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Diagnostic Tests Including Hematology in Swine Brucellosis
       and Capsule Formation in Brucella.

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DIAGNOSTIC TESTS
INCLUDING HEMOTOLOGY IN SWINE BRUCELLOSIS
AND CAPSULE FORMATION IN BRUCELLA

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INTRODUCTION

Porcine brucellosis not only causes great losses to the swine industry, but its causal agent, Brucella suis, is of considerable importance to human health because it is pathogenic for man, apparently much more so than Brucella abortus and possibly approaching Brucella melitensis in this respect. Several investigations have demonstrated that it can and does cause many abortions in swine and also frequently produces sterility in these animals. While in general it seems to be a much less widespread disease than either Brucella abortus or Brucella melitensis and is confined largely to the hog raising districts of North America and certain sections of Europe and South America the disease has rather high incidence in those places where it occurs.

In 1922, Hadley and Beach found through a questionaire that 24% of 188 pure-bred herds had abortion, while Connaway, Durant and Newman, 1921, noted infection in 28 out of 30 herds examined. There have been numerous and somewhat variable reports from sections of the Middle West, but all indicate that brucellosis of swine is a major disease affecting reproduction and, from an economic and public health standpoint, it may possibly be almost as important as the disease in cattle. The discovery of the causative organisms resulted in considerable work in the field for a period of about ten years, but since 1925 much less attention has been given the problem.

Persons in the public health field were much interested in the discovery that <u>Brucella</u> <u>suis</u> was more pathogenic for man than <u>Brucella</u> <u>abortus</u>. However, this did not stimulate the study of porcine brucellosis to any great extent because it had been demonstrated that <u>Brucella</u> <u>suis</u> rarely affected cattle, and the public health problem of the moment was the possible infection of man through the use of raw milk. Because of enormous loss to cattle producers all over the world, research work in bovine brucellosis has taken precedence over the investigations of virtually all other animal diseases for the past two decades.

The diagnostic tests for porcine brucellosis have never been entirely satisfactory. They have been adapted from those used in bovine brucellosis identification, and while they are fairly satisfactory for the disease in cattle, the results obtained in swine brucellosis diagnosis have been too variable to be adequate. With this in mind it seemed desirable to make a study of the methods of diagnosis now in vogue and to try, if possible, to introduce some new method which would be helpful in the identification of the disease.

It seemed advisable to make a study of the capsules of the Brucella group with the idea that this might throw some light on the production of antigens for the diagnostic tests to be undertaken. Hence, a study of the capsules of the entire Brucella group under varying conditions was made.

Since the blood picture has been found to be useful in the identification of some other diseases of hogs, a study of

the blood picture in normal and infected hogs was carried on, to determine whether there was enough difference in the blood pictures of the two groups to make it useful in the identification of the disease.

The work on diagnostic tests included the study of a group of those previously used and the introduction of a test new to swine brucellosis. In addition, the Neufeld reaction used in pneumococcus identification was tried to determine whether it might be useful in identifying positive sera.

HISTORICAL REVIEW

Discovery of Brucella: Brucella melitensis was the first species of the genus Brucella to be identified. It appears to have been first isolated from the spleen of patients who died of Mediterranean fever by Bruce in 1887. The causative organism was named Staphylococcus melitensis by Hughes in 1892, and Bruce, 1893, used the term Micrococcus melitensis. After Evans, 1918, studied abortus and melitensis together, she suggested that they were similar and that they be given the generic name Bacterium. Meyer and Shaw, 1920, confirming her results, suggested a more fitting name Brucella, thus honoring the discoverer of Brucella melitensis who also investigated many tropical diseases. This seems a much better designation than the overworked term Bacterium and is now in general use.

The fact that the chief host of Brucella melitensis is the milk goat was discovered by Zammit, a member of the Mediterranean Fever Commission in 1905. The organism appears to localize in the udder, the spleen, and the lymph glands in the goat and frequently in the sheep. It may, also, affect other animals and gives rise to undulant fever in man. It is widely distributed throughout the world, and reports of cattle and sheep, as well as goats, being infected with the disease have appeared from this country, Europe, and South America.

The second species of the genus, Brucella abortus (Bang), was first isolated and described by Bang of Copenhagen in 1897. Assisted by Stribolt, he isolated the organism from

the fetuses and fetal membranes of cattle that had aborted,
and they later demonstrated by a series of experiments that
this was one of the causes of abortion in cattle. By examin-
ing the milk of infected cattle, Schroeder and Cotton, 1911,
as well as Smith and Fabyan, 1912, found that the udders of
animals could be infected. The organism gives rise to acute
inflammation of the maternal and fetal placentae, produces
inflammatory conditions of the uterus and oviducts, and is
often present in the testis. It has been found to infect
other animals to a much less extent, and in man the organism
gives rise to undulant fever. It is more widespread than
Brucella melitensis and has been reported from practically
every country in the world.

The discovery of the American porcine species of the
Brucella organism from fetuses expelled prematurely from sows
was made by Traum in 1914, and thus the third member of the
genus was introduced. In Denmark Brucella suis strains have
been isolated by Thomsen, 1931 and 1934, which differ in cer-
tain respects from those found in the United States in that
they produce little if any hydrogen sulfide when grown on
suitable culture media. The porcine organism infects other
animals, such as the horse, the cow, the dog, and the fowl,
and produces undulant fever in man.

Brucella bronchiseptica and Brucella tularensis are in-
cluded by some workers in this group but are not usually so
considered. The other three organisms are closely allied and
their differentiation is difficult. While there is still some

controversy, the consensus of opinion is that they should be classified as separate species, although there are some advocates of a single species for all three groups. The terms paramelitensis, paraäbortus, and parasuis, while frequently used in the literature to refer to inagglutinable strains of Brucella, probably refer to rough variants of original smooth cultures and consequently these terms merely serve to confuse the issue.

Brucella suis is a strict parasite. Its growth is never improved by the addition of carbon dioxide and it does not develop under strictly anaerobic conditions. The American type grows more freely than the Danish type. It grows in the presence of thionin, but is inhibited by basic fuchsin, methyl violet, and pyronin. The Danish type has the same differential susceptibility as the American type, but is rather more susceptible to all dyes; consequently, when identification is made with such dyes, they should be reduced in concentration. The American type produces hydrogen sulfide on liver during the first four days of incubation. The Danish type produces no hydrogen sulfide.

Brucella suis appears to produce the same antigens as Brucella melitensis, but they are distributed in qualitative proportion nearer those of Brucella abortus. Provided absolutely smooth strains are used, they may be differentiated from Brucella melitensis, but not from Brucella abortus, by qualitative absorption of agglutinins. Brucella suis gives rise in guinea pigs to a disease closely resembling that of

Brucella melitensis. The organism is usually extremely viru-
lent and produces large lesions in spleen, liver, lymph glands
and testicles.

Agglutination and complement fixation bodies are very
prevalent in breeding boars and the disease apparently is fre-
quently transmitted by them. They often show necrotic inflam-
matory areas in the genitals, especially the epididymis, but
also in the testicles and seminal vessels in varying degrees.
In later stages of the disease the organs atrophy. The causa-
tive organism can readily be isolated from the tissues of
these organs. In the sow organisms are present in the uterus
as numerous, well-defined yellow nodules located in the deeper
layers of the mucous membrane but protruding above the sur-
face. It may be possible that sows abort more frequently than
is supposed, as they are capable of eating the fetuses. The
organisms may be present in the milk, but they do not seem to
give the disease to suckling pigs.

Diagnostic Tests: It is quite possible in some cases of
brucellosis of swine to make the diagnosis by cultivation of
Brucella suis directly from the animal material or after
guinea pig inoculation; and, of course, this is the easiest
method and furnishes positive proof that the disease is pres-
ent. The organisms are often found in the stomach contents,
the lungs, and other organs of aborted fetuses. It may also
be obtained from fetal membranes and from the genitals of
boars.

Four tests have been utilized for the diagnosis of

porcine brucellosis: the complement fixation test, the agglutination test, the precipitin test, and a group of allergic reactions.

Good and Smith, 1916, Hayes, 1922, Cotton, 1922, Hadley and Beach, 1922, Schroeder and Cotton, 1925, Thomsen, 1931 and 1934, have made studies of the agglutination test and have found that with swine blood the presence of infection is indicated by a slow reaction and a somewhat lower titer than in cattle.

Cotton and Buck, 1932, report that they were sometimes able to demonstrate Brucella suis in the blood of sows that only showed titers of 1:50. Bianchini, 1930, found brucellosis in swine relatively frequent without positive serological reactions. Connoway, Durant and Newman, 1921, have found negative reactions in sows which had aborted ten days before, or within a few days after the serological tests. Thomsen, 1934, found agglutinins appeared earlier than complement fixation bodies, and he has frequently found that animals abort when neither complement fixation or agglutination titers are present. These frequently appear much later. He has occasionally found an infected animal showing only the presence of agglutinins or, sometimes, complement fixation bodies alone.

Hayes, 1922, reports some observations on the production of agglutinins. He found them to appear seven days after intravenous injections and three to four weeks after administration by mouth. However, within a month he discovered considerable shifting in the positive picture, and some of the

animals which had been positive became negative and some of those which had been negative became positive.

Johnson and Huddleson, 1931, say the disappearance of agglutinins altogether, or their tendency to disappear in 45 out of 62 hogs that reacted to the agglutination test would strongly suggest that the organisms in most instances do not remain in the body of the hog longer than from three to five months.

Hayes, 1922, however, followed the course of the disease in a naturally infected herd of 60 hogs by means of agglutination tests. In the herd which he studied, it appeared that there was a tendency for more animals to react over a longer period of time. However, he noted that there was a general tendency for the agglutination titers to fall off after one year.

Howarth and Hayes, 1931, in a series of systematic examinations of reacting animals over a considerable period of time, found a decrease in the antibodies at the time of parturition, after which there often came a new rise in titer up to about the original level. They found, however, in repeated tests that the reactions were subject to great individual variation from time to time.

Thomsen, 1934, believes that the reaction power of an infected animal gradually subsides more or less completely in spite of persistent infection.

Gwatkin, 1931, suggests the adoption of the positive agglutination reaction in swine which the official American Committee

has laid down for cattle: 1:100 and higher is positive; 1:50, suspicious; 1:25, of no significance. Thomsen, 1934, suggests that a positive complement fixation test with 1:1000 of serum or a smaller amount should be considered a positive reaction. He believes that such an animal is either infected at the present time or has previously been infected and feels that this rule holds good even though the complement fixation test is only partial. Agglutination titers of 1:20 in an infected animal, or one of those suspected of infection, should be considered a suspect and a thorough examination should be undertaken. Thomsen feels that animals showing agglutination in dilutions of 1:50 should be designated as reacting, and infected if such animals are found in an infected herd. If agglutination occurs in dilutions of 1:100 or higher, it should be looked upon as decidedly positive. Thomsen has found, however, that unspecific reactions may go even higher than this, and consequently a combination of positive complement fixation and agglutination titers are not a perfect solution to the problem.

Higginbotham and Heathman, 1936, also feel that the complement fixation tests are not as clear-cut as the precipitin test and for that reason are not satisfactory. Hayes, 1922, also feels that the complement fixation test has no advantage over the agglutination test.

Investigators have made almost no use of the precipitin test for the diagnosis of brucellosis. Higginbotham and Heathman, 1936, while using these tests experimentally in studying the disease, think that they have no advantage over

the agglutination test as a routine procedure. The time and
cost of preparing extracts make it disadvantageous for gen-
eral diagnostic procedures. However, they think it is satis-
factory to use in research. Stubbs and Live, 1942, in study-
ing the sonic filtrate of Brucella abortus, used the precipi-
tin tests almost exclusively and found them quite satisfactory.

Very little work has thus far been published on the use
of allergic tests in the diagnosis of porcine brucellosis,
but various extracts have been tried on other experimental
animals. McFadyen and Stockman, 1913, used an allergic agent
prepared in a way similar to tuberculin and injected it both
subcutaneously and intravenously in cattle. Reichel and
Harkins, 1917, also used heat-killed suspension of organisms
which they injected intravenously into the caudal fold in
cattle. Fleischner and Meyer, 1918, and Stafseth, 1920, used
heat-killed suspensions in salt solution injected interder-
mally into infected guinea pigs and found the test was a reli-
able index of infection.

Gordiano, 1929, used a heat-killed suspension and found
favorable reactions in man. Thomsen, 1934, used the intra-
cutaneous test with a preparation of killed organisms made
in the same general manner as tuberculin and feels that this
method of diagnosis may be serviceable as an experimental test.
He found that local swelling was produced, often to a very
considerable extent, within 24 to 48 hours, whereas animals
from the healthy herd were not affected by the injection.

Burnett, 1922, using filtrates of broth cultures, also

obtained satisfactory allergic results in man. Rainsford, 1935, and Foshay, 1936, used specific bacterial antiserum intradermally for detecting residual antigen present in the skin. Pennell and Huddleson, 1937, prepared "endo-antigen" by digesting ground Brucella with trypsin. This gave specific skin reactions in sensitized animals. Another preparation produced by Stahl, Pennell and Huddleson, 1939, used protein nucleate of Brucella to determine its allergic sensitivity on sensitized rabbits. Morales, Otero and Gonzales, 1936, prepared a purified protein derivative which elicited skin reactions in infected guinea pigs. They found, however, that the nucleic acid portion plays no part in the production of the allergic reaction. Huddleson's Brucellergin, which is an extract of ground, dried organisms from which the soluble liplods are extracted, proved fairly effective in animals tested. Stubbs and Live, 1942, in studying the sonic method of disintegrating Brucella, used their product to produce allergic response when sensitized rabbits and guinea pigs were injected intracutaneously with the protein concentration of the filtrate. Their results seem promising.

The concensus of opinion seems to be that the agglutination test is probably the best single test for the diagnosis of porcine brucellosis, but it is far from satisfactory since the agglutinins develop somewhat late in the disease and disappear rather early. The chief difficulty in the use of any of the allergic products in the diagnosis of the disease is that those animals which have recovered from the infection

will give a reaction similar in nature to that of animals actively infected. The results of this test are, so far, very contradictory and to date no satisfactory agent for tests of this nature has been developed.

Capsule Formation: McAlpine, Plastridge and Bingham, 1929, and Plastridge and McAlpine, 1930, were the first to report on a mucoid type of organisms in the genus Brucella and suggested that these organisms were encapsulated. Huddleson, 1940, found capsules on all smooth strains of the three species of Brucella. The rough strains, as well as the smooth, also show a capsule. He found that a large proportion of the cells of rough strains emerge from the capsule when suspended and allowed to stand in distilled water at 37° C. Capsules may also be removed by boiling in a hot chloroform-ether mixture acidified with hydrochloric acid. This treatment also destroys that part of the organism which is made visible by the usual staining methods. He believes the capsules consist of lipoids in close combination with polysaccharids.

One of the outstanding characteristics of the mucoid forms, according to Plastridge and McAlpine, 1929, 1930, is their marked inagglutinability in the presence of antiserum prepared by injecting rabbits with ordinary antigen. Mickle, 1940, confirmed these results and found the mucoid strains highly agglutinable in serums prepared against homologous organisms, and vice versa. Plastridge and McAlpine, 1929 and 1930, and also Mickle, 1940, found it difficult to

produce agglutinin with mucoid forms as compared with the ease with which high titer serums were obtained from ordinary antigens. Mickle, in studying 27 strains of Brucella which were obtained from cases of Hodgkin's disease and from cases of brucellosis, together with three standard strains from Huddleson's laboratory, found that five of these strains would produce demonstrable capsules; and five others showed serological and cultural characteristics similar to the encapsulated strains, but did not produce demonstrable capsules. He observed that in general the organisms producing capsules were isolated from Hodgkin's disease, and the unencapsulated organisms came from cases of brucellosis. He found that the encapsulated organisms served as poor antigens, but he was able to denude the organisms of their capsules by cultivation in beef extract broth after they had been grown on liver infusion agar, and he found them indistinguishable from the encapsulated and non-encapsulated forms. They acted as better antigens than the encapsulated organisms and produced agglutinins for the encapsulated forms to high titer. The opsonocytophagic studies made by Mickle showed that there was but a slight increase when encapsulated organisms were introduced and a large increase in the index when non-encapsulated organisms were injected.

