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# A Study of Streptobacillus Moniliformis Causative Agent of Rat Bite Fever

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# A STUDY OF STREPTOBACILLUS MONILIFORMIS
# CAUSATIVE AGENT OF RAT BITE FEVER

By

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CAUSATIVE AGENT OF RAT BITE FEVER

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.

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HJB

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# INTRODUCTION

A bizarre organism was isolated in 1953 in a Castaneda blood culture flask* in an attempt to determine the cause of septicemia in a child. It was discovered that the organism causing this septicemia was morphologically and physiologically similar to one of the organisms causing rat bite fever, which to this time had never been isolated in South Dakota. It was impossible under the circumstances to make any positive identification of the orgamism. Microscopic slides were saved with the expectation that circumstances would become favorable for research with the suspected organism, Streptobacillus moniliformis. (See plate I page 2).

It was decided that a study centered around the organism and its intriguing relationship to animals and man would be appropriate for the research problem. It seemed desirable to study the localization of the bacteria in the tissues of embryos and if possible, of postnatal animals to see whether any light could be shed on the role of sensitization and allergy in the localization of the organisms. Certain of the symptoms described by Levaditi, Nicolau and Poincloux (11) resemble symptoms of rheumatic fever, in which sensitization and allergy are thought to play an important part. It was anticipated that in mammalian embryos and in very young animals the bacteria might show less tendency to localize than was found in mature laboratory animals. The following studies were accordingly undertaken using methods perfected by Woolpert (30) for cultivation of the streptobacilli in fetal rabbits, and various attempts

*Castaneda - this technique is a tryptose phosphate agar slant with a broth overlay of the same base medium.

PLATE I. The organism isolated from septicemia in 1953 (estimated 2000x).

were made to induce successful infection in new-born rabbits.

The methods of intrauterine inoculation are very powerful tools in the hands of a bacteriologist working with fastidious organisms, and it would constitute a valuable addition to the skills of an investigator.

## REVIEW OF LITERATURE

The first reference to the dangers associated with the bite of a rat was made by Wilcox (28) in 1839. A few years before this Professor Ives of Yale lectured on the dangers of a rat bite. H. Miyake (14) was the first to report the disease in Europe. He introduced the term Rattenbisskrankheit and is the first on record to have tried to find the etiological agent involved in rat bite fever. Ogata (17) also used the term Rattenbisskrankheit in his literature. He probably was the first to successfully grow the organism Streptobacillus. He classified the organism as a member of the Aspergillus group, because of its filamentous form and infected rabbits and guinea pigs from lymph gland biopsy material taken from a patient with rat bite fever. Hata (8) in 1912 used arsphenamine in the treatment of rat bite fever. Schottmüller (22) in 1914 published a classical report of the disease and told of the isolation of a Streptothrix from his patients, one bitten by a rat and one bitten by a ground squirrel.

In 1916 Blake (3) reported the isolation of a similar organism in the United States. Several investigators about this time report failures in their attempts to isolate organisms from patients bitten by a rat. In some cases blood smears revealed organisms which were filamentous in nature, though Litterer (12) was the first to describe the organism using the term Streptobacillus with reference to its morphology. Levaditi, Nicolau and Poincloux (11) recovered this filamentous organism from the blood of a human who had no history of rat bite, and they called the organism <u>Streptobacillus moniliformis</u>, the name which is in common use today.

Levaditi, Nicolau and Poincloux (11) diagnosed the disease as polymorphic erythemia. A review of the symptomatology includes: (1) septicemia of sudden onset, (2) several isolated attacks in the first weeks of illness with subsidence of symptoms from each attack marked by erythematous eruptions on the extremities, (3) joint pains and (4) enlarged spleen. The body showed two responses to the invasion of the blood stream, a generalized rash and localized nodules. Positive cultures were made by introducing 1.5 ml. of blood into 150 ml. of broth. Blood cultures are described as a fibrinous veil floating above the blood cells and after three days small white colonies are seen which increase in volume and finally break apart, forming fluff balls. Chains of bacillary forms make a tangle giving rise to the fluff ball appearance. Coccoid forms may appear isolated, in pairs, or in clusters. Some bacillary chains contained a thickened area in the center which give rise to the name moniliformis. The most consistent characteristic of these organisms is their pleomorphism. The broth surrounding these colonies remains clear and in some cases it is sterile other than where the fluff balls are growing.

A good growth requires at least 10 per cent serum which has been inactivated at 60 degrees centigrade for one hour and added to bouillon at a pH 7.4 to 7.8. In milk the organism grows without the benefit of serum. Upon transfer, filaments up to 20 to 30 microns long appear, or there may occur fusiform bacilli which contain chromatic material. In some media the fusiform bacilli predominate. The fusiform rods begin to appear in unfavorable media four to six hours after inoculation. They reach the maximum in 24 hours and then degenerate. Levaditi, Nicolau and Poincloux (11) considers this as a resting stage, more resistant, but

not the result of aging. The organism stains poorly with the usual aniline dyes. It is not acid fast, but weakly Gram negative and stains slowly with Giemsa. The optimum temperature for growth was found to be 37 degrees centigrade, and no growth was found to occur with ordinary media. In defibrinated blood the organisms grow on the walls of the tubes since there is no fibrin to hold them down. Ascitic fluid can be used in place of serum with equally good results. The organism grows especially well if rabbit testicle is added to the media. On agar containing blood, ascitic fluid, testicular extract, the organism grows as a tiny transparent colony. The organism will tolerate crystal violet in concentrations strong enough to inhibit growth of contaminating organisms, forming very small colonies. Streptobacillus will not grow on coagulated serum media. The vitality of this organism is a limiting factor in artificial cultivation. It is preferable to transfer every three days to fresh media if the cultures are kept at 37 degrees centigrade. They should be transferred every four days if they are grown at room temperatures. The organism is killed at 60 degrees centigrade in one-half hour. Vacuum and desiccation readily kill the organism (11).

