs, while in an inhibiting medium there was a tendency for the development of filaments.

In hanging drop preparations, the organisms showed sluggish motility.

The bacilli in the vegetative state, stained readily with the most of the ordinary staining reagents and were gram positive; but showed

a tendency, as the age of the culture increased, to lose the ability to retain the crystal violet stain.

Spores formed readily in modified brain-liver broth medium and were located subterminally. They were more oval than elliptical and distended the vegetative rod. In other kinds of media spore formation was greatly diminished or entirely lacking.

The colonies of 572 in deep agar were roughly spherical and clustered. Those of 591 were small, compact, and roughly spherical; those of 590 were small, compact and roughly spherical with woolly borders; those of 7 were small, compact and roughly spherical with woolly borders. The above description of the organisms was based upon the work of Heller (5) on the colony formation of anaerobes in deep agar.

Physiology.

Nutritional requirements.

The bacilli required anaerobic conditions, a rather narrow hydrogen ion range, and a high concentration of protein in the medium. In the preliminary work, Hall's brain medium was used but growth of the organisms was insufficient for investigational purposes. After surveying the literature relative to the conditions favoring the development of the organism causing "black disease" in sheep, thirty-four different kinds of media were inoculated and the results were noted. From these, a medium was selected which favored the maximum growth of the organisms and which later proved to be the best for practical purposes. This was determined by the variation of Breed's direct microscopic counts (3) as

stated by Schaeffer and Fulton (7) in the staining of endospores. By means of a platinum loop of .1 cc. capacity, films from the agitated culture were spread over an area of 1 sq. cm. on a slide and allowed to air dry. This was then flooded with a malachite green solution and heated to steaming three or four times within one half minute. The excess stain was washed under the tap for about one half minute. A 0.05 per cent aqueous, safranine solution was then applied for one half minute after which the specimen was washed, blotted and dried. By taking the average of both vegetative and spore forms of ten fields, it was possible to determine which medium favored sufficient growth for the study of the organisms and which did not.

It was found that the best growth occurred in a modified-brain liver broth medium. This consisted of 0.1 gm. dextrose, 50 gm. of finely minced beef brain and 100 cc. of liver broth. The pH of the medium was adjusted so as to give a pH of 7.2 - 7.4 after forty five minutes of sterilization at 115°C. at 15 pounds pressure. The finer the brain was minced, the more luxuriant the growth of the organisms. About 5 grams of the minced brain was placed in a culture tube to which 10 cc. of liver broth was added. A two inch piece of iron wire introduced into the medium was found to be a factor which affected the growth of the organisms. Scott and Brandly (9) recommended the use of reduced iron for the cultivation of anaerobic organisms. Reduced iron was substituted for the iron wire but gave negative results as did also ferric ammonium citrate. One half cubic centimeter of sterile laked sheep blood was added aseptically to each tube after sterilization, followed by an incubation test of

forty eight hours. This culture medium greatly increased the growth of the organisms, so it was used throughout the work as the basic culture medium.

Reduction of carbohydrates.

A sugar free broth was prepared by the inoculation of a flask of hormone broth with a culture of Clostridium welchii. This was incubated for a period of four days until it was thought that complete reduction had taken place. It was then clarified by the addition of a whipped egg, followed by one hour of sterilization which killed the organisms and coagulated the egg. The reaction was adjusted to give a pH of 7.2 after the final sterilization. After heating again for twenty minutes, the reduced broth was filtered through cotton and filter paper. Since the addition of the laked sheep blood, appeared necessary for active growth, the question arose as to whether the carbohydrates contained in the blood would lower the pH of the medium. This was found not to be the case. To this medium was added an iron wire, and 1/2 cc. of sterile, laked, sheep blood in Dunham's fermentation tubes for the carbohydrate tests. The sterile laked, sheep blood and 1/2 cc. of a sterile, 15 per cent solution of each carbohydrate used was added aseptically. This was then sterilized for twenty minutes at 115°C. at 15 pounds pressure. The test for sterility consisted of incubation for 48 hours, after the addition of the sugars and laked blood.

The presence of the laked blood, precluded the use of an indicator. So determination of acid production of the carbohydrates was made

on each test by use of the La Motte shell vial hydrogen ion set using the indicators necessary to cover the various hydrogen ion ranges.

Table No. I. Reduction of Carbohydrates

Carbohydrate	Strain 572	Strain 591	Strain 590	Strain 7	Control
Dextrose	+	+	+	+	-
Lactose	-	-	-	-	-
Sucrose	-	-	-	-	-
Maltose	+	+	+	+	-
Glycerine	+	+	+	+	-
Galactose	-	-	-	-	-
Levulose	+	+	+	+	-
Salicin	-	-	-	-	-
Raffinose	-	-	-	-	-
Mannose	+	-	+	+	-
Xylose	-	-	-	-	-
Dulcite	-	-	-	-	-
Arabinose	-	-	-	-	-
Mannite	-	-	-	-	-
Inulin	-	-	+	-	-
Sorbite	+	-	-	-	-

+ = acid - = no acid produced

Each test was made in duplicate with the corresponding uninoculated carbohydrate control and the experiment was repeated as a check.