Plastridge and McAlpine, 1929 and 1930, recommend India ink staining technique to show encapsulated organisms. This method is described by Hagan, 1927. Huddleson, 1940, recommends a similar technique which he found to be satisfactory

for demonstrating capsules in Brucella. He also used the Churchman and Emelianoff, 1933, technique with some prospect of success. Mickle, 1940, has used Hiss's capsular stain with good results.

Blood Picture: While there has been no work done on the blood picture of brucellosis in swine, that of normal hog blood has been variously described in textbooks and papers. In connection with his work on hog cholera, Kernkamp, 1939, gives the following analysis of normal hog blood: the hemoglobin, 9.9 gm. per 100 cc. as an average with considerable variation; erythrocytes, average 5,915,000, ranging from a low of 4,170,000 to a high of 7,800,000 per cmm.; leucocytes, average 14,000 per cmm. and range from 8,660 to 23,650; lymphocytes, 54.6%; neutrophils, 37.2%; eosinophils, 2.8%; basophils, 0.5%; monocytes, 4%. In studying the sedimentation rate, he used one centimeter of citrated blood in a Winthrobe hematrocrit tube and measured the sedimentation at regular intervals of one hour. He found the rate to be 0.04 centimeter per hour.

Lewis and Shope, 1929, analyzed the blood somewhat differently, listing erythrocytes as 6,560,000; leucocytes, 23,700; lymphocytes, 16,590; neutrophils, 4,977; eosinophils, 1,659; and basophils, none. Dukes in Physiology of Domestic Animals, 1935, lists the erythrocytes as 7,440,000; leucocytes, 17,110; the lymphocytes, 47%; monocytes, 8%; neutrophils, 41%; eosinophils, 2.5%; and the basophils, less than 1.0%.

Bell and Irwin, 1939, in studying the blood picture in

cattle, designated artificially infected animals which carried calves to term as "resistant" and those which aborted as "susceptible." They found a significant drop in the average number of leucocytes per cubic centimeter following the infection and also a reduction in the mean number of red blood cells in each group. Both groups showed an increase in the average per cent of polymorphonuclear leucocytes. In the "resistant" group there was a slight but not very significant rise in the average per cent of monocytes and a very marked one in "susceptible" animals. The average per cent of lymphocytes was in inverse proportion to the number of polymorphonuclear leucocytes. There appeared no significant change in the basophils or eosinophils.

Munger and Huddleson, 1939, in studying the blood picture of man in Brucella melitensis infection, found leucopenia with relative lymphocytosis and monocytosis. They found that the red cells tend to be slightly smaller than normal and there were pathogenic lymphocytes present in 40% of brucellosis cases. Liver damage cells were frequently found in the blood. Basophilic granules of the neutrophils seemed to differentiate Brucella melitensis from suis and abortus. Calder, Steen and Baker, 1939, in the study of blood from 300 patients infected with brucellosis, found the normal leucocyte count to be about one-half the usual number, leucopenia in one-third of the cases, and one-sixth showed leucocytosis. Active lymphocytosis was the most striking feature encountered with unusually high numbers of lymphocytes. The total number of neutrophils was

reduced and mild anemia was noted, due to maturation defects probably the result of damage to the liver. Coagulation time was slow and often incomplete, and the clot reaction was imperfect. The sedimentation rate was not high. This group of characteristics does not occur in its entirety in any other disease, so might be used for confirmatory evidence in man.

A STUDY OF THE CAPSULES OF BRUCELLA

No extensive study has been made on the capsules in the
entire group of Brucella, and no literature has appeared on
the subject except that by Huddleson and Mickle when they
reported the discovery of the capsules in 1940.

It has long been known that individual smooth strains of
Brucella vary in their pathogenicity for experimental animals,
even shortly after primary isolation; and it was hoped that
through a study of the presence, size, density, and composi-
tion of the capsules, some questions pertaining to biological
differences in cultures would be answered.

The following study was undertaken in order to see
whether it would be possible to distinguish between smooth,
intermediate, and rough strains of organisms by their micro-
scopic appearance and to determine any differences which might
occur in capsule formation under varying cultural conditions.
Agglutination and opsonocytophagic tests were used in conjunc-
tion with guinea pig inoculations to test the virulence of the
organisms. It was hoped that this study might contribute
toward the improvement of vaccines and antigens, as well as
toward the diagnosis of the disease.

Experimental Procedures

Materials: Thirty-eight strains of Brucella were used
in this study, of which eighteen were abortus, ten were suis,
and ten were melitensis. Seven of the abortus strains were

isolated from the stomach contents of aborted fetuses at this laboratory. One of the melitensis strains was isolated from the blood of a hog. The other organisms had been growing on artificial culture media for varying lengths of time and consisted of virulent, avirulent, and intermediate forms. A list of the organisms used, with a brief description of each, follows:

Brucella Abortus Strains

1. 2308, isolated from a cow at Animal Disease Station, Beltsville, and grown on artificial culture media for more than two years. A smooth, virulent organism, apparently one of the most stable in the collection.

2. Huddleson, an atypical rough strain, not virulent. Produces some antibody formation, but has not been recovered when injected into animals.

3. 19, a smooth strain of low virulence, used in vaccine production.

4. 42-20, McEwen English strain, used experimentally for vaccine production for a time, but later became atypical.

5. 7072, isolated from a fetus, smooth and extremely virulent organism.

6. 801, rough strain grown on artificial culture media for many years, has been transferred 392 times.

7. 1119, smooth strain used for antigen production at Animal Disease Station, Beltsville.

8. 2473, recent isolation from cow, smooth and virulent.

9. 3160, smooth strain, Alabama isolation and typing, from cow.

10. 3191, smooth strain, Alabama isolation and typing, from cow.

11. 456, Huddleson standard, smooth strain

12. Intermediate strain, University of Maryland collection, isolated from cow.

13. Six smooth strains isolated at College Park from aborted fetuses.

Brucella Suis Strains

1. 2872, smooth, virulent, has been on artificial culture media for more than two years.

2. 1942^1, smooth, virulent, isolated from epididymis of a boar.

3. 1942^2, smooth, virulent, from a swine infected with erysipelas.

4. 7300, smooth, very virulent, from the testicle of a boar and from blood of the same animal.

5. King 8, an intermediate strain of low virulence from Australia, being used experimentally for vaccine.

6. Indiana, atypical strain of low virulence.

7. 3164, smooth, virulent, isolated from epididymis of boar, Alabama.

8. 3168, smooth, virulent, isolated from epididymis of boar, Alabama.

9. 504, smooth, virulent, isolated from case of brucellosis in man in North Carolina.

10. 47, Huddleson's standard smooth strain.

Brucella Melitensis Strains

1. Henry, isolated from human case at Animal Disease Station, Beltsville, smooth, virulent.

2. 527, Utah strain, smooth, virulent.

3. 2330 F11, recently isolated from a cow, intermediate.

4. G1766, isolated from a goat at Beltsville, smooth, virulent.

5. P60, P61, isolated from human cases of brucellosis in North Carolina, smooth, virulent.

6. P13, P14, isolated from a case of Hodgkin's disease, North Carolina, smooth, virulent.

7. G131, isolated from blood of patient with brucellosis, Georgia, smooth, virulent.

8. 428, Huddleson's standard smooth strain.

All of these organisms had been typed either at the Animal Disease Station at Beltsville, Maryland, or at other reputable institutions.

Staining Methods: The most satisfactory staining technique for showing capsules of Brucella seems to be a modification of Huddleson's India ink capsular stain. This is described in detail in Huddleson's report, 1940, on the presence of capsules in Brucella. After some slight modifications of Huddleson's method, the technique used in this laboratory was as follows:

It is absolutely essential for the success of the technique that perfectly clean slides be used. New slides, dipped in alcohol and burned, give excellent results, as do new slides which have remained for some time in ether-alcohol and are then polished dry. A large loop of a dilute suspension of organisms in physiological salt solution, distilled water, or broth is placed at one end of the slide. The drop is bisected with another slide and the suspension is drawn across the original slide in a manner similar to that used in making a blood film. It is frequently found that drawing the film back and forth several times across the slide will produce a more even smear. The slide should be dried rapidly in a current of air. A large loopful of India ink (almost any India ink will do if the particles are small and it is bacteria free) diluted one-third with water is drawn over the smear of organisms in the same manner as before and allowed to dry.

The size of the drop should be such that the India ink film thins rapidly as the slide is drawn across, so that no part of the drop remains at the end of the slide. It is then placed in methyl alcohol (pH 6.8) for two or three minutes and transferred to a 25% solution of crystal violet. This is an important part of the procedure because, if the slides are not allowed to remain long enough in methyl alcohol, living organisms will be present on the finished slide. Clearing the slides in xylol helps produce a more clear-cut capsule after staining.

It has been found that by placing slides on damp paper toweling, they can be handled more readily without contaminating the hands, as the slides do not slip when the blood or India ink smears are drawn across them. If it is desired to examine the organism without staining for capsules, the India ink need not be drawn to the entire end of the slide, and those organisms will appear stained simply with crystal violet and can readily be compared with those stained for capsules.

Cultivation of Organisms: All organisms were grown on liver infusion agar, potato agar, tryptose agar, liver infusion broth, tryptose broth, liquid potato media, and beef extract broth, under varying conditions. All media used were adjusted to a final pH of 6.8. Serum from rabbits immunized against Brucella abortus, strain 2308, was added to all types of culture media in dilutions of 1:10, and Strain 2308 was grown on this media to test the effect of the serum on capsule production. A study was made of organisms growing on all types

of media at the end of 1, 2, 3, 4, 5, 9, 12, 15, 20, 25, and 30 days at room temperature and also at 37° C. Organisms have been kept on liquid media at room temperature without transfer for a year and stained and tested for viability. In addition, organisms have been kept at 5° C. on solid media for periods of 30, 60, 90, 120 days, and up to periods of 18 months without transfer and tested for viability, appearance, and antigenic characteristics. All organisms have been plated out at varying intervals and examined for appearance of rough and smooth colonies, and these have been picked off and inoculated into media and examined.

Serological Tests: Agglutination tests were carried out on all strains at various periods of growth, using rabbit sera prepared with 2308 abortus, 428 melitensis, and 47 suis, as well as other animal sera which came into the laboratory from time to time. Opsonocytophagic tests were made in conjunction with the agglutination tests.

The technique used for the opsonocytophagic test was a modification of that followed by Huddleson and described in his book, Brucellosis in Man and Animals, 1939, and in more detail by Huddleson and Meyer, 1936. This method, in turn, was a modification of the Leishman-Veitch (1905, 1908) technique of a much earlier period. The method, as adopted and used in this study, is described briefly as follows:

Whole citrated blood is essential for the technique. A 20% sodium citrate solution has been found satisfactory and is used in 0.2 cc. amounts to every 5 cc. of blood drawn and

this gives a final dilution of sodium citrate in the blood of 0.8%. The antigen is prepared for the blood of cattle by using a solution of 0.5% barium chloride and 0.6% sodium chloride. The antigen for all other types of blood is prepared in physiological salt solution. All antigens are adjusted by means of an electrophotometer to correspond with tube 8 MacFarland standard. This differs materially from Huddleson's technique in that he suggests using tube 16 MacFarland nephelometer.

Small tubes are employed, similar to those used in other serological tests, and 0.1 cc. of citrated blood is mixed with 0.1 cc. of antigen. The tubes are gently shaken and incubated for 30 minutes in a 37° C. water bath. Upon removal they are shaken and two large loopfuls of the mixture are placed on a clean slide and drawn across with another slide in the same manner as that used in making blood films. If a heavier film is desired, the smearing slide may again be drawn back to the starting point, raising it very slightly from the slide which is being smeared. It is essential that the slides be dried very rapidly in a current of air in order that there be no distortion of the blood cells. They are then stained with toluidine blue, using chloroform for fixation. This produces a deep blue color in the organisms and leaves the granules in the leucocytes colorless.

