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# Serological Activity of Adsorbed Leptospiral Antigen

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# SEROLOGICAL ACTIVITY OF ADSORBED LEPTOSPIRAL ANTIGEN

BY

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

Thesis Advisor

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# INTRODUCTION

Leptospirosis is not a single disease but a group of diseases caused by a variety of leptospiral serotypes. Investigations during the past seventy years have shown that leptospirosis occurs in humans and animals in all areas of the world. Much of our knowledge regarding the epidemiology, public health importance, and distribution of these diseases in the United States has been gained during the last ten to twelve years.

Serological diagnostic procedures have been the principle means of identifying leptospirosis in humans and domestic animals. The agglutination test is a valuable diagnostic procedure for the identification of infections, such as leptospirosis, when it is not practical to isolate the causative organism from suspected cases.

One of the earliest serological tests for the identification of leptospirosis was the agglutination-lysis test (24). This test has often been used as a basis for evaluating other leptospiral serological tests.

Muraschi (14) prepared an antigen consisting of leptospiral components adsorbed onto latex particles, for a tube agglutination test to detect leptospiral antibodies in serum. This was a modification of a procedure Singer and Plotz (21) used for the serologic diagnosis of rheumatoid arthritis.

The studies described herein pertain to tube agglutination reactions with homogenized leptospiral cell antigens adsorbed onto particulate organic carriers for use as antigen in antibody-antigen reactions. The high cost of leptospiral whole cell antigen suggests

the need of a search for a more economical use of this expensive material.

## LITERATURE REVIEW

The disease of humans now called hemorrhagic jaundice, was first described by Weil in 1898, and was subsequently found to be caused by a spirochaete. The organism was classified in the genus Spirochaeta until later studies by Noguchi in 1917 showed it to be sufficiently characteristic to justify the creation of a new genus, which was names Leptospira. Knowledge of the genus Leptospira has increased greatly in the last four decades.

Particular species of Leptospira are called serotypes because they are usually differentiated from each other by serologic means. Thirty-two serotypes are listed (2). Each leptospiral serotype is usually thought to have a primary animal host, but it may infect other animals also.

Serological testing methods have played an important part in the advance of knowledge of leptospirosis. Three general types of serological tests have been developed.

One type is the agglutination-lysis test (24), which makes use of living leptospiral organisms. A constant supply of the living organisms must be kept on hand if this procedure is to be used for routine testing. This one factor hinders its use in most diagnostic laboratories.

Another type of test is the complement-fixation test. Randall et al. (16) have described one such test using sonically disrupted leptospiral cells for the antigen. The cells were grown on artificial media enriched with serum. This method is difficult and complicated as results can be obtained only in twenty four hours or longer.

Schneider (19) used a chemical separation procedure which yielded four leptospiral cell fractions. The four complement-fixing fractions were pentose-containing materials. York (25) described the use of embryonating hen eggs for the propagation of leptospiral cells for complement-fixation tests.

Ezell et al. (7) were able to demonstrate a complement-fixing antigen from organism-free leptospiral culture liquor from the several serotypes studied. They found the antigenic material to be heat stable and non-protein in nature. Muraschi et al. (15) obtained a colloidal aqueous solution from leptospirae by ethylene-glycol extraction which showed promise in complement-fixation tests.

The third type of serological methods is the microscopic and macroscopic agglutination tests which make use of killed suspensions of leptospiral cells as the antigen. These tests are used most frequently for routine testing. The antigens can be stored for long periods of time without deteriorating. Glycerine has been used by Galton (8) as a preservative for slide antigen as suggested by Terzin (23).

Stoenner (22) describes a method for the preparation of a rapid macroscopic test antigen for use in the rapid plate test or a capillary tube agglutination test. Hoag (9) also describes a macroscopic plate agglutination test.

McAdaragh (12) used Stoenner's rapid plate test in conjunction with the agglutination-lysis test for studies on the incidence of bovine and porcine leptospiral infections in South Dakota.