In their test for virulence Levaditi, Nicolau and Poincloux (11) found that the most sensitive laboratory animal was the rabbit. Levaditi's method for rabbit inoculation is as follows:

1. Inoculation intratesticulary and orchitis develops with a generalized purulent inflammation.
   a. Smears and cultures from the involved organs are positive.

2. Intravenous inoculation which gives rise to three possibilities.
   a. The animal may survive and be immune.
   b. May develop arthritis.
      1) may survive and be immune
      2) may die with enlarged lymphoids and have positive blood culture.

3. Inoculate, then shave the flanks: an erythema is developed in 48 hours with nodules the size of lentils. Often the bacteria can be isolated from the lesions. The animal may survive and be immune or it may die from a generalized infection.

4. Inoculate on the shaved skin and a scabby erythema develops and the animal survives and is immune.

The work on virulence proved among other things that the organism has an affinity for rabbit tissue. It also proved that the organism is antigenic and the animals can develop an immunity.

The first American group to discuss the organism from a medical standpoint were Place, Sutton and Willner (19). Two cultures were taken from the joint fluid of the human cases by aspiration. From this joint fluid culture pleomorphic Gram negative rods were isolated. At the time of their first publication the organism had not been isolated, but the epidemic was recognized as a new disease and was called "Haverhill Fever", although the symptoms were very similar to those described by Levaditi, Nicolau and Poincloux (11) in France.

The report of the Boston City Hospital (18) followed the article discussed above and the organism was then definitely established as Streptobacillus moniliformis.

The first case of Haverhill Fever caused by Streptobacillus associated with the bite of rat was reported by Scharles and Seastone in 1934 (21). The patient's serum agglutinated the organism in high titer. It was pointed out by Hazard and Goodkind (9) that even in the absence of antisera the organism has such specific characteristics that its identification is possible by morphological and physiological means.

By 1935 the taxonomists were concerned with the problem of properly classifying this versatile organism. There were 22 names given to the organism by various workers. These names included those listed in Table 1.

TABLE 1

Various names used to designate Rat Bite Fever organism

| Name of Organism | Man | Reference | Year |
|---|---|---|---|
| Streptothrix muris ratti | Scottmueller | 22 | 1914 |
| Streptothrix | Tunnicliff | 26 | 1916 |
| Streptothrix longus | Litterer | 12 | 1917 |
| Streptothrix brevis | Litterer | 12 | 1917 |
| Streptothrix putorii | Dick and Tunnicliff | 6 | 1918 |
| Bacillus actinoides | Smith | 23 | 1918 |
| Actinomyces actinoides | Bergey | 1 | 1923 |
| Streptobacillus moniliformis | Levaditi | 11 | 1925 |
| Haverhillia multiformis | Parker and Hudson | 18 | 1926 |
| Actinobacillus actinoides | Topley and Wilson | 24 | 1929 |
| Bacillus actinoides var muris | Nelson | 15 | 1930 |
| Actinomyces muris | Topley and Wilson | 25 | 1936 |
| Actinobacillus actinoides | Bergey | 2 | 1939 |
| Actinobacillus muris | Nelson | 16 | 1940 |
| Murimyces streptobacillus moniliformis | Sabin | 20 | 1941 |
| Asterococcus muris | Heilman | 10 | 1941 |

The lack of a generally acceptable classification had led to the "discovery" of the organism many times. Since the work with this thesis does not deal with taxonomy and there is no wish to add any further names to the overgrown list the term Streptobacillus moniliformis will be used in this paper. This name is chosen because it is the most descriptive suggested, and because it appears most often in current literature. However, it has been pointed out in Bergey (2) that the genus Streptobacillus is invalid because it has been used prior to 1926 to describe an organism in the Lactobacillus group.

Topley and Wilson (26) in 1936 discussed the organism. These workers preferred the term Actinomyces muris. The most extensive description of morphology is based on growth on Loeffler's serum media. It is as follows:

> "Slender branching filaments, 0.4 microns to 0.6 microns wide growing in interwoven masses. After 18 to 24 hours fragmentation of the filaments sets in, many of the filaments are replaced by

chains of bacillary or coccoid bodies. Very marked pleomorphism. Occasional filaments show spherical, oval fusiform or club-shaped swellings occurring terminally, subterminally, etc. - hence the term moniliformis...."

".... great irregularity in staining. Non motile. Usually Gram negative but may be Gram positive in young cultures. Non acid-fast."

The pathogenicity of the organism is discussed by Wilson and Miles (29) who have found that it may give rise to an epizootic disease in mice characteristiced by swelling of the feet and hind legs, arthritis, conjunctivitus and lymphadenitis. Intraperitoneal inoculation of mice with 0.5 ml. of a serum broth culture is usually fatal in one to two days. There is no characteristic post-mortem appearance.

Heilman (10) found that the bacillus would also grow in human erythrocytes washed in saline and added to veal infusion. One egg yolk mixed with 150 ml. of nutrient agar would support growth as well as an ether extract of egg yolk or fresh cream or hot alcohol extract of Mycobacterium phei. Soluble starch contains a growth factor for this organism that cannot be replaced by celliobiose, dextrose or any carbohydrate. Ten per cent carbon dioxide gave a slight stimulation to the initial growth.