The stain originally prepared by Calmette, Negri, and Boquet, 1926, is made up as follows:

```
Toluidine blue . . . . . . . . . . . . .   0.5 gram
Ethyl alcohol 95 per cent  . . . . . .  10.0 cc.
Phenol . . . . . . . . . . . . . . . .   3.0 cc.
Distilled water q. s.  . . . . . . . . .100.0 cc.
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The smears are fixed in chloroform from 5 to 15 seconds and completely dried before being placed in the stain. They are stained from one to three minutes and then washed in distilled water and rapidly dried in air without blotting.

The size of the Brucella organism and the very marked phagocytosis which often occurs make it necessary to record the results somewhat differently from that which is usually used in such studies. A total of 25 neutrophils are examined in different parts of the slide and each cell is recorded as follows:

1. Marked, when more than 40 bacteria are present.

2. Moderate, when from 21 to 40 bacteria are present.

3. Slight, when less than 20 bacteria are found.

4. Negative, when no bacteria are present.

The different degrees of phagocytosis of Brucella suis are shown in Figs. 1, 2, 3, 4.

Huddleson emphasizes that a known smooth strain of organisms should be used in making the bacterial suspension and that this should be of the concentration of MacFarland's tube 16 for best results. We have found such a concentrated suspension to be confusing in making counts, inasmuch as it is impossible to tell whether the organisms are actually inside the blood corpuscles or lying on top of them. Moreover, the blood corpuscles of cattle, hogs, guinea pigs, and rabbits will engulf more than forty organisms even when the suspension is very slightly turbid

if opsonins are present, although this may not be true of human
blood. One of the most important points to be stressed, we
feel, is to have blood which is freshly drawn and not exposed
to extremes of heat or cold. Blood which is 5 or 6 hours old
does not appear to advantage beside freshly drawn blood. It
is also absolutely essential to have a 48-hour culture of a
smooth strain of organism which has not undergone the slightest
dissociation or loss of capsules by too rapid transfer. The
culture may be kept a week or more in the refrigerator and
still be perfectly satisfactory, but cultures which are trans-
ferred every 48 hours soon become quite unreliable.

Guinea pigs were used exclusively for animal inoculations.
They were bled regularly at one-week intervals and killed at
four weeks for appearance of lesions.

Several methods were tried for stripping organisms of
their capsules, such as allowing them to remain in distilled
water for 12 to 24 hours; very rapid transfer, using 6-hour to
one-day intervals; and the method used by Mickle with success
in which organisms were grown on liver infusion agar and trans-
ferred to beef broth.

Results

Microscopic Appearance of Organisms: A single strain of
Brucella seems to grow equally well on tryptose, potato, or
liver infusion agar; but different strains vary considerably
in the amount and rate of growth and in the size and shape of
the colonies. All the organisms studied, whether growing on
artificial culture media or in the animal body show capsules

under most cultural conditions. The usual size of a capsule varies from 1.5 to 5 microns, depending on the strain of the organism and the conditions of growth. In almost every instance the capsules and their bacterial cells retain their relative proportions. If the capsule becomes larger, the cell does also. In general, it was observed that the moisture content of the media determines, to a large extent, the size of the organism and its capsule. The addition of 10% immune rabbit serum did not affect capsule production. The capsules of smooth strains of Brucella suis and Brucella abortus, growing on solid media, are similar in appearance. They are generally ellipsoidal with a smooth and rather distinct margin, a pale pinkish purple cast, and contain a deeply staining purple bacterial cell which is centrally located (Figure 5). Considerable difference is noted in the shape and size of the capsules, even when all come from a single colony. Some tend to be almost round, and others appear to be quite elongate. These latter are probably in the process of cell division and have not as yet separated.

Melitensis capsules in the smooth strains tend to be round with the same characteristics as those observed in abortus and suis, except that the bacterial cell in general is also coccoidal (Figure 6). Here, also, are many different shapes and sizes. Melitensis in some cultures appears almost like Brucella abortus and Brucella suis with most of the organisms elongate, and is almost indistinguishable from them in microscopic examination.

Unencapsulated organisms are frequently noted in smooth cultures of the three species as small groups of navy blue organisms without capsules. They may be distinguished from encapsulated organisms even when the capsular stain is not used since they appear navy blue, while the encapsulated organisms are a deep purple when stained with crystal violet. These unencapsulated organisms are much more numerous in rapidly transferred cultures than in those which are incubated 48 hours or more.

The capsules of the rough or intermediate strains vary considerably in appearance, particularly under different types of cultivation (Figure 7). At 48 hours of incubation the capsular margin is less definite than in the smooth strains, and in some forms it is slightly irregular. The intensity of color of the bacterial cell decreases with the virulence of the organism in most forms, though there are some notable exceptions as, for example, Strain 19 Brucella abortus, which is nearly as perfect in color intensity as the most virulent strains. Huddleson's avirulent strain shows only a faint suggestion of bacterial cell body. The pale pinkish purple color of the capsule is present, however. When these organisms are grown for six days at 37° C. on solid media, large and fairly distinct cell bodies appear. They are never quite as perfect in contour and staining capacity as the smooth strains, however,.

Intermediate strains will produce well-stained, clear-cut capsules more quickly than rough strains, but three to

several days' incubation is necessary. In general, six days' incubation at 37° C. on solid media produces the largest and most distinctly staining organisms in all strains.

The smooth strains produce a fairly well-staining bacterial cell body within six hours, and 24-hour cultures show almost as large and clear-cut capsules as those incubated for six days; and by the end of 48 hours the capsule is as large and as clear-cut as it ever will become.

Organisms stained directly from tissue or the stomach contents of aborted fetuses show, for the most part, two types of organisms. Some of them are large, clear-cut, encapsulated forms, while others appear to be of the intermediate type. In the samples studied at the laboratory, there have always been a larger proportion of well-encapsulated, clear-cut organisms than of the apparent intermediate forms.

Life History Studies: In studies made at regular intervals of newly transferred organisms, capsules showed no change during the first two hours and then began to become roughened at their margin and the bacterial cell inside became violet in color. In the smooth strains the bacterial cell never wholly disappeared, but in the intermediate forms it was no longer visible. During the entire 24-hour period observed, the capsule was not lost but gradually changed from the rough-edged form with a very pale bacterial cell body to a clear-cut, smooth form. The time consumed in the process varied from six hours to six days, depending on the strain under examination.

Incubation of organisms on solid culture media for vary-
ing periods: Smooth, rough, and intermediate strains of Bru-
cella were kept at 37° C. on tryptose, potato, and liver in-
fusion agar for 30 days. When transferred to tryptose agar
plates and examined for colony formation, there seemed to be
only slight irregularity and few actual rough colonies in the
usual smooth strains. The original culture does not seem to
have changed greatly in appearance when examined microscop-
ically; but on being transferred, all cultures examined show
the characteristics of rough or intermediate strains. The
bacterial cell body is indistinct and the margins of the pale
pink capsules are slightly irregular. There is a tendency
toward chain formation and clumping (Figure 8). Rapid trans-
fers caused the organisms to regain some of their former
characteristics, but they had not completely recovered their
usual appearance after 22 transfers. One guinea pig passage,
however, restored the organisms to their former appearance.
Both smooth and rough strains began to show this capsular
variation about the twelfth day of incubation at 37° C. on
solid culture media.

Organisms kept at room temperature for 30 days show very
little change, and two or three transfers bring them back to
their original characteristics.

In an experiment using refrigerator temperature at 5° C.
for storage, it was found that organisms maintain their via-
bility and usual appearance 150 days without transfer, but
on being transferred, they had many characteristics of the

organisms maintained at 37° C. for 30 days. However, they do
regain their former appearance after three or four rapid
transfers. Organisms retain their characteristics on trans-
fer after 60 days at refrigerator temperature. Brucella which
are held at 5° C. for one year and for 18 months became smaller
but retained their staining characteristics. On first transfer
they frequently appeared to be well encapsulated, though their
serological characteristics and virulence were quite differ-
ent from the original strain, there being a tendency for con-
siderable reduction in virulence, particularly with the former
virulent organisms. Strain 2308, for example, after 18 months
at refrigerator temperature showed less virulence than did the
same strain which had been frequently transferred. Table 1
indicates the results of guinea pig inoculations made for
virulence of the three species of organisms. After being held
at 5° C. for 18 months, none of the former virulent strains
agglutinated in as high titer as they had at the beginning or
during the experiment. Table 2 shows the results of the agglu-
tination tests in detail.

Lyophilized organisms examined microscopically at the
end of 12 months and 18 months showed a slight decrease in
the size of the capsule and bacterial cell before incubation,
but otherwise they appeared as they did before freezing and
drying, the smooth and rough forms each with their own charac-
teristics. After culturing on solid culture media for 48
hours, it was found that the organisms appeared about twice
as long as before lyophilizing. Melitensis appeared very

similar in appearance to the usual abortus and suis strains.
Two further transfers produced strains having the usual
characteristics.

Incubation of Organisms on Liquid Media for Varying
Periods: When examined under the microscope, Brucella grown
on liquid media at 37° C., whether it be potato, 2% tryptose
broth, liver infusion broth, or beef infusion broth, exhi-
bited unusual characteristics within 24 hours. These changes
were seen first in the rough and intermediate strains and
occur most rapidly in liver infusion broth, but appear within
three days in the other kinds of media mentioned. The organ-
isms at first tended to form chains with each organism and
capsule a separate entity; but soon the individual cell and
its capsule was lost and several organisms appeared as a huge,
long capsule surrounding a deep red bacterial cell which lies
along one side of the capsule (Figures 9 and 10). If these
organisms are examined under a dark field, it is sometimes
possible to see the divisions between the separate organisms;
but more often no separations are evident. It has been re-
ported by several investigators that organisms grown in liver
infusion broth passed from smooth to variant forms rather
rapidly and became larger and longer in the variant form. In
from three to six days there were numerous unencapsulated
organisms to be found on all strains examined. They appear
earlier and are greater in number in rough and intermediate
forms, but they can also be seen in slides of smooth organ-
isms if growing on liquid media for several days. After

some ten or twelve days, all Brucella growing on liquid media appear encapsulated, but most of them assume a rough-edged form of capsule with an indistinct bacterial cell. Some few strains show very large multiple cells even after long periods of growth on liquid culture media (three months with several transfers). After growth on liquid culture media without transfer for twelve months, those organisms which lived appear once again to have assumed smooth forms, even though they were of the rough or intermediate type prior to transfer to liquid media. These smooth organisms, however, do not produce recognizable characteristics of brucellosis in guinea pigs. However, a titer of 1:200 developed in some instances with high opsonocytophagic activity in guinea pigs. None of these organisms proved to make a satisfactory antigen for normal sera.