Howarth (10) used formalin-killed leptospiral cell antigen in macroscopic tube agglutination studies. Grossly visible flocculation in

the bottom of cone shaped tubes denoted a positive reaction.

Another form of serological reaction which is closely related to the macroscopic agglutination reactions is hemagglutination. Chang and McComb (3), using Boyden's (1) procedure for the treatment of erythrocytes with tannic acid, were able to sensitize human type O erythrocytes with extracts of leptospirae so that they were agglutinated by anti-leptospiral antibodies.

Cox (4) used this same principle of sensitizing tannic acid-treated sheep cells with leptospiral extracts in studies on leptospirosis. The sensitized sheep erythrocytes would then be hemolyzed in the presence of homologous rabbit antiserum and complement.

Studies of leptospira by Rothstein and Hiatt (18) suggest that the organisms contain two major antigenic components. The first is a surface antigen, possibly a protein-polysaccharide complex. This is believed to be a type specific antigen. The second is a somatic antigen which is genus specific. It has been suggested that two and possibly three antigens occur in the surface layers, which may explain some overlapping serological reactions among the members of this genus.

Larson et al. (11) concluded from agar plate studies of leptospirae that colonies of the organisms resulted from a single cell. Using agar plate methods, Roth et al. (17) were able to isolate various strains of Leptospira from wild animals near Baton Rouge, Louisiana.

Morton and Anderson (13) demonstrated with the use of the electron microscope that leptospirae did not have flagella. Their study indicated that the organism did not have any internal granular structures. Czekalowski (5), however, claimed to have been able to reveal two types

of granules by the use of the electron microscope.

Schneider (20) used infra-red spectrophotometry to study several species of leptospirae. He found that each of the species studied would reveal a distinctive infra-red spectrum. This method appears to have a potential value as a rapid supplement to serological methods for distinguishing and classifying serotypes of the genus Leptospira.

## PREPARATION OF MATERIALS

### Preparation of Leptospiral Antigen

The suspension of leptospiral antigen was prepared from cultures of Leptospira pomona (Johnson strain). The primary cultures of this organism were obtained from the Department of Veterinary Science, South Dakota State College. Stuart's medium (6) was prepared and dispensed in ten milliliter amounts in culture tubes. The tubes were autoclaved for fifteen minutes at 121 C. The final pH of the medium was 7.6. One milliliter of sterile rabbit serum, inactivated at 56 C for thirty mimutes, was added to each tube. The tubes were inoculated with one milliliter each of actively growing Leptospira pomona organisms. The inoculated cultures were placed in an incubator at 28 C. Periodic darkfield microscope examinations were made during the incubation period to determine the point of maximum growth for harvesting the cells. Ten to twelve days of incubation were normally required for maximum cell growth in the tubes. The cultures which did not have a satisfactory density or which were contaminated were discarded. Formalin was added to the satisfactory cultures to make a final concentration of 0.5 per cent to kill the organisms. The formalized cultures were left to stand twenty-four hours at room temperature. The killed cultures were transferred to fifty milliliter tubes and centrifuged for ten minutes at 65 x G to remove extraneous material.

The supernatant fluid was transferred to twenty milliliter plastic tubes and centrifuged at 5200 x G for thirty minutes to pack the leptospiral cells. The supernatant fluid was carefully poured off. The

sedimented cells were resuspended in five milliliters of 0.85 per cent sodium chloride solution containing 0.5 per cent formalin. The suspension was homogenized by means of a ten milliliter syringe with a 23-gauge needle. The suspension of homogenized leptospiral cells was then adjusted with 0.5 per cent formalinized 0.85 per cent NaCl solution to an optical density of 0.5 at a wave length of 450 millimicrons using a Bausch and Lomb Spectronic 20 spectrophotometer.