In 1936 Van Rooyen (27) reported that he successfully infected the chorioallantoic membrane of the developing chick. At three day intervals he would inoculate the chorioallantoic membrane with a ground membrane emulsion. The virulence of the organism was maintained after three passages. The membranes thickened and became edematous and necrotic with hemolysis and ulceration.

In 1944, Buddingh (5) like Van Rooyen inoculated the chorio-allantoic membrane and found that the organism invaded the embryo and

became localized almost exclusively in the synovial lining of the joints where it appeared to grow mainly as an intercellular parasite.

Brown and Nunemaker (4) published the results of their extensive work with rat bite fever in 1942. They have chosen to use the term Streptobacillus to describe the organism because of its descriptive value and because the majority of bacteriologists refer to it as such. It also antedates Haverhillia multiformis. These authors do recognize the more correct taxonomic designation of the organism as Actinomyces muris.

They point out that in order to fully understand the biological complexities of this microorganism it is necessary to consider the filterable element that can be observed in all cultures of Streptobacillus. This is the L form of the bacteria. For cultivation of Streptobacillus moniliformis a departure from the standard culture procedures is necessary. The growth requirements of both the bacillary form of the organism and the L form are much the same as for Pleuropneumonia-like Organisms (PPLO). The methods of growing PPLO are not in use in the routine clinical laboratory. The materials and techniques necessary for growing the organism are not complex but they demand strict adherance to a few definite rules. In each of eight cases of rat bite fever reported blood cultures were done as they are routinely done in the ordinary clinical laboratyr and all were negative. When parallel cultures were done, in which the special techniques were used, the blood cultures were consistently positive.

Two basic types of media were used by Brown and Nunemaker (4). They were tryptose phosphate broth and dextrose starch broth (7). The preferred pH was 7.6 although there was adequate growth from pH 7.0

to 8.0. The most important item in the media is some form of serum protein. They tried a variety of serums but found horse serum to be the best. Since ascitic fluid was readily available to these workers (4) it was used routinely for all their cultures. The best growth was obtained when the fluid or serum was added to the medium immediately before inoculation. For convenience of culturing, vials containing four milliliters of the basic medium were stored in the ice box until needed, at which time the protein component was added in one milliliter amounts making a 20 per cent solution. In no instance in which ascitic fluid was used did they fail to recover organisms when they could be recovered with serum.

The organism would not tolerate the usual amount of agar in the media. The softest possible agar, 1.25 per cent, gave the best results. When the cultures were made to recover or observe the L form of growth, it was important to have a very clear medium and clear petri dishes. Examination with the low power lens of the microscope was possible through the petri dish bottom. It was unnecessary to open the plates when examined in this manner, thus eliminating surface contamination. Paraffin was used to seal all plates after inoculation as it was necessary to prevent the plates from drying.

When blood cultures were made, citrated blood was used and it was concentrated by centrifugation. Fifteen milliliters of whole blood from the patient were introduced into a small flask containing 8 to 10 milliliters of 2.5 per cent sodium citrate. Ten milliliter amounts of the citrated blood were centrifuged for 30 minutes at 2500 revolutions per minute. The supernatant was discarded, and the blood cell residue was used for culture.

The serum medium was inoculated with one milliliter of the blood cell residue. When cultures were made directly from a joint or abscessed area, the medium was inoculated directly with the purulent material. Sodium citrate does not interfere with growth of Streptobacillus moniliformis.

As a general rule subcultures must be made in 24 hours since the growth of the organism causes a drop in pH to 6.5 to 5.9 in this period. These levels of acidity will kill the organism if subcultures are not made at once.

Brown and Munemaker (4) have reported that the organism can be preserved for many months in a low temperature refrigerator (-25 degrees centigrade). After a number of subcultures the virulence dimishes. They found that the organism isolated from one of their cases became avirulent for mice after 400 subcultures. They were unsuccessful in regaining the virulence once it had been lost. Therefore it is pointed out that much of the contradiction in literature concerning the lethal effect, production of arthritis, and the effect of chemotherapy could be due to variation in attenuation through subculture. By freezing newly isolated cultures, it is possible to obtain at any time the same generation for animal pathogenic studies. They (4) believe the organism goes through some sort of cycle, but there are so many variables that the cycle is difficult to trace. The filaments have a tendency to fragment as the culture ages or as the pH changes. It is this fragmentation that has given rise to the name Streptobacillus. The fragments are not of uniform length. The most common appearance is a dot-dash effect. Along the course of the fragmented filaments, swollen or large round bodies can be observed. It is believed by this

team (4) that these bodies contain the viable elements in the cultures.

In observing the organism in its various forms Wayson's stain instead of the more common laboratory stains was used. This stain is little publicized and it was used originally for studying Pasteurella pastis. It has been referred to as Wayson's Plague stain.* An interesting effect is that viable filaments of streptobacillus will always show up blue while the nonviable elements show up red. Serum components in the background stain up pink.

Miravette and Caldern (13) working in Mexico City have described a cellophane paper technique for isolating the L form of this organism. This method is simpler than using gradocrol membrane or Berkfeld filters and has obvious advantages over using antibiotics to inhibit the bacillary form.