Since many of the results obtained in the capsule study had differed from those obtained by Mickle, it was thought that it would be interesting to compare the culture characteristics of Brucella in three other kinds of liquid media besides liver infusion broth which he had used. Ten cultures of Brucella were inoculated into liver infusion broth, potato infusion broth, beef broth, and tryptose broth and observed for 35 days. One group of samples was incubated at 37° C. and another group, at room temperature. A list of the cultures used and the results of the experiments are tabulated in detail in Table 3.

It was found that a pellicle developed on liver infusion broth in every case within the 35 day period, except for

Strain 19 _Brucella_ _abortus_, which developed no pellicle on
any media. The pellicle developed in liver infusion broth
in from four to eight days in rough strains, in eight to
twelve days in intermediate strains, and the smooth strains
required 30 days or longer for a pellicle to develop. Pel-
licles developed somewhat irregularly, if at all, in the
other three media used. Table 3 shows the development of
surface film, mucous sediment, and pellicle formation in the
four types of media.

Within one week a larger number of strains produced a
mucoid sediment at the bottom of the tube of liver infusion
broth than in any of the other media used. After the second
week, however, six strains had produced such a sediment in
both liver infusion broth and potato media; five strains, in
beef infusion broth; eight strains, in tryptose broth. Most
of the strains showed mucoid sediment by the eleventh day in
all media. This seemed to be quite irrespective of whether
the strain had been smooth, intermediate, or rough at the
beginning of the experiments. The mucoid phase described by
Plastridge and McAlpine, 1930 and 1937, and Mickle, 1940,
seems to be unrelated to the production of a pellicle, since
in many of the tests when no pellicle was present, mucoid
sediment developed.

Brucella were denuded of their capsules by growing the
organisms first on liver infusion agar for 48 hours and then
transferring them to a beef infusion broth, as was described
by Mickle, 1940. Within 24 to 48 hours all the organisms were

unencapsulated. However, within another 48 hours all organisms were again encapsulated, whether or not they had been transferred to other media. These capsules were slightly roughened and the bacterial cell body had the appearance of dissociated strains. Suspensions of these stripped organisms were agglutinated in positive serum in nearly as high titers as the same encapsulated strains. Guinea pigs were inoculated with 1) stripped organisms, 2) organisms which had been stripped but whose capsules had reappeared, and 3) a smooth strain of the organism. They were then compared, using weekly agglutination and opsonocytophagic tests. There was no marked difference in the three groups tested, as shown on Table 4. About 75% of the organisms, particularly the rough and intermediate strains, lost their capsules when suspended in distilled water for 24 hours; but on being transferred to culture media, regained them within 48 hours.

There is a tendency for unencapsulated organisms to be found in cultures growing on solid media which have been transferred rapidly. It has been noted that about 50% of the organisms are unencapsulated in cultures which have been transferred every 24 hours for a week. When the transfers have been made even more rapidly, e.g. every 12 hours, unencapsulated organisms in large numbers appear at the end of the second day.

It was never possible by either of these last described methods to remove all of the capsules, and at no time was a strain of unencapsulated organisms cultivated for more than 48 hours.

Discussion

The 38 strains of Brucella examined, whether smooth, intermediate, or rough, normally showed capsules. This is not in agreement with the results of Mickle, 1940, who found most of the organisms which he examined from cases of brucellosis to be unencapsulated; but it is in accord with Huddleson, 1940, who found capsules on rough, smooth, and intermediate strains.

The rapidity and luxuriance of growth and appearance of the organisms did not differ on the three kinds of solid culture media used. The differences between rough, smooth, and intermediate strains could be readily detected by microscopic examination of slides stained to show capsules. Smooth strains have capsules of a pinkish purple color with smooth, distinct margins. The bacterial cell body is centrally located and is a deep purple with a distinct, clear-cut margin. The rough strains have a pale pinkish capsule with a roughened margin, and the bacterial cell body appears as a heavily veiled, purplish mass in the center, quite indistinct as to margin. The intermediate types lie between the smooth and rough forms in appearance, in that the purple bacterial cell is less vague than in the rough forms but are not as clear-cut as the smooth forms. There is also a tendency toward a slight irregularity in the capsular margin.

The exact cause of these unusual appearances in Brucella stained with capsular stain is not known. All the forms stained equally well in ordinary stains, but it may be that

the charge of the India ink particles allows the penetration
of the crystal violet dye in the smooth strains, but does not
allow much penetration in the rough and intermediate forms.

Unencapsulated organisms stain navy blue with crystal
violet and then can be distinguished readily in either capsule
staining or with crystal violet alone.

Definite dissociation begins to appear in smooth strains
held at 37° C. for 12 days on solid culture media, and they
have so completely dissociated at the end of 30 days that
only guinea pig inoculation will cause them to return to their
former smooth form. Brucella may be held 30 days at room
temperature without any visible signs of change except a
decrease in size, probably due to the loss of water. The
organisms may be held at 5° C. on solid culture media for 60
days without dissociation. At 150 days there was appearance
of some dissociation, but there was rapid recovery after four
transfers. After 18 months at 5° C. these organisms lacked
virulence and did not serve as good antigens. This seems to
throw some light on the problem of maintenance of cultures
and the frequency with which they should be transferred.

It required two transfers for lyophilized organisms to
return to their original appearance after culturing, though
they showed no evidence of dissociation.

Organisms, upon being transferred to liquid media, began
at once to change in appearance. They became greatly enlarged,
formed chains, and fused into huge organisms. The rough-edged,
pale bacterial cell body form of the rough strains appeared

about the eleventh day. However, if these organisms are
allowed to remain in the liquid media for 12 months, only
smooth strains are recovered. These had lost their viru-
lence to a very marked degree. This may prove to be a good
method for the attenuation of organisms.

The development of mucoid sediment in liquid culture
media seems to be unrelated to the production of a pellicle.
The pellicle is apparently produced rapidly when rough or
intermediate organisms are grown on liver infusion broth and
other liquid media, whereas the pellicle does not develop in
smooth forms for 30 days or more. The mucoid sediment prob-
ably is produced because of the unusual chain formations and
coalescence of capsules.

Brucella have been denuded of their capsules for as long
as 48 hours if grown on liver infusion agar and then trans-
ferred to beef broth. However, after 48 hours organisms be-
come encapsulated and assume a form similar to that found on
rough strains. Rapid transfer will cause a portion of the
organisms to lose their capsules, and suspension in distilled
water for 24 hours will produce some unencapsulated organisms,
especially in rough and intermediate strains; but neither of
these methods produces permanently unencapsulated forms. The
unencapsulated strains did not show much variation from the
ordinary encapsulated strains when used as antigens. Hence,
it was impossible to reproduce the results obtained by Mickle,
who found many unencapsulated strains.

It would appear that rough, smooth, and intermediate

strains could be more readily and accurately determined by microscopic examination of the organisms stained to show capsules than by examination of the colonies on agar plates. It is important, particularly in such tests as the opsonocytophagic test, to have a perfectly smooth strain of organisms with no unencapsulated forms present; and microscopic examination is the only method for distinguishing such a culture.

These studies also indicate that Brucella cannot be kept on liquid culture media without undergoing rapid dissociation. However, if they are kept for a long period of time on this media, they produce a smooth organism of little or no virulence; and this form might prove to be an adequate organism for use in vaccination procedures.

FIGURE 1
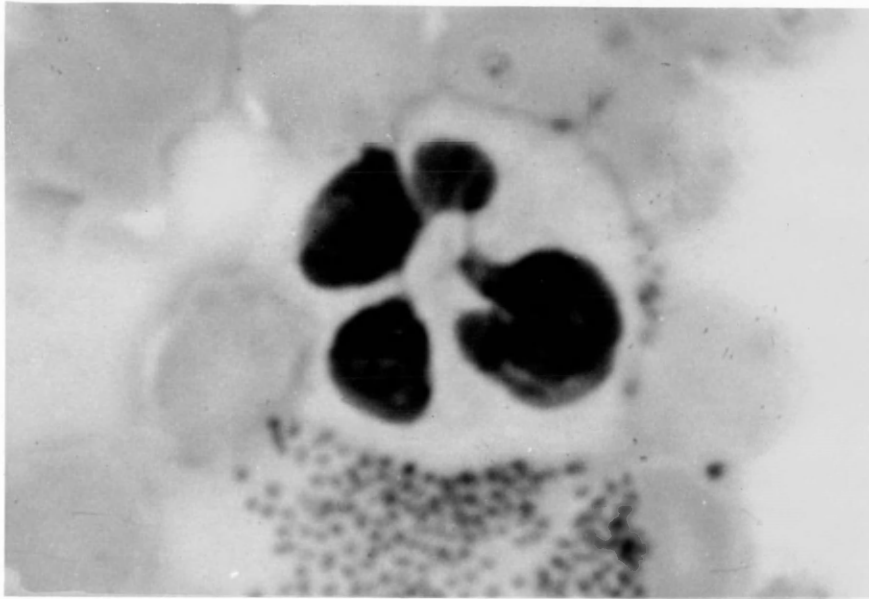
Phagocytosis of Brucella

Negative Leucocyte Showing no Phagocytosis
x 2000

FIGURE 2
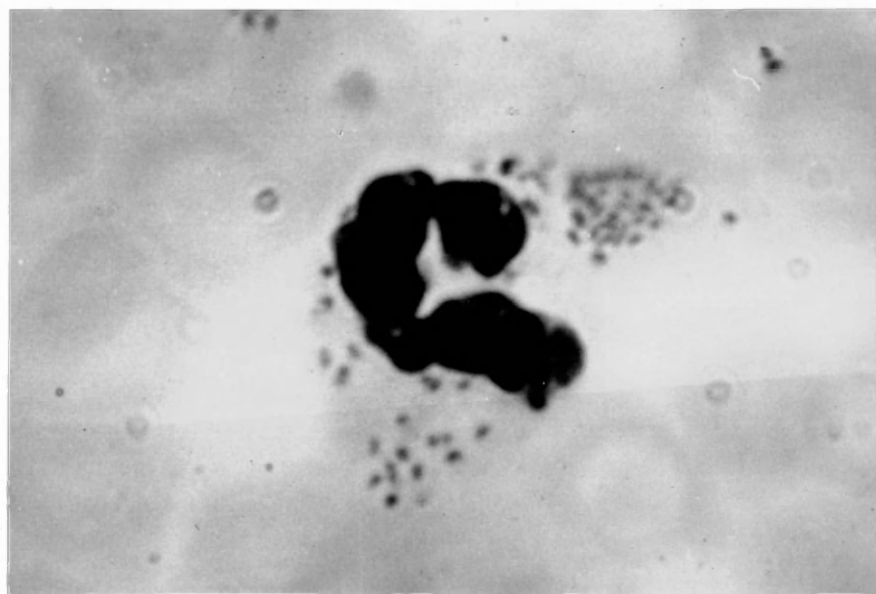
Phagocytosis of Brucella

Leucocyte Showing Slight Phagocytosis
1-20 bacteria in cell
x 2000

FIGURE 3
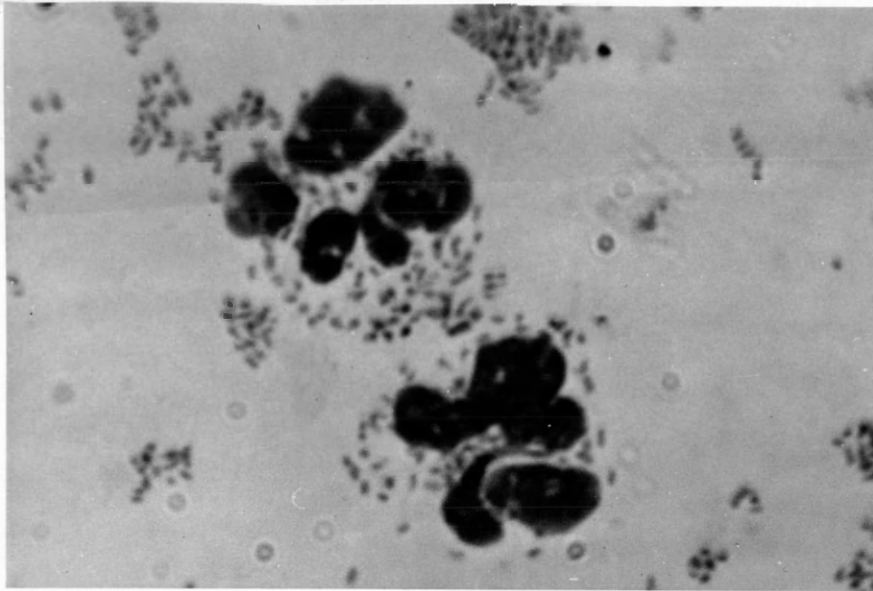
Phagocytosis of Brucella

Leucocyte Showing Moderate Phagocytosis
21-40 bacteria in cell
x 2000

FIGURE 4
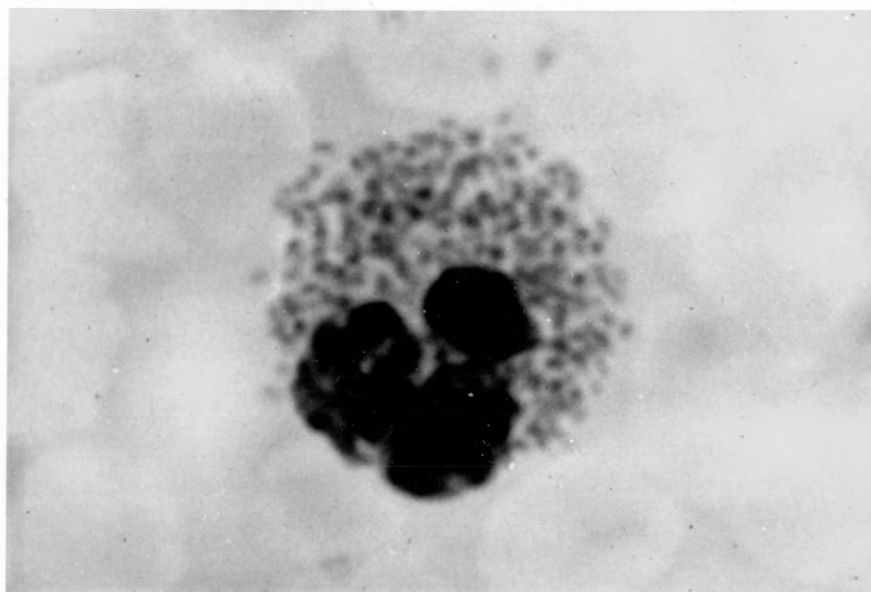
Phagocytosis of Brucella

Leucocyte Showing Marked Phagocytosis
More than 40 bacteria in cell
x 2000

FIGURE 5
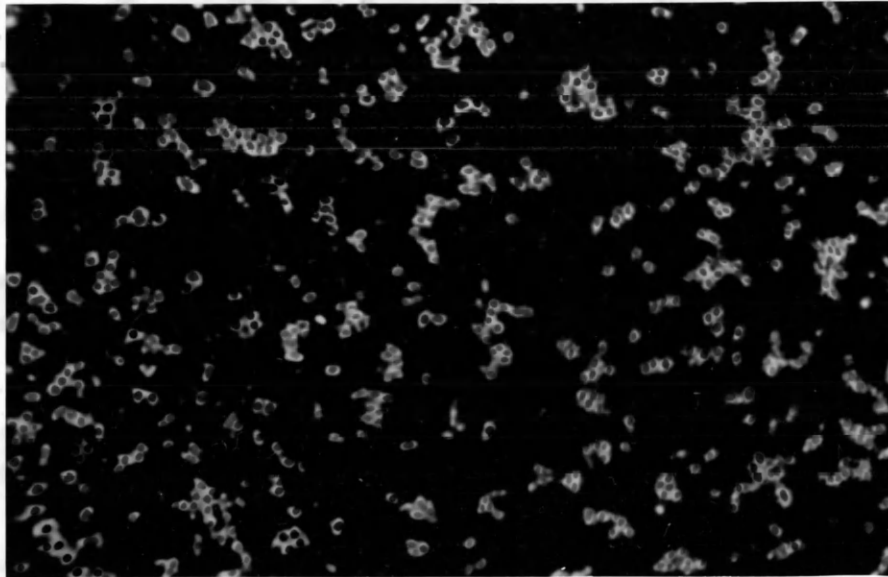
Photomicrograph of 48-hour Culture of Brucella abortus
Strain 2308, Growing on Tryptose Agar at 37° C.

India Ink - Crystal Violet Capsular Stain
x 2000

FIGURE 6
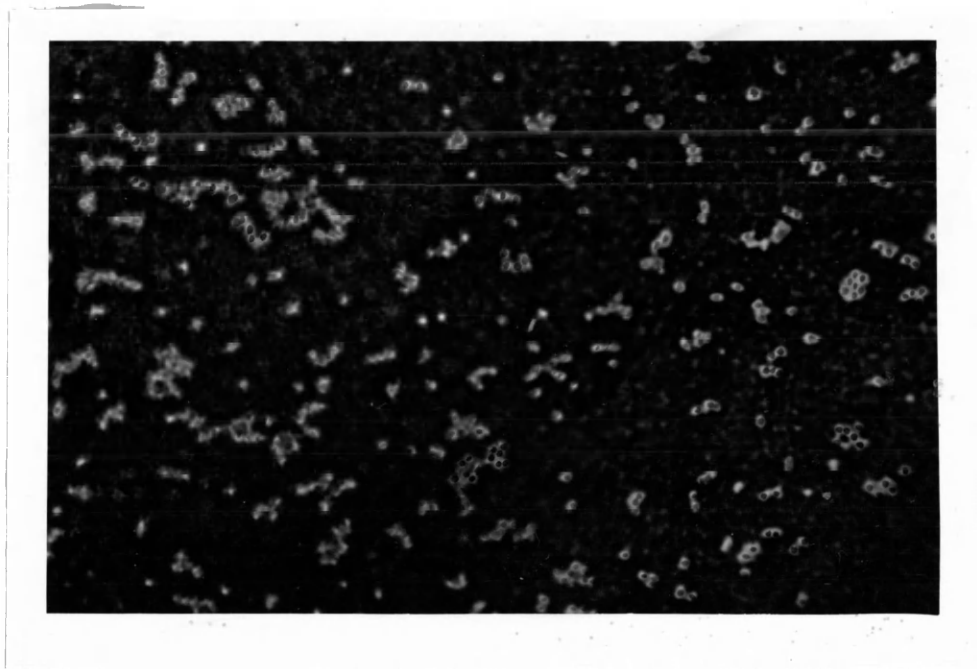
Photomicrograph of 48-hour Culture of <u>Brucella Melitensis</u>
Strain 1766, Growing on Potato Infusion Agar at 37° C.

India Ink - Crystal Violet Capsular Stain
x 2000

FIGURE 7
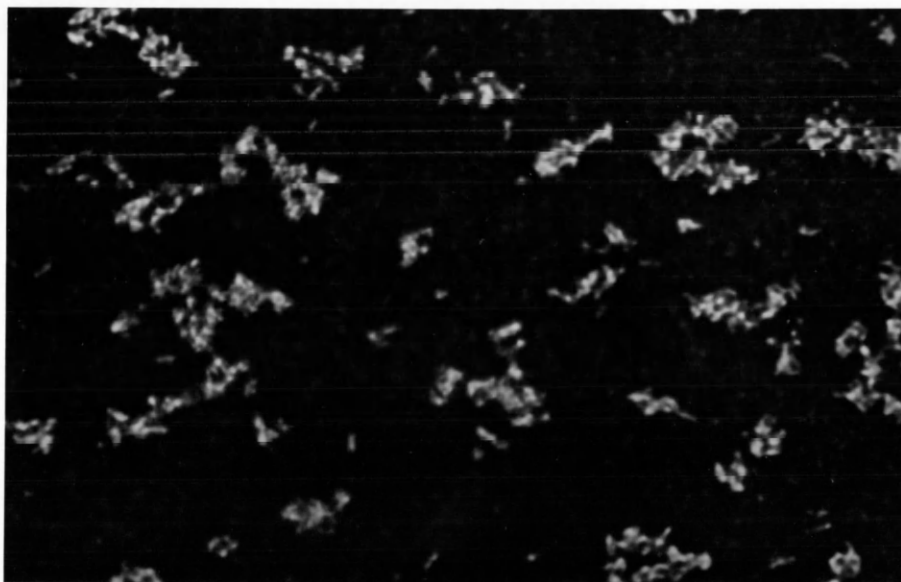
Photomicrograph of 48-hour Culture of Brucella Abortus
Atypical Strain 801, Growing on Potato Infusion Agar at 37° C.

India Ink - Crystal Violet Capsular Stain
x 2000

FIGURE 8
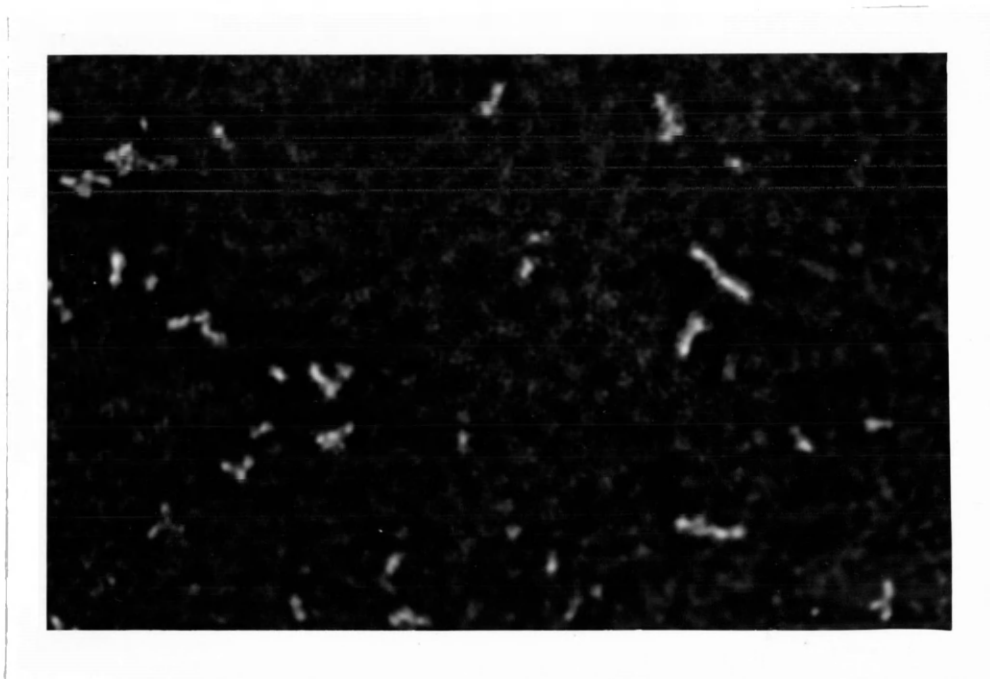
Photomicrograph of 30-day Culture of <u>Brucella</u> <u>Abortus</u>
Strain 2308 Grown on Tryptose Agar at 37° C.

India Ink - Crystal Violet Capsular Stain
x 2000

FIGURE 9
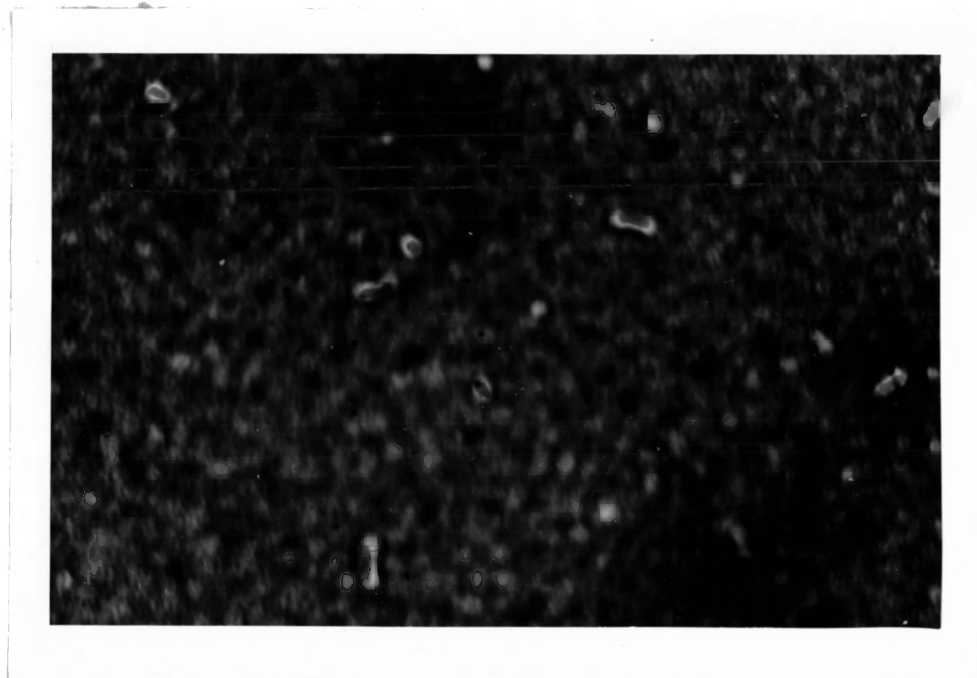
Photomicrograph of 48-hour Culture of <u>Brucella</u> <u>Suis</u>
Strain 2872 Growing on Tryptose Broth

India Ink - Crystal Violet Capsular Stain
x 2000

FIGURE 10
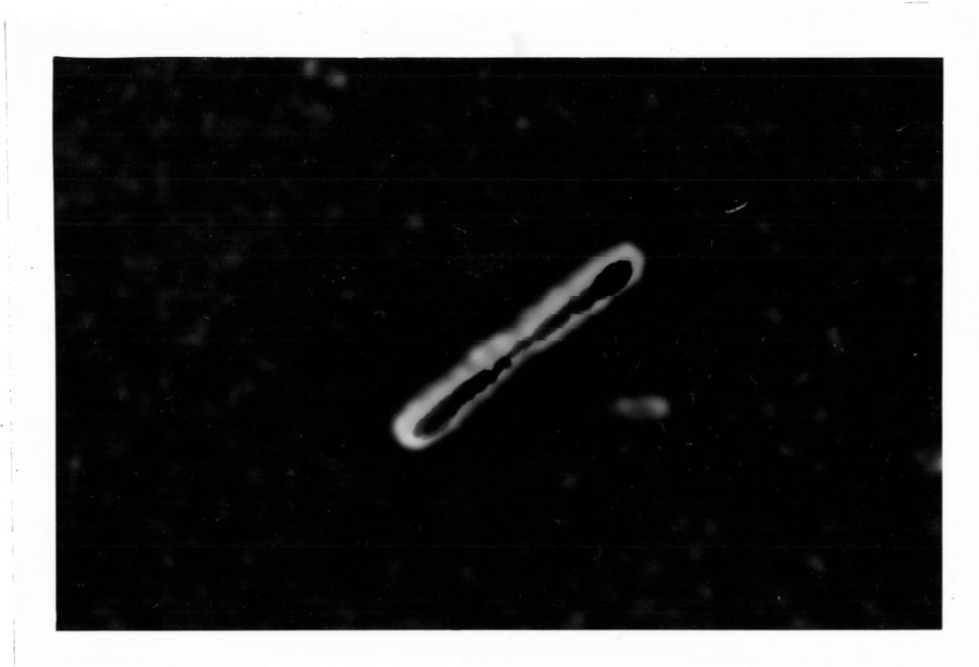
Photomicrograph of 96-hour Culture of <u>Brucella Suis</u>
Strain 2872 Growing on Tryptose Broth

India Ink – Crystal Violet Capsular Stain
x 4000

TABLE 1

Comparison of Agglutination Titers and Opsonocytophagic Indices of Guinea Pigs Inoculated with Strains of Brucella kept at 5° C. without transfer for 18 months with Young Cultures of the Same Strain

	Strains of organisms for inoculation	Pre-inoculation Tests					Post - inoculation Tests																	
							1st Week					2nd Week					3rd Week					4th Week			
		*Ag	**Opsono				Ag	Opsono				Ag	Opsono				Ag	Opsono				Ag	Opsono		
			Ma	Mo	Sl	N		Ma	Mo	Sl	N		Ma	Mo	Sl	N		Ma	Mo	Sl	N		Ma	Mo	Sl	N
Held at 5° C. for 18 mo. without transfer	Br. abortus 2308	-	0	0	3	22	-	9	10	6	0	3+	16	8	1	0	-	18	7	0	0	-	20	2	3	0
	Br. suis 2672	-	0	0	5	20	-	3	9	12	1	4+	13	6	1	0	+	15	9	0	0	-	16	9	0	0
	Br. melitensis Henry	-	0	0	9	16	-	8	7	9	1	3+	15	4	6	0	+	16	4	4	1	-	16	5	4	0
Control strains transferred regularly	Br. abortus 2308	-	0	0	8	17	+	8	7	8	2	8+	10	8	4	5	8+	6	12	7	0	8+	9	10	8	0
	Br. suis 2672	-	0	0	12	13	-	3	9	9	4	4+	9	9	5	2	8+	8	9	8	0	8+	6	9	7	1
	Br. melitensis Henry	-	0	0	10	15	+	7	9	7	2	6+	8	9	6	2	7+	7	10	6	2	7+	6	9	8	2

*Agglutination Titers

+ -- 1:25	5+ -- 1:250
2+ -- 1:50	6+ -- 1:500
3+ -- 1:100	7+ -- 1:1000
4+ -- 1:200	8+ -- 1:2000

**Opsonocytophagic Test

Ma - Marked
Mo - Moderate
Sl - Slight
N - None

On autopsy at four weeks:

Group inoculated with organisms kept for 18 months at 5° C. showed no lesions and organisms were not recovered.

Group inoculated with corresponding strain of Brucella which had been regularly transferred showed typical spleen and lymph gland lesions, and organisms were recovered from all three.

TABLE 2

Comparison of Agglutination Tests
Using Strains of Brucella
Kept at 5° C. without transfer for 18 months
with a Young Culture of the Same Strain

Antigens	Antiserums	Agglutination Titers	
		O. C.	Y. C.