Preparation of Leptospiral Antiserum

The immune serum was prepared by intravenous ear inoculation of a rabbit with successive doses of 1.0, 4.0, 4.0, and 6.0 milliliter amounts of a six day old culture of Leptospira pomona (Johnson's strain) grown in Stuart's medium enriched with ten per cent of rabbit serum and killed by overnight exposure to formalin added to a final concentration of 0.5 per cent in the medium. The four injections were given at five to seven day intervals. Two weeks after the last injection, the rabbit was bled from an incision in the ear. The whole blood was centrifuged at 1000 x G for ten minutes and the serum was decanted. The serum was centrifuged a second time at 1000 x G for ten minutes to insure complete removal of red blood cells from the serum. The serum was decanted a second time.

An estimate was made of the approximate titer of the immune serum by a plate agglutination test. Serum in quantities of 0.08, 0.04, 0.02, 0.01, and 0.005 milliliter amounts was pipetted onto separate squares of a ruled glass plate and one drop of leptospiral plate antigen, obtained from The United States Public Health Service Communicable

Disease Center, Chamblee, Georgia, was placed on each of the test areas and mixed. The plate was rotated for three minutes and agglutination reactions were read. The approximate titer was determined to be 1:612.

Normal rabbit serum was obtained by bleeding a rabbit from the heart by means of a syringe and needle. This blood was centrifuged to secure the serum in the same manner as the blood containing the immune serum. A plate agglutination test similar to that described for the immune serum was performed. There was no indication of agglutination.

Preparation of Buffered Saline

The buffered saline used in the agglutination studies was prepared by adding 8.5 grams of sodium chloride to one liter of 0.1N glycine solution. The glycine-buffered saline was then adjusted to a pH of 8.2 with 1N NaOH solution using a Beckman pH meter.

## EXPERIMENTAL METHODS AND RESULTS

During the course of this study, eight substances or extracts of substances were tested in solution for their suitability as fixation agents for homogenized leptospiral cell particles. From these eight substances, three appear to be suitable: supernatant of washed homogenized milk, fragments of yeast cells, and lecithin.

### Supernatant Fluid of Washed Homogenized Milk

One quarter of a pint of homogenized milk was portioned equally into four centrifuge tubes. After centrifugation at 250 x G for five minutes, the surface portion was removed and discarded. That portion of the supernatant fluid which remained in each tube was decanted and placed in other centrifuge tubes and centrifuged again at 250 x G for five minutes. The surface portion was again removed and discarded. That portion of the supernatant fluid which remained in each tube was decanted and placed in other centrifuge tubes. Glycine-buffered saline was added to each tube as a wash. The process of centrifugation, decanting, and washing was repeated four more times. After the last decanting, the suspension was adjusted with glycine-buffered saline to an optical density of 0.5 at a wave-length of 650 millimicrons using a Baush and Lomb spectrophotometer.

Two-fold eight tube serial dilutions from 1:4 to 1:512 in glycine-buffered saline were made of leptospiral positive rabbit serum and of leptospiral negative rabbit serum. Four racks of tubes containing dilutions of the two sera were used to test agglutination reactions at room temperature, 37 C, 45 C, and 56 C.

Two milliliters of homogenized leptospiral cell suspension was added to eighteen milliliters of the homogenized milk suspension to be used as the antigen.

Two tenths of a milliliter of antigen was added to each tube and the racks were shaken and placed in water baths at the different temperatures. One of the four racks was left a room temperature.

The macroscopic tube agglutination test using the homogenized milk suspension for the diluent for homogenized leptospiral cell particle antigen had a very encouraging result. The result is shown in Table I. This was not a rapid test. In this study, the first two tubes of the positive serum dilution cleared in one hour from their original milky appearance but there was no indication of agglutination. Plate I shows the final appearance of the tubes after eighteen hours of incubation. The last two tubes had no agglutination and the solution had a milky appearance.

A Biuret test on the homogenized milk suspension indicated that there was a peptide linkage present in the substance in suspension. A Babcock butterfat test indicated that there was no butterfat present.