The pH of each culture was determined every five days for fifteen days. No gas production by any of the strains in the different carbohydrates was noted.

Proteolysis

Hydrogen sulphide formation.

The basic medium previously described was used. A small strip of lead acetate paper, held in place by the cotton plug, was suspended in the culture tube about one centimeter above the surface of the medium.

Strains 572 and 591 blackened the lead acetate paper within 3-5 days indicating a distinct production of hydrogen sulphide. Strains 590 and 7 did not blacken the lead acetate paper within that time but showed a partial reaction 10-14 days later.

To demonstrate the blackening of brain, a medium consisting of minced beef brain cooked in an Arnold for thirty minutes was used. The water content of the medium was separated and to it 1 per cent dextrose was added. The brain and the water were then mixed and tubed. An iron wire was placed in each tube and the medium was then sterilized for forty five minutes at 115°C. at 15 pounds pressure. Strains 572 and 591 showed distinct blackening of the brain, but strains 590 and 7 did not.

A 10 per cent nutrient gelatin medium having a pH of 7.4 after sterilization was used to determine gelatin liquefaction. Tests were made at 37°C., at approximately 26°C. and at 15°C. Strains 572 and 591 liquefied the gelatin at all temperatures and turned the sediment black, while strains 590 and 7 neither liquefied the gelatin nor turned the

sediment black.

Action on litmus milk.

Twenty cubic centimeters per liter of a neutral, aqueous solution of litmus added to skimmed milk was used to determine the action of the organisms on this medium.

Strains 572 and 591 in two days reduced the litmus, coagulated the milk and showed much proteolytic action by completely digesting the curd. Strains 590 and 7 did not reduce the litmus and showed no coagulation or proteolytic effects upon the milk within 10-14 days. To determine whether growth had taken place, smears made from strains 590 and 7 showed that moderate growth was present and after two weeks some slight change to acid in the litmus milk was noted.

Coagulation serum liquefaction.

Sterile horse serum was placed in sterile, plugged tubes, and coagulated serum slants were made by heating in an inspissator. These slants were inoculated from recent transfers of the organisms. Cultures by the Wright method for anaerobiosis were made. Tests were twice repeated as checks on the first results. Strains 572 and 591 showed nearly complete liquefaction within 7-10 days while strains 590 and 7 did not.

Oxygen requirements.

All four strains were strict anaerobes. There was no growth on the surface of solid media in the presence of air.

Table No. II summarizes the morphologic and cultural characteristics of the organisms.

Table No. II.

Morphology and cultural reactions of organisms isolated from sheep dead of suspected "black disease".

Strain Number	Morphology	Staining	Spores	Motility	Deep Agar Colony	Gelatin	Litmus Milk	H_2S formation	Coagulated serum liquefaction	Blackening of brain and wire	Dextrose	Lactose	Sucrose	Maltose	Glycerine	Galactose	Levulose	Salicin	Raffinose	Mannose	Xylose	Dulcite	Arabinose	Mannite	Inulin	Sorbite
572	Rods L. 3-8.2μ W. 1μ Rounded ends	Some G - Probably due to age of culture	Oval Subterminal	+	Roughly spherical clustered appearance, Gas	Liquefied Sediment black	Coagulated. Litmus reduced Curd digested	+	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	-	+
591	Rods L. 3-10μ W. 1-1.5μ Rounded ends	Gram +	Oval Subterminal and central	+	Small, compact, roughly spherical Somewhat woolly borders	Liquefied Sediment black	Coagulated Litmus reduced. Curd digested.	+	+	+	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-
590	Rods L. 4.5-6.7μ W. .8-1.2μ	Gram +	Oval Subterminal	+	Roughly spherical clustered appearance, Gas	Liquefied Sediment not very black	No coagulation No litmus reduction. No curd digestion	- Slight blackening as the age of culture increased	-	-	+	-	-	+	+	-	+	-	-	+	-	-	-	+	-	-
7	Rods L. 1.5-5.5μ W. .8-1μ	Gram +	Oval Subterminal	+	Small compact Roughly spherical Woolly borders	Liquefied Sediment not very black	No coagulation Litmus reduction. No curd digestion.	- Slight blackening as the age of culture increased	-	-	+	-	-	+	+	-	+	-	-	+	-	-	-	+	-	-

Pathogenicity.