In searching for better methods for laboratory cultivation of Streptobacillus the technique of Woolpert (30,31) using the mammalian fetus as a media for growing various agents was discovered. Woolpert (30) and his colleagues pointed out that bacteriologists have given little thought to the use of a mammalian fetus for experimental work. It is reasonable to suppose that the mammalian fetus would serve as a good medium for fastidious bacteria since it is agreed that the fetus is sterile up to the time of birth. The placenta is impermeable to the

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* Wayson's Plague Stain - dissolve 0.2 grams basic fuchsin and 0.75 grams of methylene blue in 20 milliliter of absolute alcohol.

Add the dye solution to 200 milliliter of a five per cent solution of phenol in distilled water.

FILTER - each day before use. Stain slides for a few seconds. Wash with tap water and dry.

If you wash with 95 per cent alcohol instead of tap water the red background is eliminated.

antibodies developed by the mother. There is little or no histological response in fetal tissue which might suggest that such tissue would grow organisms even when the postnatal animal would resist them. The concept of parasitism is incomplete without considering the reactions of embryonic and fetal animals. The newborn animal is relatively mature in form and function since it has the characteristics of the species. If susceptibility in any degree parallels morphological development we may expect the fetal reactions to differ from the postnatal reactions. Such conditions appear ideal for growth of certain bacteria which are difficult to maintain in an in vitro culture. The major problems associated with embryonic cultures would be (1) inoculating the fetus without jeopardizing the life of the mother or producing abortion, (2) observing the course of events following inoculation, (3) recovering the injected tissue at the desired status. Dr. Woolpert concludes his article by assuring that "the technique is not without its difficulties, but in our hands the difficulties have not been sufficiently serious to render the technique impractical."

## CULTURAL PROCEDURES AND DATA

### A. IN VITRO CULTURE

The cultures of Streptobacillus moniliformis used in this research were obtained through the courtesy of Dr. F.R. Heilman of the Mayo Clinic, Rochester, Minnesota. The organism sent by Dr. Heilman was isolated from the blood of a patient who had been bitten by a rat.

Upon arrival the culture was immediately transferred from the serum agar slant into tubes containing four milliliters of tryptose phosphate broth (Difco) (7) to which had been added 20 per cent horse serum (Difco) (7). Also, upon arrival an intraperitoneal inoculation from the original culture was made into a white mouse.

After 24 hours incubation at 37 degrees centigrade two of the inoculated tubes had a good growth and one was sterile. In order to study the effect of different media on the morphology of the organism dextrose agar slants (Difco) (7) were prepared. After solidification in the slanting position 1.0 ml. of horse serum in the form of a serum overlay was added to each tube. These serum overlay tubes were inoculated with 0.1 ml. of inoculum taken from the tryptose phosphate broth tubes. Because of the necessity of picking up at least one of the fluff balls of growth, sterile pipettes were used in making transfers from liquid media.

By varying the conditions of growth such as changing from liquid to solid media or reducing the nutrients, it is possible to alter the morphology of the organism. In general, when the organism is grown in fluid media the filamentous forms predominate; in solid media the

shorter rod like forms predominate. If the medium is unfavorable, the streptobacillus will grow with irregular morphology, bizarre knob forms and fusiform swellings (9,10). The organism used for this study was typical in this respect. Below is a photograph of the organism when grown in tryptose phosphate broth. (See plate II page 17). Beside it, for comparison is a photograph of the same generation of the organism, when grown in a serum overlay. (See plate III page 18).

## B. DESICCATION

When determining its pathogenicity to animals or when doing comparative work, it is necessary to have a freshly isolated strain of the organism. Desiccated cultures were made from its fourth generation on artificial media, and preserved until later for study in animals. These desiccated cultures were reconstituted, and the study in animals began with the fifth generation of the organism. The undesiccated portion was used for physiological studies.

The following method was used to preserve the actively growing cultures of Streptobacillus moniliformis. Horse serum was added to the original slant. Within 24 hours, minute amounts of the suspended growth were aspirated from the culture tube into a sterile syringe. This material was carefully injected onto sterile ground glass which had been placed in the fermentation tubes. Cotton plugs were used to close the ends of the fermentation tubes. Each closed, sterile, fermentation tube was placed on $CaCl_2$ in a five milliliter tube. While on a vacuum line it was sealed by flame. The sealed evacuated tubes were placed in a refrigerator and stored until needed. To test the viability of the cultures one tube was reconstituted after one month of storage. Within