Supernatant Fluid of Fractured Yeast Cells

A culture of yeast cells grown on Sabouraud's dextrose agar (6) was harvested and suspended in 100 milliliters of glycine-buffered saline. This suspension was autoclaved for 15 minutes at 121 C. After cooling, small glass beads were added to the solution. The suspension was whipped in a Waring Blendor for 45 minutes to disrupt the yeast cells. The suspension was poured into fifty milliliter centrifuge tubes

TABLE I. HOMOGENIZED MILK SUSPENSION FIXATION REACTIONS*

| Serum Dilutions | Room Temperature | | 37 C | | 45 C | | 56 C | |
|---|---|---|---|---|---|---|---|---|
| | Immune Serum | Normal Serum | Immune Serum | Normal Serum | Immune Serum | Normal Serum | Immune Serum | Normal Serum |
| 1:4 | - | - | 2 | - | 4 | - | 4 | - |
| 1:8 | - | - | 1 | - | 4 | - | 4 | - |
| 1:16 | - | - | - | - | 3 | - | 4 | - |
| 1:32 | - | - | - | - | 2 | - | 4 | - |
| 1:64 | - | - | - | - | 1 | - | 4 | - |
| 1:128 | - | - | - | - | - | - | 3 | - |
| 1:256 | - | - | - | - | - | - | 2 | - |
| 1:512 | - | - | - | - | - | - | 2 | - |
| Saline Control | - | | - | | - | | - | |

*The numerical values represent degree of agglutination; 4 denotes the highest, 1 the lowest, and the negative sign (-) denotes no reaction.

Plate I. Leptospiral macroscopic tube agglutination reaction using homogenized milk as the fixation agent.

and centrifuged at 250 x G for five minutes. The supernatant fluid was decanted into two other 50 milliliter centrifuge tubes and centrifuged again at 250 x G for five minutes. The decanting and centrifugation at 250 x G for five minutes was repeated once again. The optical density of the final suspension was 0.09 at a wave length of 500 millimicrons.

Two-fold ten-tube serial dilutions from 1:4 to 1:2048 in glycine-buffered saline were made using immune rabbit serum and normal rabbit serum to test the yeast cell fragments as a fixing agent for antigen.

Four racks of tubes containing dilutions of the two sera were prepared. Two milliliters of homogenized leptospiral cell suspension was added to 18 milliliters of the fractured yeast cell suspension to be used as the antigen.

Two-tenths of a milliliter of the antigen was added to each of the tubes and the racks were shaken. One rack was left at room temperature. The other racks were placed in water baths at temperatures of 37 C, 45 C, and 56 C, respectively.

The results of agglutination studies using fractured yeast cell suspension for the diluent for homogenized cell particle antigen are shown in Table II. Plate II shows the appearance of the tubes after eighteen hours of incubation.

Suspension of Lecithin as a Fixation Agent

A colloidal suspension of lecithin (Central Soya Co., Inc., Chicago, Illinois) was prepared by adding 0.5 grams of lecithin to 20 milliliters of glycine-buffered saline and mixing well. The

TABLE II. FRACTURED YEAST CELL SUSPENSION FIXATION REACTIONS*

| Serum Dilutions | Room Temperature | | 37 C | | 45 C | | 56 C | |
|---|---|---|---|---|---|---|---|---|
| | Immune Serum | Normal Serum | Immune Serum | Normal Serum | Immune Serum | Normal Serum | Immune Serum | Normal Serum |
| 1:4 | - | - | - | - | 2 | - | 4 | - |
| 1:8 | - | - | - | - | 2 | - | 4 | - |
| 1:16 | - | - | - | - | 2 | - | 3 | - |
| 1:32 | - | - | - | - | 1 | - | 2 | - |
| 1:64 | - | - | - | - | - | - | - | - |
| 1:128 | - | - | - | - | - | - | - | - |
| 1:256 | - | - | - | - | - | - | - | - |
| 1:512 | - | - | - | - | - | - | - | - |
| 1:1024 | - | - | - | - | - | - | - | - |
| 1:2048 | - | - | - | - | - | - | - | - |
| Saline Control | - | | - | | - | | - | |

*The numerical values represent degree of agglutination; 4 denotes the highest, 1 the lowest, and the negative sign (-) denotes no reaction.