Br. abortus, 2308	Serum, rabbit immunized with Strain 2308 Br. abortus	1:50	1:2000
Br. suis, 2872		1:100	1:1000
Br. melitensis, Henry		1:50	1:500
Br. abortus, 2308	Serum, guinea pig immunized with Strain 2872 Br. suis	1:50	1:1000
Br. suis, 2872		1:50	1:1000
Br. melitensis, Henry		1:50	1:500
Br. abortus, 2308	Positive cattle serum I of high titer	1:100	1:2000
Br. suis, 2872		1:100	1:1000
Br. melitensis, Henry		1:50	1:1000
Br. abortus, 2308	Positive cattle serum II of high titer	1:50	1:1000
Br. suis, 2872		1:100	1:1000
Br. melitensis, Henry		1:50	1:500

TABLE 3

Study of the Development of Surface Film,
Mucoid Sediment, and Pellicle Formation
in 10 Strains of *Brucella*

	Liver Infusion Broth					Potato Liquid Media					Beef Infusion Broth					3% Tryptose Broth				
	1st wk	2nd wk	3rd wk	4th wk	5th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk
Br. suis Indiana	sf ms		p				ms	p					sf	p		ms			p	
Brucella melitensis Henry	sf ms			p			ms	growth			ms						ms			p
Br. suis 1942[1]			ms	p				ms	p							ms				
Brucella abortus 19	ms							ms			ms					ms				
Brucella abortus 45-20	ms p						ms p				ms					ms				
Brucella abortus 1119	ms				p	ms					ms p							ms		
Br. suis 2872			ms		p			ms			ms					ms				
Br. suis King 8			ms	p		ms				p			ms			ms				
Brucella abortus 2308			sf ms				p	ms												
Brucella abortus Huddleson	sf ms		p				ms	sf	p				ms	sf		ms			p	

sf -- surface film
ms -- mucoid sediment
 p -- pellicle

TABLE 4

Comparison of Agglutination Titers and Opsonocytophagic Indices
in Guinea Pigs inoculated with <u>Brucella abortus</u>, Strain 2308
a. Stripped organisms
b. Organisms stripped of capsules, later regained
c. Smooth strain of organisms

	Pre-inoculation Tests					Post - inoculation Tests																
						1st Week					2nd Week					3rd Week					4th Week				
	*Aggl	**Opsono				*Aggl	Opsono				*Aggl	Opsono				*Aggl	Opsono				*Aggl	Opsono			
		Ma	Mo	Sl	N		Ma	Mo	Sl	N		Ma	Mo	Sl	N		Ma	Mo	Sl	N		Ma	Mo	Sl	N
Stripped organisms	-	0	0	4	21	4+	8	10	7	0	8+	10	10	5	0	8+	11	10	4	0	6+	15	8	2	0
Capsules stripped but reappeared	-	0	0	9	16	3+	10	7	8	0	8+	8	8	8	1	6+	15	10	2	0	4+	15	10	0	0
Smooth strain without treatment	-	0	0	15	10	4+	9	9	7	0	8+	10	9	6	0	8+	14	10	1	0	8+	14	10	1	0

*Agglutination Titers

+ -- 1:25	5+ -- 1:250
2+ -- 1:50	6+ -- 1:500
3+ -- 1:100	7+ -- 1:1000
4+ -- 1:200	8+ -- 1:2000

**Opsonocytophagic Test

Ma - Marked
Mo - Moderate
Sl - Slight
N - None

A STUDY OF THE BLOOD PICTURE IN PORCINE BRUCELLOSIS

A study of the blood picture of healthy, infected, and vaccinated hogs was undertaken as an aid in understanding the differences in the physiological responses of hogs infected with Brucella. The total number of leucocytes and erythrocytes per unit volume of blood was determined, as well as the fluctuations in the types of white blood cells. It was hoped that there would be sufficient difference between infected and noninfected animals to make this a useful aid in diagnostic tests.

Experimental Procedures

Animal Groups: The present investigation deals with a relatively large number of swine (423) on which 1226 studies of blood pictures were made. These animals included several groups of hogs which were being used in other experiments in brucellosis at the Animal Disease Station, National Agricultural Research Center at Beltsville, Maryland. For convenience in tabulation, the animals were divided into five groups:

Group A consisted of 176 adult animals which were negative to the agglutination test at the time they were tested. From one to three studies of the blood were made on each of the animals.

Group B included 89 adult animals which had been infected, according to the agglutination test, from one month to one year or more. These animals all had a titer of 1:100 or over

at the time the tests were made. Two to five blood studies were made of each animal.

Group C was made up of 53 animals which had shown titers of 1:100 or more in the first test, but whose titers were 1:50 or less on subsequent tests. Two or more studies were made on each animal.

Group D was comprised of 58 gilts which showed low titers (1:25 or less) or none, in the agglutination tests, but which had been in an infected herd. They had, however, been segregated from the rest of the herd. Two studies were made of each animal.

Group E contained 47 animals vaccinated subcutaneously with 5 cc. of King 8 strain of Brucella suis (a strain of reduced virulence) and studies were made of this group at weekly intervals.

Methods of collecting samples: The problem of drawing blood from swine is a difficult one, since the usual method of procuring it from the jugular vein is practically impossible. The blood will be more nearly sterile if drawn from the superficial ear veins, but this is a difficult procedure for one not experienced in handling hogs. It can be drawn more conveniently by nicking the tail which must later be ligated to prevent continued bleeding. Both methods were used in obtaining the blood samples for these tests, though an attempt was made in every case to draw the blood from the ear.

Since it is difficult to obtain the same amount of blood

from the ear of each animal, the dilution in a 20% solution
of sodium citrate varied somewhat; but the blood was meas-
ured and the variations taken into account. It was found
that 0.1 cc. of 20% sodium citrate would keep 10 cc. of por-
cine blood from clotting if the blood was thoroughly mixed
with the citrate immediately upon being collected; but in
general it proved to be more satisfactory to use 1 cc. of 20%
citrate for this amount of blood since the bleeder was not
always able to shake the tube at once. Even with this pre-
caution there was considerable difficulty with clotted blood,
particularly when it had to be held several hours before the
blood cells were counted.

Techniques of counts and tests: The studies included
an erythrocyte count, total leucocyte count, differential
leucocyte count, grams of hemoglobin per 100 cc. of blood
and the sedimentation rate.

In the erythrocyte studies red cell counts were made
from the same pipette on the two sides of a double hemocy-
tometer slide, the two enumerations checking within 100,000
cells. Counts of the white blood cells were compared on both
sides of a hemocytometer slide and either checked within 500
cells or were repeated. Slides for differential counts were
stained with Wright's, Giemsa, or Hasting's stains and in
every case 100 cells were classified.

The hemoglobin was measured with a Dare hemoglobinometer
and the grams per cubic centimeter in each animal was checked
with the Tallquist technique. A few were also checked with an
electrophotometer.

Sedimentation rate was measured in a Cutler blood sedimentation pipette in those samples which were received within a short time after collection, since a delay in setting up the test decreases the rate and many of the samples were too old to obtain accurate results.

Agglutination tests were done on all the blood on which blood studies were made.

Results

Hemoglobin: The values obtained for hemoglobin in the five groups of animals were not significantly different. The uninfected Group A had an average of 12.21 gm. of hemoglobin per 100 cc. of blood. The range was from 8.53 to 15.14 gm. of hemoglobin per 100 cc. of blood.

The infected animals in Group B averaged 11.569 gm. per 100 cc. blood with a high of 14.59 and a low of 7.28. Seven animals in the group which had been infected for a year or more showed only 10.32 gm. per 100 cc. of blood. This was noted in three examinations.

Groups C and D were not tested for hemoglobin.

The vaccinated Group E showed an average of 12.26 gm. per 100 cc. of blood on the prevaccination test and 11.40, 10.74, 10.32, and 11.42 gm per 100 cc. of blood on four postvaccination tests at weekly intervals (Table 5). A high of 14.32 gm. per 100 cc. and a low of 8.12 were noted in the group.

Erythrocytes: The erythrocytes, like the hemoglobin, did not vary significantly in the different groups. There

was wide variation even in the same animal from time to time,
but this has been found to be true in normal hog blood, as
described by Kernkamp, 1939, and others. The average number
of erythrocytes for the 176 uninfected animals in Group A was
6,418,000 with deviations from 4,372,000 to 8,650,000.

The infected Group B averaged 5,770,000 with animals
ranging from 3,537,500 to 6,860,000. Sixteen animals had less
than 4,000,000 cells per cmm.

Animals in Groups C, D, and E varied little from those
in Group A, with averages of 6,400,000 in C; 6,060,000 in D;
and 6,254,000 in E.

In animals where several tests were made, it was usual
to find that the results did not remain uniform when several
weeks or months elapsed between tests. However, where they
were carried on weekly, as in Group E, there was considerable
uniformity; and those animals which were relatively low at
first remained low for the month during which the animals
remained under experimentation.

Leucocytes: The greatest variation between the infected
and noninfected swine occurred in the total leucocyte count.

The negative group of 176 animals showed an average o
of 14,887. The counts did not vary as extensively as has
been reported in other papers, being from 10,200 to 21,200.

The positive group of 89 animals shows a somewhat higher
average of 16,620; the more recently infected group of eight
animals (infected less than 1 week) showed the somewhat high
average of 19,400. Seven animals infected more than a year

with titers of 1:50 to 1:200 have an average of 17,200 per cmm. (Table 5 and Graph I) Vaccinated animals of Group E that had 14, 200 leucocytes in the pre-vaccination test, had a high average at the end of one week of 27,140, but this decreased to 17,300 at the end of the second week, and by the third week the average was 14,400 where it remained for the fourth week. (See Table 6)

Group C animals showed little variation in the leucocyte counts made while the titer dropped. The average number of leucocytes present per cubic millimeter, when the animals had high titers, was 18,200. Three months later, when the titers had dropped, the count was 17,200.

Gilts in Group D showed a somewhat lower average number of leucocytes than the adult animals, with an average of 11,600 per cmm. This remained the same on the second test three months later.

Lymphocytes: The increase in the total number of leucocytes appears to be due, at least largely, to the increase of lymphocytes both in per cent and in actual numbers.

In Group A the uninfected animals showed an average percentage of 52.8 lymphocytes, or 7,863.3 per cmm. The infected animals in Group B, with the exception of seven which had been infected a year or more, showed an average percentage of 65.2 lymphocytes and an average number of 10,823.2 per cmm. The seven hogs which had been infected a year or more showed an average percentage of 52 per cmm. and an average actual number of 8892 (Table 5 and Graph II).

Group C animals, whose titers had been lost during a period of three months, showed 64% lymphocytes or 11,520 per cmm., and at the end of the three-month period there was a drop to 59.3% or 10,494.

The group of gilts showed 69.9% lymphocytes per cmm. or 8108.4. It should be remembered that this group of animals had been in an infected herd and although they were segregated at an early age, some of them did at one time show a titer of 1:25.

Group E vaccinated animals showed an average lymphocyte count of 50.3% or 7,132 actual lymphocytes per cmm. prior to vaccination. This increased 64% or 15,269 actual lymphocytes per cmm. within a week and remained at that level for four weeks. The total white cell count decreased, however, to 17,200 at the end of the second week, so that the actual number of lymphocytes per cmm. is 11,008 (Table 6).

Numbers of what probably were immature cells because of the basophilic granules in the cytoplasm were noted in some of the blood with high lymphocyte counts, probably indicating the stimulation of lymphopoietic centers.

Monocytes: In general there seemed to be no definite change in the number of monocytes. The average per cent for uninfected animals was 4.6 or 655. The infected group showed 4.9% or 814.3. Group C showed 4.4% and Group D, the gilts, showed 4.7%.

Neutrophils: The total number of neutrophils was only slightly reduced in most cases of animals infected with the

disease. The average per cent of neutrophils for the 176
uninfected animals was 39.1% or 5820.8. The average per
cent of neutrophils in Group B infected animals was 20.4%
or 3390.4. Group C animals showed 32% or 5670, and 31.5%
or 5418 three months later when the titers had dropped. (See
Table 5)

Gilts of Group D had 39.5% neutrophils or 4582 with
little change on the second test. The vaccinated animals of
Group E showed 42% or 5964 on the pre-vaccination test, and
19.1% or 5184 average neutrophils at the end of one week. At
the end of two weeks the count averaged 41.1% or 7069. At
the end of the fourth week the count had become 44.9% or 6645,
