# AGGLUTINATIONLYSIS TEST AND THE AGGLUTINATION TEST FOR LEPTOSPIRA POMONA INFECTION

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# EFFECT OF VARIOUS FACTORS UPON THE AGGLUTINATION-LYSIS TEST AND THE AGGLUTINATION TEST FOR *LEPTOSPIRA POMONA* INFECTION

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# CHAPTER I

## INTRODUCTION

Human and animal leptospiral infections have, in recent years, received an increasing amount of attention because of their significance as a world wide veterinary and public health problem. This has resulted in an increased interest in the serological diagnosis of leptospirosis.

The following methods have been employed for diagnosis: complement fixation test,[2, 3, 27] adhesion test,[4] modified macroscopic agglutination tests,[6, 25, 32] capillary tube test,[39] urine fraction test,[31] hemagglutination test,[9] hemolytic test,[12] and the microscopic agglutination-lysis test.[36]

However, the microscopic agglutination-lysis tests are considered to be most accurate and are used extensively by research groups. Macroscopic or plate agglutination tests are favored by diagnostic laboratories in many states.

Up to now, no standard procedure has been established to determine a means of standardization of techniques and interpretations for the various procedures. Many laboratories have been using different interpretations of the results of serological methods, and have also employed different techniques in the preparations of antigens, dilutions of the serum, and the time and temperature of incubation.

There is a lack of information on the comparison of controlled

conditions as they apply to the microscopic agglutination-lysis tests. The purpose of this research was to ascertain the effects of living and formalin killed antigens, various incubation temperatures and periods of time upon the sensitivity of the microscopic agglutination-lysis tests using Leptospira pomona antisera obtained from man, cattle, sheep, goats, pigs, dogs, guinea pigs and hamsters.

## CHAPTER II

## REVIEW OF THE LITERATURE

Costa and Troisier[10] examined the reactions of the Wassermann test on five sera from patients who had "ictero hemorragic spirochetosis." They concluded that the reaction of the test might be positive with "spirochetosis."

Martin and Pettit[27] found confirmation in their studies with human sera concerning the fixation of complement in "ictero hemorragic" infection. The reaction was positive with the Wassermann antigen and a positive spirochetosis serum. The reciprocal was also true. They used, as antigen guinea pig liver rich in "spirochetes," but positive results were obtained with "spirochetosis" serum as well as with a strongly positive syphilitic serum.

Schuffner and Mochtar[36] developed the microscopic agglutination-lysis reaction using living leptospirae as an antigen. Incubation was at room temperature and the tests were read employing dark ground illumination.

Brown and Davis[4] applied the phenomenon of adhesion onto colloidal particles as means of differentiating serological types of leptospirae.

Bessemans and Nelis[2] found that fixation of complement was specific. They used thirteen sera from human syphilitics with strong positive Wassermann reactions. These always gave negative results when used against antigens prepared from pure cultures of leptospirae,

but poor results when used with alcoholic antigen extracts of guinea pig livers.

Gaehtgens[17] tested 70 sera of patients showing symptoms suggestive of Weil's disease using the agglutination and complement fixation tests. Their results showed fairly close agreement between the two methods. However, as a rule, agglutination titers were higher than complement fixation titers.

Brown and Camb[5] demonstrated the sensitivity of the adhesion test and compared it with the agglutination test. They found that adhesion disappears at about the same dilution as the end point of agglutination and hence concluded that adhesion test could be specifically used for leptospirosis diagnosis.

Erber[16] found centrifugation of older antigen cultures removed debris and facilitated the reading of agglutination lysis tests. The culture and serum were mixed, saline added and incubated at 37 C for one hour.

Pot[32] employed a macroscopic agglutination test in cases of Weil's disease using killed antigen. The antigen was treated with phenol which disrupted the leptospirae. He centrifuged and resuspended the sediment in a fluid free of serum protein. This solution was brought to required density by addition of saline and 0.2 per cent formalin. He tested 26 human sera which were positive for Schuffner's agglutination-lysis test. Positive results were obtained in 25 cases.

Using 100 human sera, Pot and Dornickx[33] made a comparison between the complement fixation test and agglutination-lysis test

results. They concluded that low titers, approximately 1:100, with the agglutination-lysis test were without diagnostic value and might lead to incorrect conclusions.

Smith and Tulloch[37] studied the macroscopic agglutination test. They used 4 - 10 day old leptospirae cultures as antigen, to which were added various quantities of formalin. They also observed the influence of heat on the effect of agglutination. They stated that optimal results required incubation at a temperature between 30 and 37 C. Human sera, urine, as well as guinea pig sera were employed.

Brown[6] employed a rapid presumptive serological test for the diagnosis of Weil's disease. A dense saline suspension of Leptospira icterohaemorrhagiae was placed on a slide. This suspension was formolized to a concentration of 0.2 per cent. Serum dilutions were then added. After 10 minutes at room temperature readings were made. Brown's technique for antigen preparation was modified by Lederle Laboratories[1] in 1941 and was adapted for the plate agglutination test. This test is not used at the present time.

Boerner and Luckens[3] have reported a method of preparing antigen for the complement fixation test using broth cultures of Leptospira icterohaemorrhagiae and Leptospira canicola. These preparations showed no anticomplementary effects. A comparison was made between the complement fixation and agglutination-lysis tests. The complement fixation test demonstrated leptospiral antibodies when the agglutination-lysis test results were as low as 1:300.