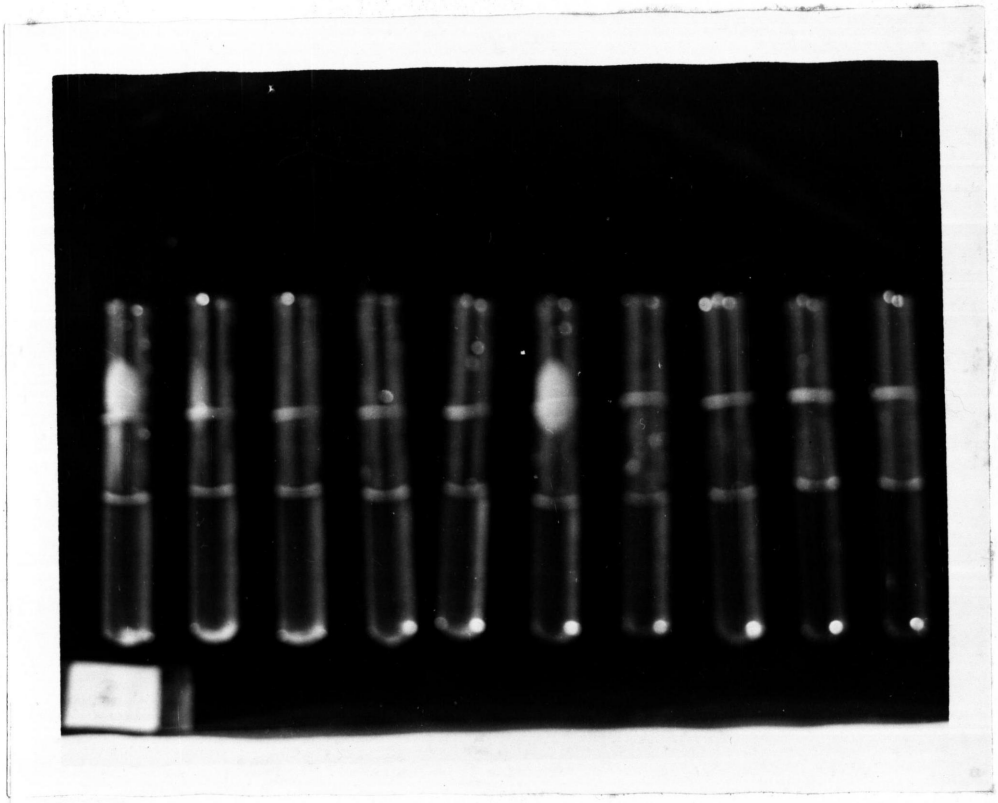

Plate II. Leptospiral macroscopic tube agglutination reaction using fractured yeast cells as the fixation agent.

suspension was filtered through #40 Whatman filter paper to remove extraneous material that appeared to be present. The suspension was then adjusted with glycine-buffered saline to an optical density of 0.2 at a wave length of 600 millimicrons.

Ten tube two-fold dilutions from 1:4 to 1:2048 in glycine-buffered saline were made using immune rabbit serum and normal rabbit serum to test the lecithin suspension as a fixing agent for the antigen.

Four racks of tubes containing dilutions of the two sera were prepared. Two milliliters of homogenized leptospiral cell suspension was added to 18 milliliters of the lecithin suspension and mixed well.

Two tenths of a milliliter of the antigen was added to each of the tubes and the racks were shaken. One rack was left at room temperature. The other racks were placed in water baths at temperatures of 37 C, 45 C, and 56 C, respectively.