so that while there was a reduction in the total number of
leucocytes, the actual number of neutrophils did not change
to any appreciable extent (Table 6).

Eosinophils: The eosinophils for Group A infected ani-
mals averaged 3.075% or 458. The infected group of 89
was 9.6% or 1595. Group C animals showed 8.1% or 1458 eosin-
ophils on the first test and three months later 12% or 2064.
In Group D the gilts had an eosinophilic percentage of 7.2%
or 835. The vaccinated animals of Group E showed a percent-
age of 1.8 or 488 at the end of one week and 8.2% or 1400 at
the end of two weeks. By the end of four weeks the percent-
age had risen to 11% or 1528 (Tables 5 and 6).

Basophils: There was no appreciable variation in the
basophil count in any of the groups. It averaged less than
one at all times, usually in the neighborhood of 0.1% and

so is not listed individually (Tables 5 and 6).

Sedimentation rate: The rate of speed of the settling of blood cells in anti-coagulated blood was determined in some of the animals in each group. The intervals of measurement were one hour. There was no appreciable difference in the sedimentation rates in the five groups, the average being 0.05 cm. and the range from 0.002 to 0.12 cm. These variations occurred in all groups and the average was about the same.

Discussion

Certain measurable changes apparently do occur in the blood of hogs infected with swine brucellosis, as shown by the results of this study, though whether they will be particularly useful in the diagnosis of the disease is somewhat questionable because of the very wide variations normally occurring in the blood of healthy hogs.

The results of the study made of the hemoglobin of the five groups of animals would indicate very definitely that a qualitative measurement of the hemaglobin of Brucella-infected hogs is of no particular value as an aid in the clinical diagnosis of brucellosis in swine.

The erythrocytes, likewise, did not vary sufficiently in the five groups to be a reliable index for any type of diagnostic work. There is apparently a slight anemia which is evident in the smaller number of red cells and in the reduced amount of hemoglobin in the infected group of animals, but the difference is not sufficient to make it possible to

distinguish infection in a herd where there is so much normal variation in hemoglobin and erythrocytes.

Considerable deviation occurs in the number of leucocytes in the infected and non-infected groups, but here, again, it is probably not great enough to be significant because of the variation in leucocyte counts in apparently perfectly normal animals. In early stages of the disease the leucocyte count frequently rises enormously and, with other tests, might aid somewhat in the diagnosis of the disease. It appears to return to normal, however, after the infection has been present for a time.

In so far as the blood picture is concerned, the enumeration of lymphocytes and eosinophils offers the best method for identification of the disease. There is a distinct rise in the number of lymphocytes, particularly in recently infected animals, and this seems frequently to remain high over a considerable period of the infection. However, when animals have remained infected for a long period of time, the lymphocytic picture seemed to return to normal.

The eosinophilic leucocytes show about a three-fold increase in infected animals. This increase is not apparent in the early stages of the disease but appears to be a part of the blood picture which occurs during recovery. It must be borne in mind, however, that little attempt was made to eliminate the possibility of other infections in the animals studied which might have influenced the blood picture, particularly that of the eosinophils, since this is most frequently

produced by parasitic conditions which are often present in hogs.

The neutrophils present in the blood stream of both infected and noninfected animals showed little variation in actual numbers, though the percentage decreased tremendously because of the great increase in the number of lymphocytes in infected animals. There was little fluctuation of the neutrophils in the five groups of animals studied.

There was no appreciable variation in the basophils, monocytes, or sedimentation rate of infected and healthy animals.

A comparison of the results of the pre-vaccination and post-vaccination tests in a group of animals vaccinated with a porcine strain of low virulence showed no change of consequence in the erythrocytes after vaccination. The average leucocyte count doubled during the first week after vaccination and gradually decreased until by the end of the third week it was back to normal for the group. The lymphocytes more than doubled in number the first week after vaccination, but fell back materially during the second week and were normal in number by the end of the third week. There was no significant change in the number of neutrophilic leucocytes. The eosinophils increased in numbers each week, doubling between the first and second weeks. There was a slight increase in the third and fourth weeks so that the number of eosinophils was greatest at the end of the fourth week, when the agglutination titer was receding.

If vaccination can be considered to produce a mild form of the disease with a typical blood picture, as the evidence here presented would tend to indicate, the significant aspects of the blood picture in porcine brucellosis would be a marked increase in the total number of leucocytes, an even more marked increase in the lymphocytic picture, with 65% or more lymphocytes, and during recovery a marked increase in the number of eosinophils.

While a high percentage of lymphocytes would not appear to be very conclusive evidence that porcine brucellosis was present in a herd of animals, it would be an aid in determining the presence of the disease, particularly in its early stages. The presence of a large number of eosinophils might also indicate recovery if the presence of parasitic diseases was eliminated from the picture.

TABLE 6

Comparison of Average Blood Picture
of Adult Infected and Uninfected Swine

	Group A Average of 176 adult animals uninfected	Group B Average of 89 adult animals infected
Erythrocytes	6,418,000	5,770,000
Leucocytes	14,867	16,620
Lymphocytes	52.8% or 7863.3	65.2% or 10,823
Neutrophils	39.1% or 5820.8	20.4% or 3390.4
Eosinophils	3.075% or 458	9.6 or 1595
Basophils	0.1 or 14.8	0.21 or 34.9
Monocytes	4.6 or 655	4.9 or 814
Hemoglobin	12.21 gm. per 100 cc. blood	11.569 gm. per 100 cc. blood

GRAPH 1.
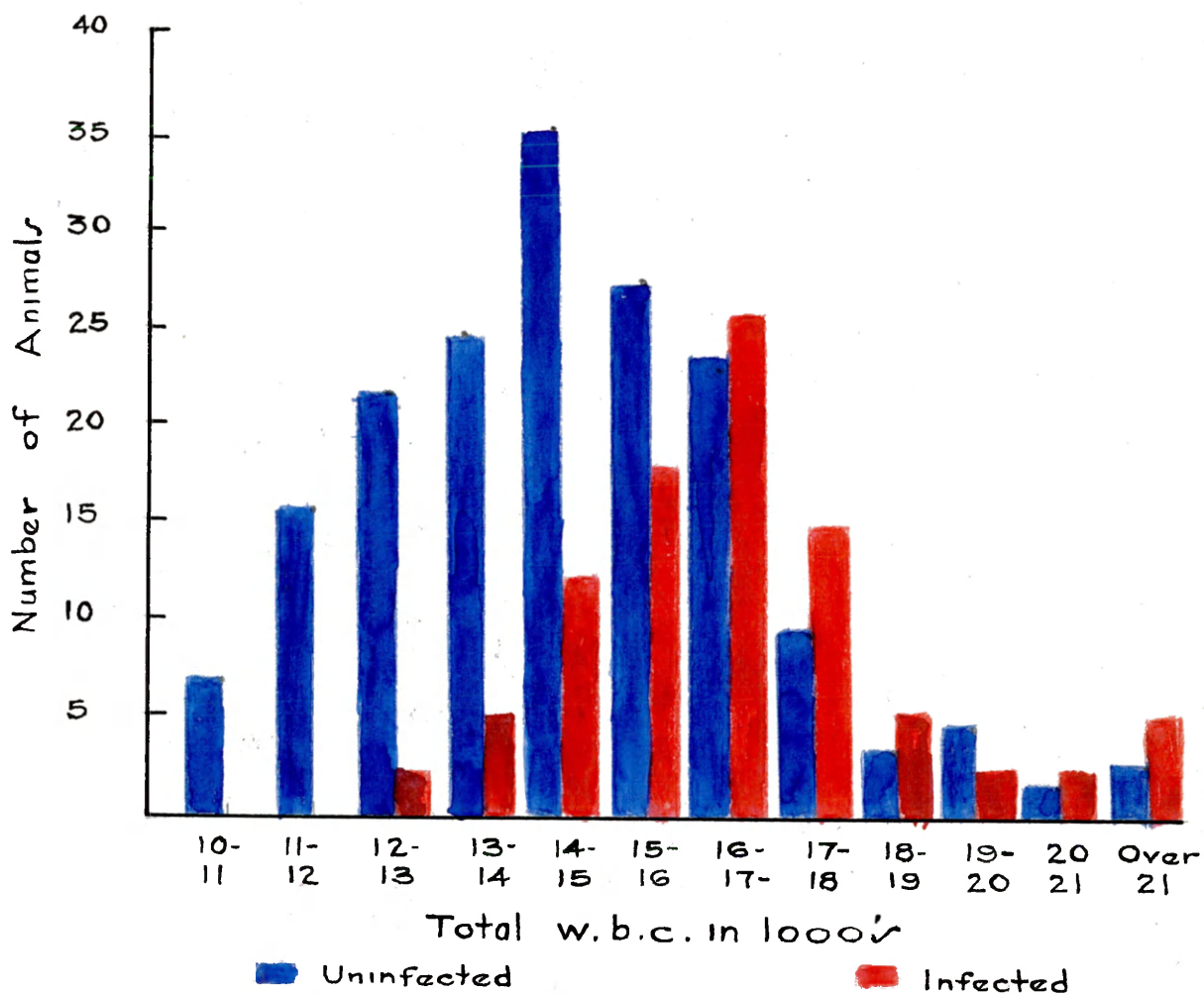
TOTAL LEUCOCYTES IN INFECTED AND
NONINFECTED ANIMALS.

GRAPH 2.

Total Lymphocytes in 1000's

■ Uninfected   ■ Infected
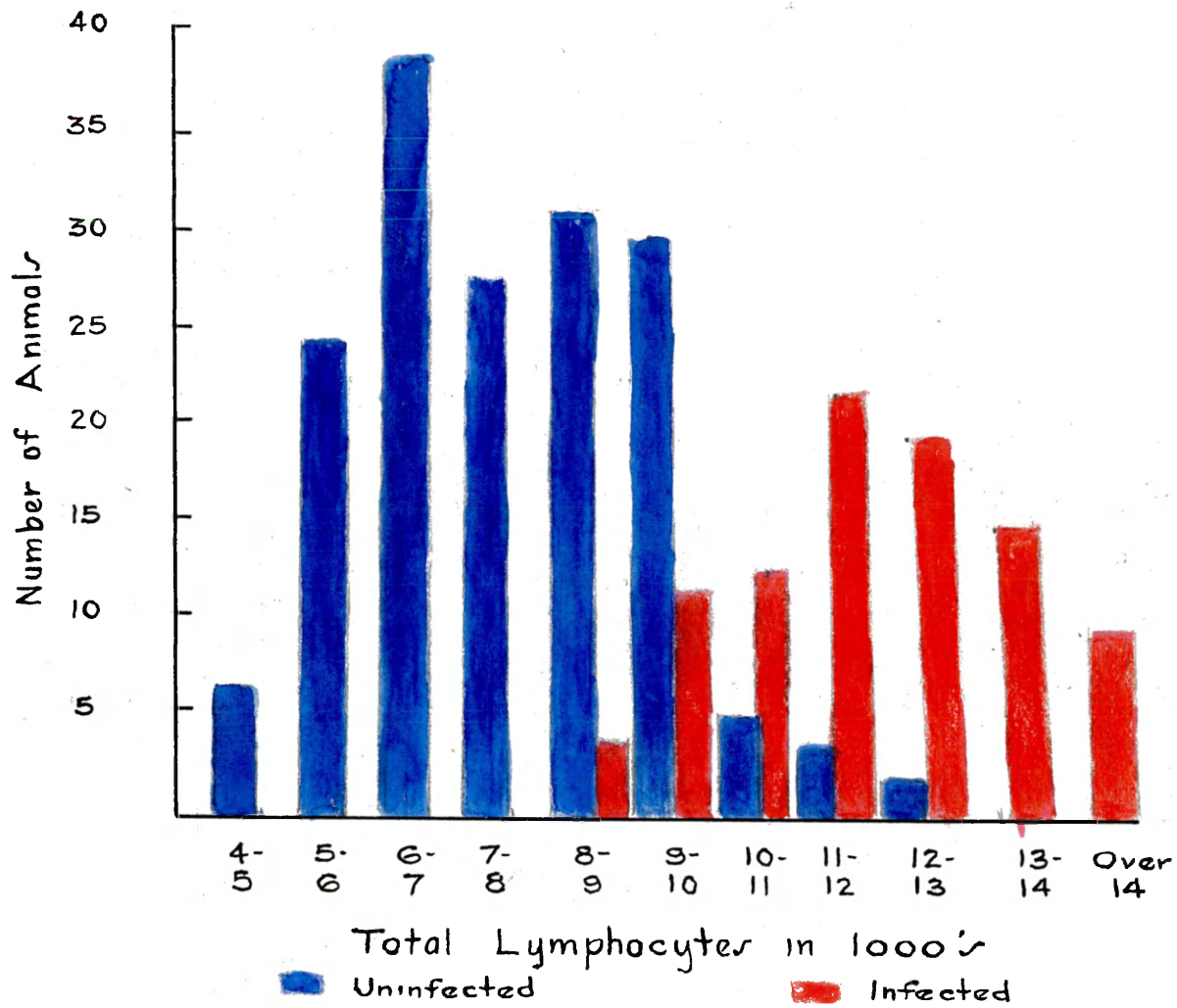
TOTAL LYMPHOCYTES IN INFECTED AND NONINFECTED ANIMALS

DIAGNOSTIC TESTS IN PORCINE BRUCELLOSIS

In investigating the diagnostic tests now in use, the one most generally relied upon, the agglutination test, was used extensively in these studies. However, an attempt was made to correlate this test with the complement fixation test, and the opsonocytophagic test was introduced for the first time into work on the diagnosis of porcine brucellosis.

Experimental Procedures

Agglutination Tests: The 1228 samples of hog blood which came into the laboratory were tested by the agglutination method commonly used for studying the blood of cattle. The test was set up in the following dilutions: 1:12½, 1:25, 1:50, 1:100, 1:200, and those samples which reacted in 1:200 dilutions were further tested in the higher dilutions of 1:250, 1:500, 1:1000, 1:2000, and in one case as high as 1:4000.

Strain 1119 Brucella abortus antigen prepared by the Bureau of Animal Industry, United States Department of Agriculture, was utilized for the tests. In some instances a porcine strain was used to make the antigen, but inasmuch as there seemed to be no difference in the reaction, most of the animals were tested with the usual bovine strain.

Complement Fixation Tests: The complement fixation test was set up in the manner similar to that described by Kolmer and Boerner, 1939, for the complement fixation tests for syphilis.

The antigen was made up according to the method described by Thomsen, 1934, except that the organisms were grown for four days on tryptose agar instead of liver infusion agar, as he suggests. The organisms were washed off the slant with a small amount of sterile distilled water and heated for three hours at 100° C. and then refrigerated for ten days with occasional shakings. The suspension was centrifuged at low speed to throw down the greater portion of the bacteria and the supernatant fluid, still turbid, was used with 0.5% phenol added.

The usual Kolmer kilutions for bacterial antigens were made up with saline, and antigenic and anti-complementary titrations were made. A dose of bacterial antigen equivalent to one-third of the anti-complementary unit was used.

The tests were incubated two hours in a water bath and the readings made ten minutes after complete hemolysis of the serum control, where complete hemolysis occurred. Otherwise, they were read at the end of the two-hour period.

Opsonocytophagic Tests: These were carried out on 434 porcine blood samples, and an agglutination test for each sample was also made. The opsonocytophagic test was carried on in the same manner as that described on pages 24 and 25, except that the suspensions of organisms were made up in physiological salt solution instead of 0.5% barium chloride and 0.6% sodium chloride.

A comparison of agglutination and complement fixation tests was made, using the blood of 42 animals. The technique

for the agglutination tests was similar to that described above, with dilutions of 1:12½, 1:25, 1:50, 1:100, 1:200.

Neufeld Reaction: The Neufeld reaction, similar to that described by Sabin, 1933, Armstrong, 1932, and Cooper and Walter, 1935, for pneumococci was used to determine whether specific immune sera produces a "quelling" reaction. Two large loopfuls of rabbit serum from animals which had been previously immunized against Brucella was placed on a clean slide. An equal quantity of a suspension of organisms in distilled water was added to it and the two were mixed by tilting the slide, as described in capsule staining technique on page 21, and stained with India ink and crystal violet. Variations of this technique were used, when the organisms were suspended in physiological salt solution instead of distilled water. Very small quantities of organisms and large quantities of sera were used, as suggested by Sabin, 1933, and Armstrong, 1932, for the pneumococci.

Results

Agglutination Tests: In a herd of 149 infected animals where two tests were made three months apart, Brucella suis had been isolated from the blood of several animals. Of these, 60 were mature sows with three of them in advanced pregnancy, 11 were mature boars, and 55 were gilts which had been segregated from the rest of the herd for some time. Only one gilt showed a titer of 1:12½ and the rest were negative. The number of animals with their agglutination titers is listed as follows:

No. of Animals	Agglutination Titers
1	1:4000
1	1:2000
8	1:1000
13	1:500
18	1:200
20	1:100
17	1:50
9	1:25
10	1:12½

Some of the high reactors had been sold between the first and second tests. Of the 139 remaining animals tested three months later, 51 of which were young gilts, two showed a titer of 1:200; two, of 1:100; two, of 1:50; one, 1:25; and sixteen, 1:12½. Three of the animals which reacted with a titer of 1:12½ were young gilts; the rest were adult animals. Many of the animals had lost their titers within the three months.

It was found, in examining blood from infected animals, that many of them which showed a titer of 1:1000 or above were apparently in the height of the disease. Animals which had the disease for some time did not, for the most part, show titers of more than 1:200, and in many cases they were less than 1:50. In animals which had just been infected, the titers were frequently as low as 1:25 or 1:50.

Agglutination and Complement Fixation Tests: A comparison of the two tests on 42 animals is summarized in Table 7. There are some discrepancies in the results of the two tests, but in general they agree fairly well.

Opsonocytophagic Tests: In a group of hogs which was tested prior to vaccination with King 8 strain of Brucella suis,

the average opsonocytophagic reaction was low. There were, however, three animals which showed a moderate opsonocytophagic index in this group. Table 8 shows the opsonocytophagic indices for these three strains, as well as the average and lowest indices. After vaccination with King 8 the indices began to rise, reaching their height at the second week period and remaining so through the fourth week. The results of this test are summarized in Table 9.