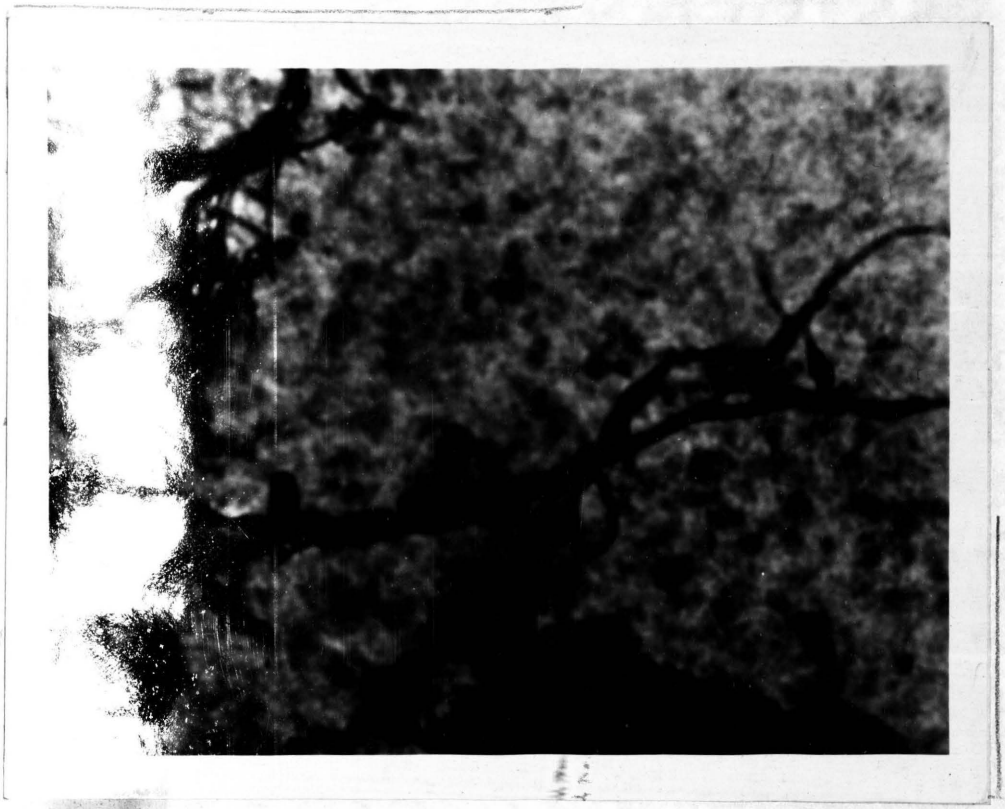

PLATE II. Filamentous form of the organism most frequently seen in tryptose phosphate broth (estimated 1500x).

PLATE III. Large bacillary form of growth at times revealing transverse bands; most frequently observed in serum overlay tubes (estimated 2000x).

twenty-four hours after reconstituting the culture in tryptose phosphate broth plus 20 per cent horse serum, the filamentous form of streptobacillus was actively growing and it exhibited the same physiological characteristics as the original cultures. This method of storing streptobacillus proved satisfactory. The rest of the cultures were not reconstituted for several months, yet they were equally as viable as the original cultures, and all had the same physiological characteristics. In two cases, an attempt was made to reconstitute the cultures with human serum. Both attempts failed to produce growing streptobacillus from the human serum enrichment media. The horse serum reconstituted cultures were readily transferred to 20 per cent human serum and produced luxuriant growth.

## C. IN VIVO CULTURE

In three days the mouse which had been injected with the original culture began to show signs of a generalized infection. It became inactive, its coat became ruffled, respiration increased and appetite was lost. However, after four days the mouse looked better and by the end of eight days its general appearances were normal. Other mice were injected intraperitoneally from one of the actively growing cultures. On the third day following inoculation, these mice began to show increased respiration and jerky movements. By the fourth day the symptoms were pronounced. One milliliter of blood was aspirated from the heart of one of these mice after it had been anaesthetized. This heart blood was introduced into tryptose phosphate broth media plus 20 per cent horse serum. Within 24 hours a pure culture of the long filamentous form of the organism was actively growing. The core of the

experimental work for this thesis involves the study of the organism as it grows in mammalian tissue, the in vivo culture served to make certain that this particular strain was capable of surviving and producing a pathogenic condition in the living animal. The application of the Woolpert technique for growing bacteria is more complicated and for practical reasons it was necessary to determine the ability of a particular organism to grow in living tissue before the Woolpert technique was applied.

## D. L. FORM

Many bacteria give rise to an L form; Streptobacillus is unique only in the ease and regularity with which its L form can be called into being. An actively growing bacillary culture was submitted to conditions necessary for production of the L form. Most favorable media and procedure for the production of L form coincides closely with these desirable for the cultivation of the Pleuropneumonia-like Organisms (4). A liquid medium was used which contained ten milliliters of Bacto PPLO Broth Base (Difco) (7) with one per cent Bacto PPLO Serum Fraction (Difco) (7). One thousand units of penicillin per milliliter and 0.01 grams of crystal violet dye per liter were used as bacteriostatic agents. These cultures were incubated at 38.5 degrees centigrade for a period of three days. Then 0.02 ml. from these liquid cultures were pipetted onto a plate of Bacto PPLO Agar containing one per cent Bacto PPLO Serum Fraction (Difco) (7). The liquid was spread over the plate with a bent glass rod and the plates were sealed with wide rubber bands to prevent excessive loss of moisture. The plates were incubated at 38.5 degrees centigrade. On the fourth day of incubation microscopic examination of the plates revealed many L-type colonies. Two agar "cut outs" were made

and stained. A section of agar containing the colonies was cut out and placed on a glass slide. A cover slip was stained with an alcoholic solution of azure and methylene blue and allowed to air dry before it was placed over the agar block. These sections were sealed with paraffin and examined with a microscope. When this preparation was examined under the oil immersion lens the L form of the bacteria showed up as granular colonies pitted into the media.

A much simpler method for the propogation of the L form of streptobacillus was discovered. It was called the cellophane paper technique (13). It consists of depositing strips of sterile cellophane paper on the surface of serum agar media after the strips had been submerged in sterile physiological saline. The inoculum from a solid or liquid culture was streaked onto the cellophane paper. Cultures were incubated at 37 degrees centigrade for 48 hours and then microscopically examined. The bacillary form of the organism grew on the upper surface of the cellophane paper. When the cellophane paper was peeled off from the agar the L form of the organism was revealed as growing pitted into the agar in pure culture.

## B. BIOCHEMICAL REACTIONS

In order to study the biochemical reactions it was necessary to add at least 20 per cent horse serum, human serum, rabbit serum, or ascitic fluid to each medium under consideration. For the fermentation tests Seitz-filtered carbohydrates were added to a serum-peptone medium. After the incubation period brom-thymol blue was added and the pH was estimated optically (10). Reactions are recorded in Table 2.