The results of agglutination studies using a lecithin suspension as the diluent for homogenized leptospiral cell antigen are shown in Table III.

Tests with Bovine Sera

Leptospiral tube agglutination tests were performed on ten bovine sera to test the homogenized milk suspension antigen. The bovine sera were secured from the South Dakota Livestock Sanitary Board Laboratory at Pierre. Macroscopic plate agglutination test performed at that laboratory showed that three of the sera were known to contain leptospiral antibodies. The other seven sera had not been tested for the presence of leptospiral antibodies. Macroscopic plate agglutination

TABLE III. LECITHIN SUSPENSION FIXATION REACTIONS*

| Serum Dilutions | Room Temperature | | 37 C | | 45 C | | 56 C | |
|---|---|---|---|---|---|---|---|---|
| | Immune Serum | Normal Serum | Immune Serum | Normal Serum | Immune Serum | Normal Serum | Immune Serum | Normal Serum |
| 1:4 | - | - | - | - | 3 | - | 4 | - |
| 1:8 | - | - | - | - | 3 | - | 4 | - |
| 1:16 | - | - | - | - | 3 | - | 4 | - |
| 1:32 | - | - | - | - | 2 | - | 4 | - |
| 1:64 | - | - | - | - | 1 | - | 3 | - |
| 1:128 | - | - | - | - | - | - | 2 | - |
| 1:256 | - | - | - | - | - | - | 1 | - |
| 1:512 | - | - | - | - | - | - | - | - |
| 1:1024 | - | - | - | - | - | - | - | - |
| 1:2048 | - | - | - | - | - | - | - | - |
| Saline Control | - | | - | | - | | - | |

*The numerical values represent degree of agglutination; 4 denotes the highest, 1 the lowest, and the negative sign (-) denotes no reaction.

tests had been performed for the presence of brucellosis antibodies and found to be negative. Immune and normal rabbit sera were tested at the same time as the ten bovine sera for leptospiral agglutination.

Two-fold ten tube dilutions from 1:4 to 1:2048 were made in glycine-buffered saline of each of the ten bovine sera and of the two rabbit sera.

One and one-half milliliter of homogenized leptospiral cell suspension was added to 28.5 milliliters of homogenized milk suspension. This was the antigen used.

Two tenths of a milliliter of the antigen was added to each of the tubes and the racks were shaken. The racks of tubes were incubated in a 56 C water bath for eighteen hours.

The results of the macroscopic tube agglutination test on bovine sera using homogenized milk suspension antigen are shown in Table IV.

Cross-agglutination Studies

Agglutination studies were conducted to determine if there was cross-agglutination be sera containing antibodies against organisms other than leptospira with leptospiral antigen. Four human sera used in these studies were obtained from the South Dakota State Health Laboratory at Pierre. One human serum was reactive to the Kolmer complement-fixation test for syphilis, one was non-reactive to the Kolmer test, one was positive for brucellosis by the plate agglutination test and one was negative for brucellosis. Two bovine sera were used. One was leptospiral immune serum and the other was normal serum. Two rabbit sera were used. One was leptospiral immune serum and the other

TABLE IV. BOVINE SERA AGGLUTINATION STUDIES*

| Serum Dilutions | Positive Bovine Sera | | | Unknown Bovine Sera | | | | | | | Rabbit Immune Serum | Rabbit Normal Serum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | | |
| 1:4 | 4 | 4 | 4 | - | - | - | - | - | - | - | 4 | - |
| 1:8 | 4 | 4 | 4 | - | - | - | - | - | - | - | 4 | - |
| 1:16 | 4 | 4 | 4 | - | - | - | - | - | - | - | 4 | - |
| 1:32 | 4 | 4 | 3 | - | - | - | - | - | - | - | 4 | - |
| 1:64 | 4 | 4 | 2 | - | - | - | - | - | - | - | 4 | - |
| 1:128 | 3 | 4 | 1 | - | - | - | - | - | - | - | 3 | - |
| 1:256 | 1 | 2 | - | - | - | - | - | - | - | - | 2 | - |
| 1:512 | - | 1 | - | - | - | - | - | - | - | - | 2 | - |
| 1:1024 | - | - | - | - | - | - | - | - | - | - | 1 | - |
| 1:2048 | - | - | - | - | - | - | - | - | - | - | - | - |
| Saline Control | | | | | | | | | | | | - |