In a group of 21 animals which showed a titer of 1:200 or more, the opsonocytophagic index was moderate, and in 21 animals which had recently recovered from porcine brucellosis, the index was considerably higher. In 25 animals which had high titers, but whose titers had been reduced over a period of twelve months to 1:25 or 1:12½, the opsonocytophagic index was also high. The indices of these groups are indicated in Table 10.

Thirty-eight gilts from an infected herd, which had been removed to separate quarters soon after birth, showed low opsonocytophagic indices. In a group of 51 negative animals, most of which were presumably unexposed, the opsonocytophagic test was low. Six of these animals had relatively high indices with thirteen or more marked cells. These animals may have acquired some immunity, since they nursed positive brood sows for a time.

Neufeld Reaction: The Neufeld reaction did not appear to produce any "quelling" in Brucella when equal or larger quantities of serum were mixed with the organisms.

Discussion

The agglutination tests of the 1228 samples appear to show that infected hogs do not always react in low dilutions, as the literature would seem to indicate. In the recently infected animals the titers did not rise much above 1:200, but those in the height of the disease showed titers of 1:500 and 1:1000, and some were as high as 1:4000. When the animals had been infected a year or more, the titer was frequently as low as 1:25 or 1:50.

These results would seem to indicate that the disease is a self-limiting one; that the agglutinins appear rather slowly, reach their height within a three-month period, and gradually recede. There were, however, some exceptions to these findings.

It would seem from the relatively few results obtained on the comparison of agglutination and complement fixation tests of 42 animals, that there was not enough difference in the readings obtained in the two tests to warrant the use of the complement fixation test in routine laboratory procedures, since it is somewhat difficult to handle.

In uninfected animals the opsonocytophagic index was usually low. The few showing moderate indices were gilts which may have picked up some immunity from the infected sows during the nursing period.

Those animals which showed a titer of 1:200 or over in the agglutination test had moderate indices which is usual of the disease in man, according to Huddleson, 1939, though

In cattle Rabstein and Cotton, 1942, found many high indices in infected animals.

The opsonocytophagic test was high (15 or more marked cells) in those animals which had recovered, or were recovering from the disease. Huddleson, 1939, suggests that 15 marked leucocytes in the 25 counted is indicative of immunity.

A group of animals vaccinated with King 8 _Brucella_ _suis_ showed a rising opsonocytophagic index during the second week and this continued high for the four weeks of the test period, thereby indicating in all probability that vaccination with King 8 _Brucella_ _suis_ produced some immunity. Whether this or any other immunity in porcine brucellosis is lasting, only future exposure tests will tell.

While the opsonocytophagic test is not the answer to the diagnostic problem in porcine brucellosis, it appears to be very helpful in conjunction with other tests in determining the stage of the disease and the manner in which the animal is reacting to the infection.

While the Neufeld reaction did not appear to show "quelling" in Brucella, it would be relatively difficult, in any case, to judge with certainty whether there was any capsular swelling, inasmuch as the organisms must be stained before the capsule can be seen. Since there are variations in the normal size of the organisms and frequently large organisms appear in groups of otherwise small ones, it would be difficult to distinguish quelling unless it was very marked, as in the pneumococci.

TABLE 7

Comparison of 15 Typical Agglutination and Complement Fixation Tests in a Group of 42 Animals

	Agglutination Tests	Complement Fixation Tests
1	4+	2+
2	8+	4+
3	10+	4+
4	2+	+
5	7+	4+
6	+	±
7	6+	4+
8	2+	+
9	+	±
10	6+	4+
11	5+	4+
12	-	-
13	-	+
14	+	+
15	-	-

Agglutination Test Dilutions

+ - 1:12½   6+ - 1:250
2+ - 1:25   7+ - 1:500
3+ - 1:50   8+ - 1:1000
4+ - 1:100   9+ - 1:2000
5+ - 1:200   10+ - 1:4000

Complement Fixation Test

- complete hemolysis
± very slight inhibition of
+ 25% inhibition of hemolysis
2+ 50%   "   "   "
3+ 75%   "   "   "
4+ 100%   "   "   "

TABLE 8

Variations in Opsonocytophagic Indices in Healthy Swine

	Marked	Moderate	Slight	None
Average for 40 animals	0.4	2	9.8	12.7
3 animals	9	10	6	0
showing moderate	4	4	11	6
indices	3	15	7	0
Lowest index 1 animal	0	0	2	23

TABLE 9

Average Opsonocytophagic Indices in 40 Hogs
Before and After Vaccination
With King 8 Strain of Brucella Suis

	Average Pre-vaccination Test	Average Post-vaccination Test			
		1st wk	2nd wk	3rd wk	4th wk
Marked	0.425	4.2	17.1	17.3	16.9
Moderate	2.05	10.2	3.6	4.3	5.4
Slight	9.85	9.15	5.2	3.2	2.6
Negative	12.72	0.2	0.1	0.2	0.1

TABLE 10

Comparison of the Average Opsonocytophagic Indices
of Groups of Animals in Various Stages
of Porcine Brucellosis

	Marked	Moderate	Slight	None
Average index for 21 animals with titer of 1:200 or more	11.2	3.0	5.5	4.7
Average index for 25 animals whose titer had recently dropped to 1:25 or less	18.9	3.3	2.6	0.2
Average index for 21 animals recently recovered from the disease	16	2.3	4	2.6

DISCUSSION

The study of the capsules of Brucella in connection with
the investigation of porcine brucellosis would indicate that
microscopic examination of organisms stained to show capsules
is easier, less costly, and more accurate than plating the
organisms and examining the colonies to determine whether
they are rough, smooth, or intermediate. This should be a
valuable aid in keeping cultures in good condition for the
preparation of vaccines, antigens, and the like. It should
also be borne in mind that Brucella grown in liquid culture
media are rapidly changed and, hence, are unsuitable for use
in any of the serological tests. Strains of Brucella, when
transferred too frequently, introduce large numbers of unen-
capsulated organisms which produce inaccurate results in tests
like the opsonocytophagic test.

It is quite possible that a strain of Brucella which is
capable of developing marked antigenic responses in animals
but is incapable of producing more than a very mild attack of
the disease, could be developed for the vaccination of swine.
The results of long incubation on both liquid and solid media
indicate that these methods would produce attenuated strains
similar to the one now used for vaccination of cattle. Hence,
it might be possible to find a smooth strain of Brucella suis
which, when cultivated in a similar way, would produce a sat-
isfactory organism for the vaccination of swine.

The diagnosis of porcine brucellosis--always a problem--
can be aided by the study of the blood picture of the animals
in question, since it is one of the few diseases in which
lymphocytosis is a characteristic of the blood picture. The
results obtained in this study indicate that the blood picture
in swine follows rather closely that found in man, but it is
probably not as reliable an index because the normal blood
picture in hogs varies to such a marked extent.

Eosinophils increased during recovery and, while this is
characteristic of a number of diseases, it does not seem to
have been previously reported for brucellosis in any animal.

The findings with regard to the total number of erythro-
cytes and the quantity of hemoglobin did not reveal that an
evaluation of them would be a benefit or aid in the diagnosis
of the disease.

Of the diagnostic tests now in use, the agglutination
test seems to be the most satisfactory if only a single test
is used to determine the presence of the disease. The inter-
pretation of low titer reactions which occur frequently in
hogs is always a problem. Here the opsonocytophagic test
should be helpful in determining the stage of the disease,
inasmuch as most of the animals in its early stages had low
indices and those which were recovering from the disease had
high opsonocytophagic reactions, whereas in such cases the
agglutination titers were low. It has been thought by some
workers that all animals with low titers should be looked
upon as potential spreaders of infection, but it appears that

frequently such animals, when placed by themselves, will recover from the disease and their titers become negative. For this reason a study of the history of the herd is desirable, along with any tests that may be given.

SUMMARY

A. Capsular Study

1. Thirty-eight strains of Brucella abortus, Brucella suis, and Brucella melitensis were examined microscopically, culturally, and serologically in studying capsule formation.

2. All strains examined were normally encapsulated, whether they were smooth, rough, or intermediate.

3. While it was impossible to distinguish the species of organisms microscopically, in capsule study the differences between rough, smooth, and intermediate strains could be readily detected by microscopic examination. Smooth strains of organisms have a distinct pink capsule with a regular margin and a centrally placed, deep purple bacterial cell. Rough strains show only a vague suggestion of a bacterial cell body and the capsular margin is roughened. Intermediate strains lie between the two in appearance.

4. Dissociation begins to occur at twelve days in smooth strains held at 37° C. on solid culture media, although the organism may be kept at room temperature for thirty days and at 5° C. for sixty days with no signs of change. After eighteen months at 5° C. formerly virulent organisms made poor antigens and had lost their virulence for guinea pigs.

5. All Brucella examined underwent change within 24 hours after being transferred to liquid media, becoming larger and forming chains with some fusion of bacterial cells and

capsules. This is apparently the cause of the mucoid sediment produced in liquid culture media. In most strains by the eleventh day the cells have become sufficiently dissociated so that they have the characteristics of rough strains. When allowed to remain in liquid media for twelve months, only smooth, virulent organisms were recovered. These do not produce recognizable characteristics of brucellosis in guinea pigs, although titers of 1:200 developed in some of the animals with high opsonocytophagic activity present.

6. Unencapsulated organisms are found in strains of Brucella that have undergone rapid transfer. These appear as navy blue when stained with crystal violet, while the encapsulated forms are a deep purple. Smooth strains of organisms were denuded of their capsules by growing them on liver infusion agar and transferring them to beef broth, but the organisms regained their capsules within 48 hours under all conditions to which they were subjected. Rough strains of organisms may lose some of the capsules if allowed to remain in distilled water for twelve hours. No encapsulated strain of Brucella was cultivated for more than 48 hours.

B. Blood Picture

1. Studies of the blood picture were made of 1228 healthy, infected, and vaccinated animals.

2. Mild anemia appears to be a characteristic of brucellosis as shown by the reduced hemoglobin and erythrocytes, but was not sufficiently great to be a reliable index in the diagnosis of the disease.

3. There was a considerable rise in the total leuco-
cyte count; but here, again, because of the variation in nor-
mal hog blood, it is not dependable as a measure of infection.

4. Active lymphocytosis was the most striking feature
encountered and was evidenced by an increase in both the per-
centage values and the actual numbers of lymphocytes.

5. Little change or, in some cases, a slight reduction
was found in the actual number of neutrophils.

6. The percentage, as well as the actual number, of
eosinophilic leucocytes is more than trebled in most animals
during the period of recovery from brucellosis.

7. There is no appreciable variation in basophils,
monocytes, or the sedimentation rate of blood in healthy and
infected swine.

C. Diagnostic Tests

1. Agglutination tests on 1228 healthy, infected, and
immune animals show that, while most of the swine react in
lower dilutions than cattle, there are some which show high
titers. The titers tend to be low in the early and late
stages of the disease and quite high during its acute stages.

2. A comparison between the results of the complement
fixation test and the agglutination test in 42 animals showed
little variation between the two tests.

3. The opsonocytophagic index was low in uninfected
animals, moderate in animals showing a titer of 1:200 or more,
and high in animals recovering from the disease. Vaccinated
animals showed a rising opsonocytophagic index during the

second week after vaccination, and this continued high for the four weeks of the test period.

    4. The Neufeld reaction did not appear to show "quelling" in Brucella.

LITERATURE CITED

1. Armstrong, R. R., 1932. Intermediate pneumococcal typing. British Medical Journal, 1: 187-188.