TABLE 2

Physiological studies of Streptobacillus moniliformis

| MEDIA | INCUBATION PERIOD | RESULTS |
|---|---|---|
| Dextrose | 3 days | acid produced, pH 6.0-7.6 |
| Lactose | 3 days | growth, no acid |
| Levulose | 3 days | acid produced, pH 6.0-7.6 |
| Maltose | 3 days | acid produced, pH 6.0-7.6 |
| Mannitol | 3 days | growth, no acid |
| Rhamnose | 3 days | growth, no acid |
| Saccharose | 3 days | growth, no acid |
| Salicin | 3 days | acid produced, pH 6.0-7.6 |
| Gelatin | 3 days | no liquefaction |
| Nitrates | 2 days | no reduction |
| Indol | 2 days | not produced |
| Methylene blue | 18 hours | reduced |
| Milk | 2 days | growth, no change |

The addition of soluble starch to the media greatly accelerated growth. For rapid growth dextrose starch broth (Difco) (7) plus 20 per cent serum surpassed all other media. A disadvantage of this medium was the rapid drop in pH caused by the metabolism of the organism. During the growth process a drop in pH from 7.6 to 6.0 occurred in 24 hours. The more highly buffered tryptose phosphate broth delayed such pH changes for about 36 hours. A good practice was to subculture as soon as fluff balls appeared in the media. The biochemical reactions cited above were confirmed as typical reactions of Streptobacillus moniliformis as previously described (4,10,29). It was possible to study the physiological effect of many antibiotics on this organism. Literature that was reviewed with this study gave little information about the organism's response to antibiotics. Disks containing the antibiotic were placed in tubes containing two milliliters of serum tryptose phosphate broth. One tenth milliliter of inoculum was pipetted from an actively growing

cultures into each of the tubes containing an antibiotic. The results of the test are shown in Table 3.

TABLE 3

Effect of antibiotic on Streptobacillus

| ANTIBIOTIC | CONCENTRATION PER MILLILITER | RESULTS |
|---|---|---|
| Bacitracin | 1 unit | complete inhibition |
| Albamycin | 15 micrograms | complete inhibition |
| Aureomycin | 5 micrograms | complete inhibition |
| Furadantin | 15 micrograms | partial inhibition |
| Chloromycetin | 5 micrograms | partial inhibition |
| Penicillin | 0.5 units | partial inhibition |
| Panmycin | 15 micrograms | no effect |
| Terramycin | 15 micrograms | no effect |
| Polymycin B | 50 units | no effect |
| Erythromycin | 15 micrograms | no effect |
| Tetracycline | 15 micrograms | no effect |
| Dihydrosteptomycin | 5 micrograms | no effect |

Controls were set up for each of the antibiotics tested to prove that the organism would have grown in the media without the antibiotic. A second control was included to determine the sterility of the media and the antibiotic disk. This control was accomplished by adding only the antibiotic disk to the tryptose phosphate broth and 20 per cent serum. These tubes were incubated with the inoculated tubes and checked for sterility. In all cases the sterility of these controls was observed and typical growth occurred in all cases tested without the presence of the antibiotic.

This organism also resisted the effect of the sulfonimides and the arsonicals. A differential medium was made using tryptose phosphate broth and 20 per cent horse serum plus increasing amounts of sulfanilamide. This medium was found useful in controlling contamination in stock cultures, where asepsis was made difficult because of the addition of

blood serum. This medium also allowed the isolation in pure culture of small, fastidious cocco-bacilli from the lung tissue of normal rats. Of eleven cultures obtained from 23 rats, only two showed contaminants during the three transfers through which these organisms remained viable.

## F. HISTOLOGICAL REACTIONS

The surgical procedure used in this study was that of Woolpert (30). A female rabbit 21 days pregnant was anaesthetized with nitrous oxide. The abdomen was shaved and disinfected, the animal was draped with sterile towels, and a median incision two inches long was made low in the abdomen with the aid of a grooved director. Bleeding, if it occurred, was controlled by artificial hemostasis.

The eight embryos were easily felt and manipulated through the incision. Their skulls were easily palpated. The feti were inoculated intracerebrally with 0.2 ml. of a well-shaken 24 hour serum broth culture of the organism, changing needles between filling the syringe and injecting the embryos. Two attempts were made to inoculate intraperitoneally, but subsequent evidence of successful inoculation was not found. Only the most distal and the third of the embryos in each horn were inoculated. (See plate IV page 25).

The uterus was returned to position and the peritoneum was closed with interrupted linen sutures one-half inch apart. The abdominal wall was closed with continuous stitches of linen thread and sealed with a sprayed-on film of collodion. Recovery was rapid and uneventful.

The doe was sacrificed after 40 hours, the injected feti were removed into 40 per cent formaldehyde for 24 hours and dissected.