Strains 572 and 591 were at all times nonpathogenic for guinea pigs while strains 590 and 7 were pathogenic.

Pathogenic strains were kept available by increasing the pathogenicity of the cultures by guinea pig inoculation. The organisms were recovered after the death of the animals, purified and many cultures sealed for storage. Sealed cultures in storage retained their pathogenicity, but those repeatedly transferred on artificial media lost it.

Guinea pigs inoculated intramuscularly with .2 cc. to 1 cc. of a twenty-four hour culture in the modified brain-liver broth medium usually succumbed in 18 to 24 hours. Smaller doses prolonged the time of death. There was pronounced swelling, the edema extending from the inoculated hind leg to the sternum in some cases. There was no evidence of gas in the tissues. The subcutaneous tissues of the inoculated leg and the abdomen were infiltrated with a clear jelly-like exudate. Some cases showed a slightly blood tinged exudate near the site of inoculation. The abdominal viscera were congested. The stomach and small intestines were inflamed and congested, with small hemorrhagic areas. The spleen and liver appeared normal, but the organism generally could be recovered from the liver. It was readily obtained near the site of inoculation. There was little excess of pleural fluid and the lungs appeared to be normal with no hemorrhages visible.

Toxin production.

Filtrates were prepared from cultures of strains 590 and 7 grown

in peptic digest broth. This was prepared by mincing 250 gm. of pig's stomach, 250 gm. of beef, and 250 gm. of beef liver. A liter of water plus 2 per cent hydrochloric acid was added to this mixture. This material was thoroughly mixed and held at 56°C. for 24 hours, and then raised to 80°C. for fifteen minutes. The increased temperature inactivated the enzymes present. The flask of material was then removed from the broth and allowed to stand for two hours. The clear fluid was then poured off and the pH adjusted to 7.4. After heating for fifteen minutes this was filtered through paper.

Three filtrate cultures were made of each strain, using for the toxin producing media, peptic digest broth, modified brain-liver broth and hormone broth plus 0.1 per cent dextrose. Forty-eight hour cultures were filtered through Berkefeld V candles. After sterility had been proven, six 250 gm. guinea pigs were inoculated intramuscularly with the filtrates in 1 cc. doses. Guinea pigs were inoculated with filtrates from cultures of both strains 590 and 7 grown in each of the three media.

All tests for toxic effects of the above strains on guinea pigs were negative. The experiment was repeated using the same filtrates. White mice were inoculated subcutaneously with 1/2 cc. of the filtrates produced from each strain. No toxic effects were noted and the results for toxin production were recorded as negative.

Immunity.

Formolized whole-culture bacterins were prepared from active cultures of strains 590 and 7. At intervals over a period of three weeks,

cultures were made from the bacterin to ascertain if the added 0.5 per cent formolin had killed the organisms. When the organisms no longer grew on a suitable medium, and therefore considered dead, 250 gm. guinea pigs were given repeated injections. The initial dosage of 0.25 cc. of the bacterin was increased to 1 cc. Four injections at five day intervals were given. Following this treatment the two groups of injected guinea pigs were inoculated with .05 cc. and .025 cc. of virulent culture respectfully. Controls were given .025 cc., the amount previously shown to be more than the minimum lethal dose. All the bacterinized guinea pigs and the controls died within 48 hours. There did not seem to be any difference between the bacterinized and control groups. These results led to the conclusion that the methods of immunizing and the type of immunizing material used gave no protection to the animals.

Identification of the organisms.

The first step was to separate the proteolytic, from the non-proteolytic strains. Strains 572 and 591 were proteolytic while strains 590 and 7 were nonproteolytic. Identification of the organisms was based on the following: morphology, character of the vegetative and spore forms, motility, cultural reactions, action on proteins, action on the carbohydrates, and pathogenicity. Identification was based on the chart given below which was taken from Hall's sporulating anaerobic key (4).

"(Spores subterminal <u>rarely central</u>; always when mature swelling the rods into clostridiums.

Motile rods.

Coagulated albumin liquefied; brain blackened; gelatin liquefied.

Lactose fermented ---------- B. aerofetidus.

Lactose not fermented.

Filtrates toxic for guinea pigs on feeding ---- B. botulinus.

Toxic for chickens ---------- type A.

Slightly or non toxic for chickens ---------- type B.

Filtrates non toxic on feeding:

Cultures pathogenic on injection of 1 cc. or less; lytic action peculiar to this species ---------- B. histolyticus.

Cultures non pathogenic except in large doses -- B. sporogenes.

Coagulated albumin not liquefied; brain not blackened.

Gelatin liquefied; usually pathogenic for guinea pigs (see B. novyi).

Lactose fermented.

Saccharose fermented; salicin not fermented ---- B. chauvaei.

Saccharose not fermented; salicin fermented --- Vibrion septique.