2. Bang, B., 1897. The etiology of contagious abortion. Zeitschrift für Tiermedizin, 1: 241-265.

3. Bell, F. N. and M. R. Irwin, 1938. Variation of blood cells of cattle in health and during Brucella infections. Journal of Infectious Diseases, 63: 251-262.

4. Bianchini, L., 1930. Bang bacillus infection in swine. La Clinica Veterinaria, 109.

5. Bruce, D., 1887. Notes on the discovery of a microörganism in Mediterranean fever. Practicioner, 39: 161-165.

6. Bruce, D., 1893. A new form of fever found on the borders of the Mediterranean. Annalis de l'Institut Pasteur, 7: 289-302.

7. Burnett, Et., 1922. Studies on Mediterranean fever. III. The diagnosis of Mediterranean fever by intradermal test. Archives de l'Institut Pasteur de Tunis, 2: 165-172.

8. Calder, R. M., C. Steen, and L. Baker, 1939. Blood studies in brucellosis. Journal of the American Medical Association, 112: 1893-1898.

9. Calmette, A., L. Negré and A. Boquet, 1926. Manual of microbiological and serological technique. Masson et Cie, Paris. 392 pages.

10. Churchman, J. W. and N. V. Emelianoff, 1933. A study of the bacterial capsule by new methods. Journal of Experimental Medicine, 57: 485-486.

11. Connaway, J. W., A. J. Durant and H. G. Newman, 1921. Infectious abortion in swine. Missouri Agricultural Experiment Station, Bulletin No. 187.

12. Cooper, G. and A. Walter, 1935. Application of the Neufeld reaction to the identification of the types of pneumococci. Journal of the American Public Health Association, 25: 469-474.

13. Cotton, W. E., 1922. The character and possible signi-
    ficance of the Bang abortion Bacillus that attacks
    swine. Journal of the American Veterinary Medical
    Association, 62 (n.s. 13): 35-46.

14. Cotton, W. E. and J. M. Buck, 1932. Brucella abortus in
    the blood stream of swine. North American Veterin-
    arian, 13: 35-48.

15. Dukes, H. H., 1935. Physiology of Domestic Animals.
    Comstock Publishing Company, Ithaca, New York.
    762 pages.

16. Evans, A. C., 1918. Further studies on Bacterium abortus
    and related bacteria. II. A comparison of Bacterium
    abortus with Bacterium bronchicepticus and with the
    organism which causes Malta fever. Journal of Infec-
    tious Diseases, 22: 580-592.

17. Favilli, G. and G. Biancalani, 1932. Studies on carbo-
    hydrates of bacteria of the Brucella group. Lo Speri-
    mentale Archivo di biologia normale e patologica,
    86: 357-364.

18. Favilli, G. and G. Biancalani, 1934. Studies on carbo-
    hydrates of bacteria of the Brucella group. Lo Speri-
    mentale Archivo di biologia normale e patologica,
    88: 337-344.

19. Fleischner, E. C. and K. F. Meyer, 1918. The bearing of
    cutaneous hyper-sensitiveness on the pathogenicity of
    the Bacillus abortus bovinus. American Journal of the
    Diseases of Children, 16: 266-297.

20. Foshay, L., 1936. The nature of the bacterial-specific
    intradermal antiserum reaction. Journal of Infectious
    Diseases, 59: 331-397.

21. Gwatkin, R., 1931. Brucella abortus agglutinins in the
    blood of sows slaughtered in Toronto. Cornell Veter-
    inarian, 21: 77-81.

22. Gwatkin, R., 1935. A study of various fractions of
    Brucella abortus. Canadian Journal of Research,
    12: 115-164.

23. Good, E. S. and W. V. Smith, 1916. Bacillus abortus (Bang)
    as an etiological factor in infectious abortion in
    swine. Journal of Bacteriology, 4: 415-427.

24. Giordiano, A. S., 1929. Brucella abortus infection in man,
    intradermal reaction as an aid to diagnosis. Journal
    of the American Medical Association, 93: 1959-1965.

25. Hadley, F. B. and A. B. Beach, 1922. An experimental
    study of infectious abortion in swine. Research
    Bulletin 55, University of Wisconsin Agricultural
    Experiment Station.

26. Hagan, W. A., 1927. A method for the demonstration of
    capsules in bacteria. Science, 69: 173.

27. Hayes, F. M., 1922. Some studies on swine abortion.
    Journal of the American Veterinary Medical Associa-
    tion, 60 (n.s. 13): 435-452.

28. Hershey, A. D., I. F. Huddleson and R. B. Pennell, 1935.
    The chemical separation and biological activity of
    the polysaccharide constituent in Brucella cells.
    Journal of Infectious Diseases, 57: 183-191.

29. Higginbotham, Margaret and Lucy Heathman, 1936. Precipi-
    tin and complement fixation reactions of polysaccharide
    extracts of Brucella. Journal of Infectious Diseases,
    57: 30-34.

30. Howarth, J. A. and F. M. Hayes, 1931. Brucelliasis in
    the swine of the University of California. Journal
    of the American Veterinary Medical Association,
    78 (n.s. 31): 830-846.

31. Huddleson, I. F., 1939. Brucellosis in Man and Animals.
    The Commonwealth Fund, New York, New York. 331 pages.

32. Huddleson, I. F., 1940. The presence of a capsule on
    Brucella cells. Journal of the American Veterinary
    Medical Association, 96: 708-709.

33. Huddleson, I. F., 1940. Demonstration of a capsule on
    Brucella cells. Proceedings of the Society of Experi-
    mental Biology and Medicine, 43: 773.

34. Hughes, M. L., 1892. The natural history of certain
    fevers occurring in the Mediterranean. The Mediter-
    ranean Naturalist, 2: 325-355.

35. Huston, R. C., I. F. Huddleson and A. D. Hershey, 1934.
    The polysaccharide fraction in Brucella. Technical
    Bulletin No. 137, Michigan Agricultural Experiment
    Station.

36. Johnson, H. W. and I. F. Huddleson, 1931. Natural Bru-
    cella infection in swine. Journal of the American
    Veterinary Medical Association, 31: 849-862.

37. Kernkamp, H. C. H., 1939. The blood picture in hog cholera.
    Journal of the American Veterinary Medical Association,
    95: 525-529.

38. Kolmer, J. A. and F. Boerner, 1941. Approved Laboratory Technique. Third Edition. D. Appleton-Century Company. 1267 pages.

39. Lewis, P. A. and R. E. Shope, 1929. The diagnosis of hog cholera. Journal of the American Veterinary Medical Association, 74: 145-152.

40. McAlpine, J. S., W. N. Plastridge, and G. D. Bingham, 1929. Studies of the abortus-melitensis group: Five factors influencing sugar utilization. Journal of Infectious Diseases, 45: 485-489.

41. McFadyean, J. and S. Stockman, 1913. Abortion in sheep. Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to inquire into epizootic abortion. Appendix to Part III. London: His Majesty's Stationery Office.

42. Meyer, K. F. and E. B. Shaw, 1920. A comparison of the morphologic, cultural, and biochemic characteristics of Br. abortus and Br. melitensis. Studies on the genus Brucella. Journal of Infectious Diseases, 27: 173-178.

43. Mickle, W. A., 1940. Capsule formation by members of the Brucella group. Journal of Infectious Diseases, 66: 271-277.

44. Morales-Otero, P. and L. M. Gonzales, 1938. Purified protein antigen from Brucella. Proceedings of the Society of Experimental Biology and Medicine, 38: 703-709.

45. Munger, M. and I. F. Huddleson, 1938. A preliminary report on the blood picture in brucellosis. Journal of Laboratory and Clinical Medicine, 24: 617-619.

46. Pennell, R. B. and I. F. Huddleson, 1937. Chemical constitution and biological properties of the endo-antigen of the Brucella group of microorganisms. Technical Bulletin No. 156, Michigan Agricultural Experiment Station.

47. Plastridge, W. N. and J. G. McAlpine, 1930. Microbic dissociation in the abortus melitensis group. Journal of Infectious Diseases, 46: 314-323.

48. Rabstein, M. and C. Cotton, 1942. Bang's disease immunity tests and a new vaccination method. Proceedings of the 46th Meeting of the U. S. Livestock Sanitary Association, Chicago, Ill.

49. Rainsford, S. G., 1935. Laboratory methods of diagnosis of Brucella infections. Journal of the Royal Naval Medical Service, 10: 1-10.

50. Reichel, J. and J. M. Harkins, 1917. The diagnosis of infectious abortion of cattle with special reference to the intradermal test. Journal of the American Veterinary Medical Association, 1: 846-854.

51. Reiter, D. O., 1936. Studies on the extraction of a precipitable substance from the genus Brucella. Journal of Infectious Diseases, 58: 45-53.

52. Sabin, A. B., 1933. Immediate pneumococcus typing directly from sputum by the Neufeld reaction. Journal of the American Medical Association, 100: 1584.

53. Schroeder, E. C. and W. E. Cotton, 1911. 11. The Bacillus of infectious abortion found in milk. 28th Annual Report of the Bureau of Animal Industry, U. S. Department of Agriculture, 139-146.

54. Schroeder, E. C. and W. E. Cotton, 1925. Recent Bureau of Animal Industry Experiment Station bovine infectious abortion studies. Journal of the American Veterinary Medical Association, 67 (n.s. 25): 550-561.

55. Smith, T. and M. Fabian, 1912. The pathogenicity of Bacillus abortus, Bang. Centralblatt für Bakteriologie (Abt. I) 61: 549-562.

56. Stahl, W. H., W. H. Pennell and I. F. Huddleson, 1939. A study of the protein-nucleates of the genus Brucella. Michigan Agricultural Experiment Station, Technical Bulletin No. 168.

57. Starseth, R. J., 1920. Studies in infectious abortion. Michigan Agricultural Experiment Station, Technical Bulletin No. 49.

58. Stubbs, E. L. and I. Live, 1942. Studies on the allergic and antigenic activity of sonic filtrate of Brucella abortus. American Journal of Veterinary Research, 3: 146-153.

59. Thomsen, A., 1931. An enzootic of infectious abortion of swine in Denmark. Revue générale de médecine vétérinaire, 8: 233-251.

60. Thomsen, A., 1934. Brucella infection in swine. Studies from an epizootic in Denmark, 1929-1932. Acta Pathologica et Microbiologica Scandinavica, Supplement 21.

61. Traum, J., 1914. Annual Report of the Chief, Bureau of Animal Industry, U. S. Department of Agriculture, p. 30.

62. Zammit, T., 1905. A preliminary note on the examination of the blood of goats suffering from Mediterranean fever. Reports of the Commission on Mediterranean Fever, Part 3: 83-96. Harrison and Sons, London.

TABLE 1

Comparison of Agglutination Titers and Opsonocytophagic Indices of Guinea Pigs Inoculated with Strains of Brucella kept at 5° C. without transfer for 18 months with Young Cultures of the Same Strain

	Strains of organisms for inoculation	Pre-inoculation Tests			Post - inoculation Tests																
		**Ag	*Opsono			1st Week					2nd Week					3rd Week					4th Week				
			Ma	Mo	Sl	N	Ag	Ma	Mo	Sl	N	Ag	Ma	Mo	Sl	N	Ag	Ma	Mo	Sl	N	Ag	Ma	Mo	Sl	N
Held at 5° C. for 18 mo. without transfer	Br. abortus 2308	-	0	0	3	22	-	9	10	6	0	3+	16	8	1	0	-	18	7	0	0	-	20	2	3	0
	Br. suis 2872	-	0	0	5	20	-	3	9	12	1	4+	18	6	1	0	+	16	9	0	0	-	16	9	0	0
	Br. melitensis Henry	-	0	0	9	16	-	8	7	9	1	3+	15	4	6	0	+	16	4	4	1	-	16	5	4	0
Control strains transferred regularly	Br. abortus 2308	-	0	0	8	17	+	8	7	8	2	8+	10	8	4	3	8+	6	12	7	0	8+	9	10	6	0
	Br. suis 2872	-	0	0	12	13	-	5	9	9	4	4+	9	9	5	2	8+	8	9	8	0	8+	6	9	7	1
	Br. melitensis Henry	-	0	0	10	15	+	7	9	7	2	6+	8	9	6	2	7+	7	10	6	2	7+	6	9	8	2

*Agglutination Titers

+ -- 1:25   5+ -- 1:250
2+ -- 1:50   6+ -- 1:500
3+ -- 1:100   7+ -- 1:1000
4+ -- 1:200   8+ -- 1:2000

**Opsonocytophagic Test

    Ma - Marked
    Mo - Moderate
    Sl - Slight
    N - None

On autopsy at four weeks:

Group inoculated with organisms kept for 18 months at 5° C. showed no lesions and organisms were not recovered.

Group inoculated with corresponding strain of Brucella which had been regularly transferred showed typical spleen and lymph gland lesions, and organisms were recovered from all three.

TABLE 8

Study of the Development of Surface Film, Mucoid Sediment, and Pellicle Formation in 10 Strains of Brucella

	Liver Infusion Broth					Potato Liquid Media					Beef Infusion Broth					2% Tryptose Broth				
	1st wk	2nd wk	3rd wk	4th wk	5th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk	1st wk	2nd wk	3rd wk	4th wk	5th wk
Br. suis Indiana : sf : ms			p			ms	p						sf	p		ms				p
Brucella melitensis: sf : Henry : ms				p		no growth						ms								
Br. suis 1942 : sf : ms			ms	p			ms	p				ms								
Brucella abortus 19 : ms	ms						ms					ms								
Brucella abortus 45-20 : ms : p	ms						ms	p				ms								
Brucella abortus 1119 : ms	ms			p		ms		p			p	ms			ms					
Br. suis 2872 : ms			ms	p		ms					ms					ms				
Br. suis King 8 : ms			ms	p				p												
Brucella abortus 2308 : sf : ms			sf : ms			p : ms						ms					ms			
Brucella abortus : sf : ms Huddleson : ms : p			p			ms : sf	p			ms : sf						ms : sf			p

sf -- surface film
ms -- mucoid sediment
p -- pellicle

TABLE 5

Average Blood Picture of 47 Animals Vaccinated with King 8 Strain, Brucella Suis

	Pre-vaccination Picture	Post-vaccination Pictures			
		1st Week	2nd Week	3rd Week	4th Week
Erythrocytes	6,557,000	6,490,000	6,295,000	6,000,000	6,254,000
Leucocytes	14,200	27,140	17,200	14,400	14,880
Lymphocytes	50.3% - 7132	68% - 18,455	61% - 10,492	57.7% - 8208	58% - 8630
Neutrophils	42% - 5964	19.1% - 5184	27% - 4644	28.5% - 4104	26.6% - 3858
Basophils	0.1	0.2	0.2	0.3	0.2
Eosinophils	2.8% - 398	3.2% - 648.4	6% - 1032	9.4% - 1353	11% - 1528
Monocytes	4.2	3.9	4	4.1	4.2
Hemoglobin per 100 cc. blood	12.28	11.40	10.74	10.52	11.42