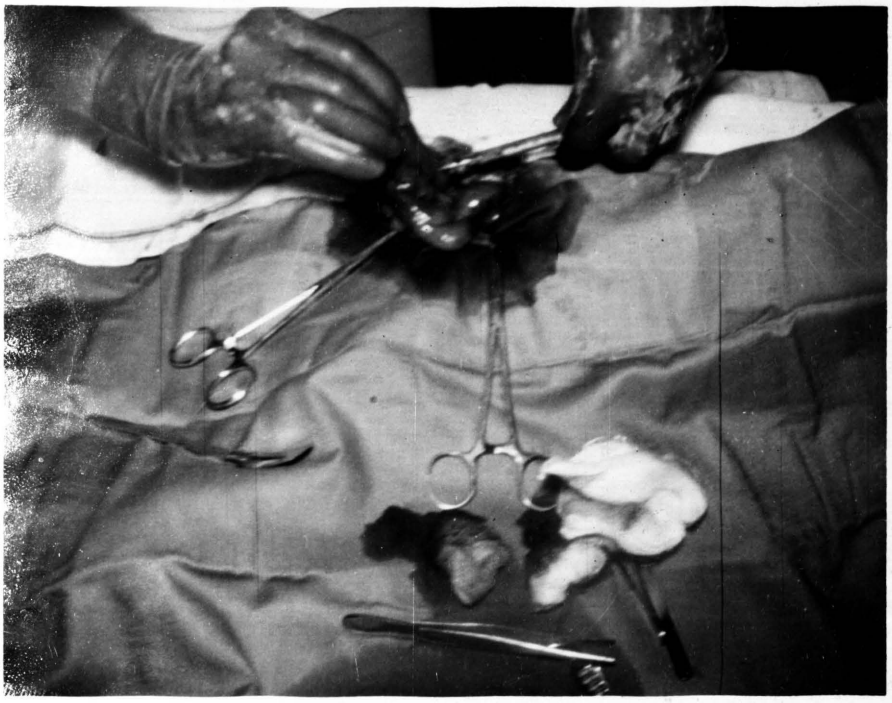

PLATE IV. Inoculation of fetus in utero.

Tissues were mounted in paraffin blocks by standard histological procedures and sections 12 microns thick were placed on albumin-glycerol coated microscope slides. The slides were stained with Wayson's Plague Stain or with hematoxylin-eosin. They were examined, with the collaboration of Dr. John Elston, Pathologist, for bacteria and for evidence of inflammatory reaction. Literature (4,10,18) had forewarned that in tissue and other very favorable media, only the rod form of the organism would be found. Although a culture consisting entirely of fine filaments was injected into the brain tissue of the embryo, only rod forms were observed in histological slides made after 48 hours incubation. Bits of brain tissue removed from the embryo prior to fixing in formaldehyde and cultured in the usual media gave rise to "typical" filamentous forms. In embryonic tissue the organism grew uninhibited by any sort of histological or inflammatory response and there was no infiltration of phagocytes. (See plate V page 27).

A mature young male rabbit was inoculated intratesticularly with the same passage of the streptobacillus, and was sacrificed after 48 hours. Sections of the testicular tissue revealed focal inflammation around the bacteria, marked accumulation of histiocytes and infiltration with neutrophils and a fair proportion of eosinophils. (See plate VI page 28).

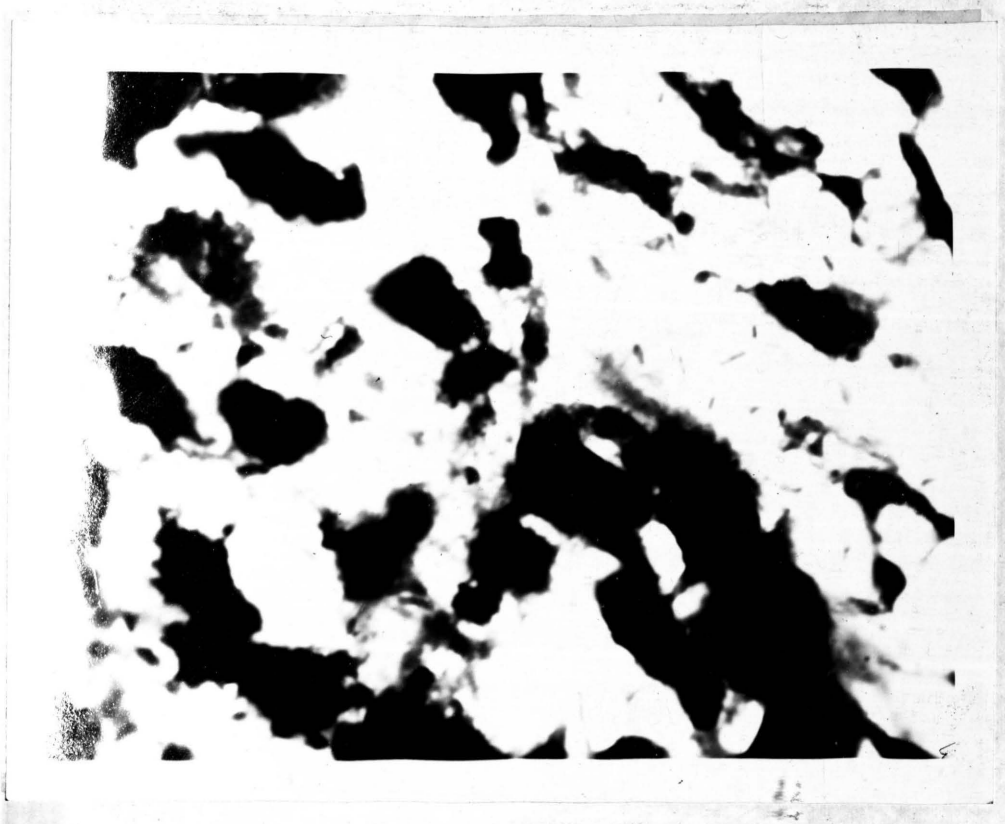

PLATE V. S. moniliformis growing in an embryonic brain showing no histological reaction (estimated 1300x).