*The numerical values represent degree of agglutination; 4 denotes the highest, 1 the lowest, and the negative sign (-) denotes no reaction.

was normal serum.

Two-fold ten tube dilutions from 1:4 to 1:2048 were made in glycine-buffered saline of each of the eight sera.

One milliliter of homogenized leptospiral cell suspension was added to nineteen milliliters of homogenized milk suspension for the antigen.

Two tenths of a milliliter of the antigen was added to each of the tubes and the racks were shaken. The racks were placed in a 56 C water bath for eighteen hours.

The results of the cross-agglutination studies with the eight sera are shown in Table V.

Agglutination Study of Globulin Fraction

An agglutination study was made of the globulin fraction of leptospiral immune and normal rabbit sera. Eight and one half milliliters of saturated ammonium sulfate solution was added to eight and one half milliliters of each of the rabbit sera to precipitate the globulin. The two sera were centrifuged at 1600 x G for ten mimutes. The supernatant fluids were removed and discarded. Half-saturated ammonium sulfate solution was added to each of the tubes. The solutions were centrifuged again at 1600 x G for ten minutes. The supernatant fluids were decanted and discarded. The coagulated protein in each tube was reconstituted to eight and one half milliliters with distilled water. The liquids were pipetted into two visking casings which were sealed at the bottom. The visking casings were sealed at the top and immersed in a beaker containing 0.85 per cent NaCl solution.

TABLE V. CROSS-AGGLUTINATION STUDIES*

| Serum Dilutions | Human Syphilitic Serum | | Human Brucellosis Serum | | Bovine Leptospiral Serum | | Rabbit Leptospiral Serum | |
|---|---|---|---|---|---|---|---|---|
| | Immune | Normal | Immune | Normal | Immune | Normal | Immune | Normal |
| 1:4 | - | - | - | - | 4 | - | 4 | - |
| 1:8 | - | - | - | - | 4 | - | 4 | - |
| 1:16 | - | - | - | - | 4 | - | 4 | - |
| 1:32 | - | - | - | - | 4 | - | 4 | - |
| 1:64 | - | - | - | - | 4 | - | 4 | - |
| 1:128 | - | - | - | - | 3 | - | 3 | - |
| 1:256 | - | - | - | - | 1 | - | 2 | - |
| 1:512 | - | - | - | - | - | - | 2 | - |
| 1:1024 | - | - | - | - | - | - | 1 | - |
| 1:2048 | - | - | - | - | - | - | - | - |
| Saline Control | | | | | | | | - |

*The numerical values represent degree of agglutination; 4 denotes the highest, 1 the lowest, and the negative sign (-) denotes no reaction.

This was placed in a refrigerator. The visking casings were placed in fresh saline each day. Daily tests were made of the saline with a five per cent barium chloride solution to test for the presence of sulfate. After five days of dialysis, no sulfate was found to be present in the saline. This indicated that the globulin solutions in the visking casings were free of sulfate.

Two-fold ten tube dilutions from 1:4 to 1:2048 were made of the sera in glycine-buffered saline for the two globulin fraction solutions, rabbit leptospiral immune and normal whole sera.

Five tenths of a milliliter of homogenized leptospiral cell suspension was added to nine and one half milliliters of homogenized milk suspension for the antigen.

Two tenths of a milliliter of the antigen was added to each of the tubes and the racks were shaken. The racks were placed in a 56 C water bath for eighteen hours.