Lactose not fermented ---------- B. novyi)"

Strains 572 and 591 may, by the use of this key, be identified as Clostridium sporogenes while strains 590 and 7 fall under the classification of Clostridium novyi.

DISCUSSION

Since strains 572 and 591 have been determined to be Clostridium sporogenes, they may be considered of no significance in relation to black disease. Strains 590 and 7 were considered to be same species with some slight differences in the fermentation of the carbohydrates. The cultural characteristics of both organisms were identical, and the only variation in the fermentation reactions was that strain 590 fermented inulin and 7 did not.

Strain 590 was slightly longer than strain 7. Otherwise no differences in morphology were noted.

Both strains were subjected to the different tests at the same time. Duplicate tests and controls were made in each case. Much of the cultural work was based on the work of Turner (10, 11, 12) and Hall (5). The 590 strain compared favorably with the strains isolated by Turner and Hall in respect to cultural reactions, but appeared to be smaller in size. There were some differences in the fermentation reactions between strain 590 and the organism isolated by Turner. Strain 590 fermented dextrose, maltose, glycerin, levulose, mannose, and inulin, while the strain (B. D. 8) of Turner of Clostridium oedematiens fermented glucose, maltose, levulose, and galactose, with no fermentation of glycerine.

Table No. III shows the comparison of the 590 strain with other strains of similar organisms. According to Turner (12) the strains listed in this table include one human ("Weinberg"); one equine ("B.

novyi 139"), and eight strains isolated from "black disease", whose reactions were identical and are listed as B.D. 8. Other strains recorded for comparison are, one strain described by Edgar (B.D. bacillus), two strains described by Zeissler (B. oedematiens and B. gigas), Clostridium oedematiens described by Bergey (2) and a strain described by McEwen (6) (B. paludis) isolated from sheep.

Table No. III.

	Dextrose	Lactose	Sucrose	Maltose	Glycerine	Galactose	Levulose	Salicin	Raffinose	Mannose	Xylose	Dulcite	Arabinose	Mannite	Inulin	Sorbite
New Zealand Strain 7	+	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-
Montana Strain 590	+	-	-	+	+	-	+	-	-	+	-	-	-	-	+	-
"Weinberg"	+	-	-	+	+	wk	-	-	0	0	0	-	0	-	-	0
Hall "Novyi 139"	+	-	-	+	-	wk	wk	-	0	0	0	-	0	-	-	0
"B.D. 8"	+	-	-	+	-	+	+	-	0	0	0	-	0	-	-	0
New South Wales Edgar. B.D. bacillus	+	-	-	+	0	+	+	-	-	0	+	-	0	-	0	0
Zeissler. B. oedematiens	+	-	-	±	+	-	-	-	-	0	0	-	0	-	-	0
Zeissler. B. gigas	+	-	-	-	-	±	+	-	-	0	0	-	0	-	-	0
Bergey's Manual of Determinative Bacteriology: Clostridium Oedematiens	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-
McEwen B. Paludis	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

+ = definite acidity. - = no acidity. 0 = not tried.

wk. = weak acidity production.

From the above table it may be seen that the Montana 590 strain was nearly identical in reaction with some that are listed, but differs in others. According to Bergey (2) Clostridium oedematiens ferments dextrose, maltose, galactose and levulose. The 590 strain did not ferment galactose. In addition to fermenting dextrose, maltose, and levulose, it fermented glycerine, mannose and inulin.

Scott (8) (9) stated that the different media that have been recommended for the production of toxins by anaerobes have failed to reveal the presence of such toxins. This may explain why the 590 strain did not produce a toxin.

From a survey of the literature concerning the cultural and fermentation reactions of Clostridium oedematiens, it is to be noted that strain 590 compared favorably with the cultural reactions stated by other investigators, but that there was some difference in the saccharolytic properties.

SUMMARY AND CONCLUSIONS

1. Three anaerobes isolated from sheep suspected of having died of a disease resembling "black disease" were studied. These were compared with a New Zealand strain 7 which had been isolated from a known case of "black disease".

2. A brain-liver broth medium with a pH of 7.2 - 7.4, consisting of minced beef brain, liver broth plus 0.1 per cent dextrose, an iron wire, and .5 cc. laked sheep blood, was found to produce optimum growth.

3. Optimum growth conditions were determined by direct counts of bacteria in definite quantities of culture media.

4. A medium composed of a sugar free broth, laked sheep blood and an iron wire was found to favor optimum growth in carbohydrate test cultures.

5. Two strains (572 and 591) proved to be Clostridium sporogenes, while strain 590 was Clostridium oedematiens and identical with strain 7.

6. It could not be demonstrated that immunity could be produced by bacterins prepared from strain 590 of Clostridium oedematiens.

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