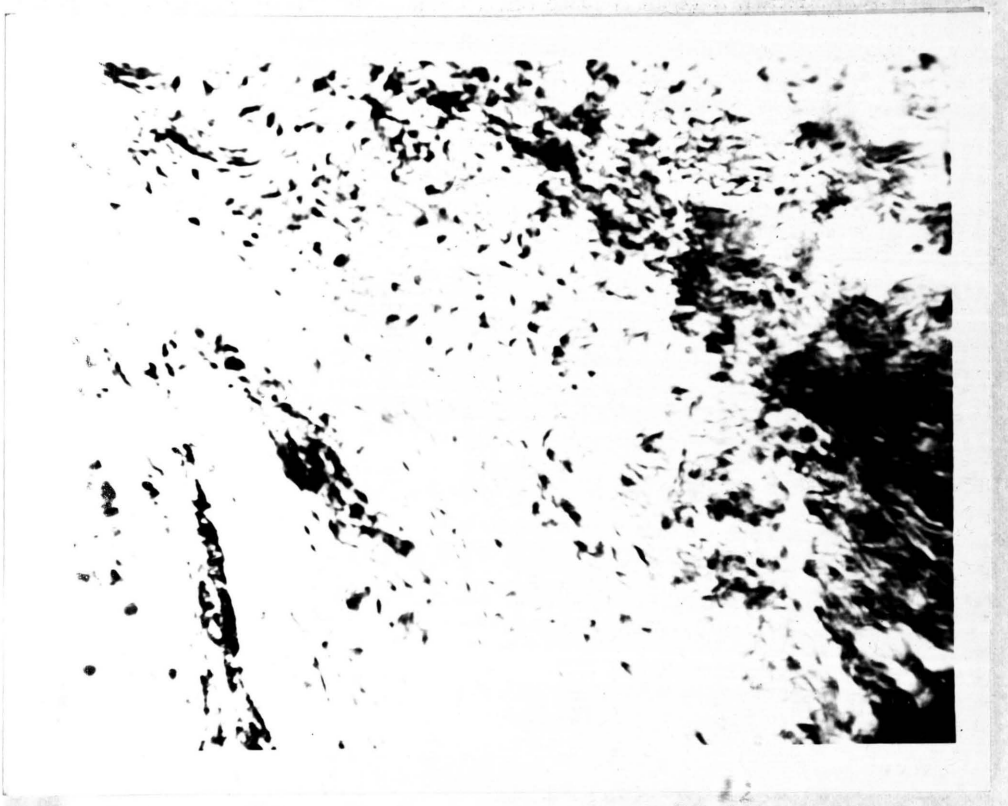

PLATE VI Histological response shown to bacterial growth in testicular tissue (estimated 200x) normal tubules on left inflamed area center and right.

## DISCUSSION

Successful attempts were made in preserving the cultures of Streptobacillus moniliformis for many months in vacuo although, previous workers had met with failure when desiccating cultures of this organism. The discrepancy could arise from different techniques or because of the viability of two different strains. The organism grew well on solidified media in this experiment. Not all workers have been successful in cultivating the organism in other than liquid media. These discrepancies are not easy to account for, but it is possible that there could have been differences in the physiological state or nature of the various strains under study. There must be a correlation between the numerous physiological differences and the variations in pathogenicity and variation in morphology that is observed in different cultures.

The use of the Woolpert technique with this bacterium established a difference in the localization of the organism as between fetal and adult tissues; as in the fetal brain diffuse infection occurred with no inflammatory response; in the testicle of the adult rabbit few organisms survived and these were walled off into minute foci with surrounding infiltration of granulocytes. It is felt that the histological studies have shown the first steps of the gradual building up of tissue response which culminates in allergic reactions to the invading organism. While the histological studies probably did not make any great contribution to our knowledge of the pathogenesis of rat bite fever, some of the other things that were done in this investigation seem worthy of note in closing this report. For example, there is to

my knowledge no published report of the sensitivity of the organism to various antibiotics other than references to the production and isolation of the L form. Tests were run showing the sensitivity of Streptobacillus moniliformis to a wide variety of these agents, and showed that they are resistant to most of them. The tests used for this sensitivity analysis are entirely comparable to the standard tests for antibiotic sensitivity that are used in all clinical bacteriological laboratories. The only differences are the uses of 20 per cent serum enrichment used in the media and the type of controls necessary in this case. A differential media was made involving the sulfanilamide inhibitor which allowed for control of contamination in stock cultures, and which allowed the growth of the fastidious cocco-bacilli from the lungs of normal rats. It is not claimed that these organisms were Streptobacillus moniliformis, but it is possible that successful growth on artificial media might be related to ability to grow in species other than the natural host and had an attempt been made to establish these unidentified isolates in mice or rabbits they might have proven to be Streptobacillus moniliformis.

## SUMMARY

(1) Morphological and physiological studies of *Streptobacillus moniliformis* were done in vitro on a strain of the organism isolated from a human case of rat bite fever.

(2) The organism was studied for its sensitivity to various antibiotics and sulfonamide drugs.

(3) A suitable method for preserving the organisms in their virulent state was developed.

(4) Organisms resembling the species under study were isolated from rat lungs and carried in artificial media for three serial transfers.

(5) A differential medium for isolating certain fastidious organisms in their virulent state was developed.

(6) The Woolpert technique for inoculation of feti in utero was applied for the first time to *Streptobacillus moniliformis*.

(7) Histological studies were made of tissue reaction of fetal brain and adult testicle to the inoculation of virulent *Streptobacillus moniliformis*.

(8) The significance of the histological findings in relation to the pathogenesis of Streptobacillus infections is discussed.

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## LIST OF PLATES