The results of the globulin fraction studies are shown in Table VI.

TABLE VI. GLOBULIN FRACTION STUDIES*

| Dilutions | Globulin Fractions of Rabbit Sera | | Whole Rabbit Sera | |
|---|---|---|---|---|
| | Immune | Normal | Immune | Normal |
| 1:4 | 4 | - | 4 | - |
| 1:8 | 4 | - | 4 | - |
| 1:16 | 4 | - | 4 | - |
| 1:32 | 4 | - | 4 | - |
| 1:64 | 4 | - | 4 | - |
| 1:128 | 4 | - | 3 | - |
| 1:256 | 3 | - | 2 | - |
| 1:512 | 2 | - | 2 | - |
| 1:1024 | 1 | - | 1 | - |
| 1:2048 | - | - | - | - |
| Saline Control | | | | - |

*The numerical values represent degree of agglutination; 4 denotes the highest, 1 the lowest, and the negative sign (-) denotes no reaction.

## DISCUSSION

The homogenized milk suspension and colloidal lecithin fixation studies of leptospiral antigen appeared to produce better results than the fractured yeast cell suspension. Initial studies were made by using whole yeast cells. It was found that the yeast cells settled spontaneously from the suspension and this made results of agglutination tests indescernible. The decision was made that possibly some portion of the yeast cells could be used for a suspension.

An attempt was made to make a suspension of cholesterol to use in agglutination studies but efforts to produce a satisfactory suspension failed. Also an attempt to make a satisfactory suspension by using whipping cream and half and half cream failed to produce satisfactory results. Another suspension was tried using finely ground silica but the silica settled rapidly and was discarded as unsatisfactory.

The 56 C incubation temperature appeared to produce a higher titer in the agglutination studies. Singer and Plotz (21) did a study on temperatures of incubation for tube agglutination tests. They report that the best results were obtained at 56 C.

The use of homogenized milk suspension antigen in tube agglutination tests showed that it produces as satisfactory results on bovine serum as on rabbit serum for detecting leptospiral antibodies. The cross-agglutination studies on the few sera used indicated that there is a possibility that there is no cross-agglutination of leptospiral antigen with antibodies against other organisms. The globulin fraction

studies indicated that the leptospiral antibodies are present in the globulin part of the serum. A slightly higher titer was obtained from the globulin fraction than from the whole serum.

Homogenized leptospiral cell adsorption onto particulate carriers would result in considerable extension of whole cell antigen. The homogenized cell suspension is diluted considerably even before the cell particles are adsorbed onto the particulate carriers. One could estimate that the antigen is extended ten times.

There is a great potential field for additional studies of adsorbed leptospiral antigens or adsorbed antigens for the diagnosis of other infectious diseases of man or animals. These adsorbed antigens could possibly be adapted for use in rapid plate agglutination tests or capillary tube tests.

## SUMMARY AND CONCLUSION

Macroscopic tube agglutination studies were described for Leptospira pomona. Various fixation agents for leptospiral antigens were tested.

The conclusions that may be drawn from this work are as follows:

1. A diluted suspension of washed and centrifuged homogenized milk appears to be a satisfactory diluent for homogenized leptospiral cells in macroscopic tube agglutination tests.
2. Fractured yeast cell suspensions appeared to be less satisfactory as the diluent for homogenized leptospiral cells in macroscopic tube agglutination tests.
3. Colloidal lecithin seemed to be as satisfactory a substance as homogenized milk as a diluent for homogenized leptospiral cell antigen in macroscopic tube agglutination tests.
4. The homogenized milk suspension of leptospiral antigen appeared to produce as satisfactory agglutination results on bovine sera as on rabbit sera.
5. Cross-agglutination is possible but none occurred with the sera tested.
6. Antibodies of leptospirosis appeared to be present entirely in the globulin fraction of the immune rabbit serum.

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