Starbuck and Ward[38] made a comparative study of the macroscopic agglutination test with the standard microscopic test. Using positive human and rabbit sera against Leptospira icterohaemorrhagiae, it was found that the titers obtained with the macroscopic test were always

much lower than those obtained with the microscopic test.

Gardner and Wyle[19] examined 1120 human sera utilizing the macroscopic tube agglutination method. The antigen used was a suspension of formalinized (0.25%) killed organisms. Positive sera gave visible results after standing overnight at room temperature.

Gardner[20] described a simple rapid microscopic technique for leptospiral agglutination. He compared the macroscopic agglutination test with the rapid microscopic technique using killed cultures as antigen. The ratio of positive to negative results was very similar for both tests. A similar method was employed by Kruger.[26]

Randall et al.,[35] employed sonic vibrated leptospirae as antigen in the complement fixation reaction and made a comparison with the microscopic agglutination test on human sera, using living antigen. Their results indicate that there is a parallelism between the amount of the complement fixing antibody and agglutinins; however, they noted that it was necessary to multiply the indicated dilution of the sera in the complement fixation test by six for obtaining a comparison with the dilution in the agglutination test.

Newman[30] used killed antigen (formalin 0.15%) and incubated at 37 C for 3 hours. In this study the use of the agglutination test for laboratory diagnosis of leptospirosis in dogs was evaluated. During the second week of infection the test was only 50 per cent accurate; however, after the third to fourth week the test was 100 per cent accurate. This indicated that the accuracy of the agglutination test increases with chronicity of infection. Even though the test is rapid and reasonably accurate its usefulness is limited.

York[41] described the complement fixation test for bovine leptospirosis using antigen prepared from embryonated eggs. He also made a comparison between the complement fixation test and the agglutination-lysis test with 55 bovine sera. He obtained a close correlation between dilution end points for the two tests. However, he mentioned several advantages that the complement fixation test has over the agglutination-test such as: a more objective test, distinct difference between positive and negative sera, and a more useful method when a laboratory has equipment for routine complement fixation tests.

Hoag et al.,[24] described a method for the preparation of macroscopic leptospiral agglutinating antigens, prepared in a manner that they became more sensitive, specific and stable under longer periods of storage. These workers made a comparison with microscopic agglutination-lysis tests using sera from cattle, pigs and dogs. It was found that the degree of correlation between these methods, at significant antibody levels, was approximately 70 per cent.

York and Johnson[44] compared three basic tests: agglutination-lysis, plate agglutination and complement fixation. They used sera from cattle infected with Leptospira pomona and dogs infected with Leptospira canicola. For the agglutination-lysis test, the mixed antigen and serum were incubated at 37 C for one hour. In the complement fixation test the antigen was mixed with the serum and incubated at 37 C for one hour. The hemolytic system was then added. The authors found that the agglutination-lysis and complement fixation tests were equally reliable and sensitive for diagnostic work.

However, they also found that the plate agglutination test is less sensitive, but with commercially prepared antigen false positive reactions were absent.

Stoenner[39] evaluated the technique of the capillary tube test employing sera from human, cattle and dog. The antigen used was a suspension of formalin killed leptospirae in hypertonic buffered sodium chloride solution. Serum dilutions and antigen were mixed in capillary tubes. This method was compared with the standard microscopic agglutination-lysis and was found to be favorable in sensitivity and specificity.

Gochenour et al.,[21] studied 29 human cases of acute leptospirosis and they found that serological examination by the complement fixation test using sonic vibrated antigen gave good results.

Stoenner[40] evaluated the plate test and capillary tube technique on 7066 bovine sera and found both compare favorably with the agglutination-lysis test in specificity and sensitivity.

The hemagglutination procedure developed by Chang and McComb[9] produced lower titers than those obtained with the agglutination lysis test.

Stoenner[41] stated that incubation of serum and antigen varies from laboratory to laboratory from one hour at 37 C to overnight at room temperature. Usually sera are diluted in tenfold increments, but in some laboratories the sera are tested by either 2, 4, or 5 fold dilutions. In most instances, cultures with four to five days growth are used as antigen, but in some cases cultures grown for 30 days have been used and produced favorable results. The use of

various media influence the number of leptospirae which can be cultivated; therefore, when using undiluted cultures the numbers of organism used as sources of antigen may vary due to the dark field method of evaluating cell content. In a series of studies comparing the effect of some variables of the agglutination lysis test on titers of sera he reported that of all the variables and modifications, the most important factors having effect on the titers, were density of antigen and method of preparing serial dilutions. Of lesser importance, but not insignificant, was the variation due to strain of Leptospira, time of serum-antigen incubation or the age of culture. He stated, "In tests in which antigen of low cell content and tenfold serial dilutions of serum were used, geometric mean titers were thirty-eight fold greater than titers obtained in tests in which dense antigen and twofold serial dilutions were employed." The author states that the technique of agglutination-lysis test should be standardized so that results from various laboratories will be comparable.

Nowicki[31] demonstrated that the urine of humans or animals infected with leptospirae contains a specific lytic agent. When the urine was concentrated ten times, the lytic agent was recovered very easily and was used for <u>in vitro</u> tests. Positive results were obtained in 20-30 hours after infection. Cultures of living leptospirae or those preserved with Chinosol were used as antigen. The advantages of this test are: ease of technique, early diagnosis and differentiation between active infection and recovered cases.

Following the work of Chang and McComb[9], Cox[11] found that leptospirae extracted with ethanol could sensitize sheep erythrocytes to agglutination with homologous leptospirae antisera from rabbits. It was also noted that sensitized sheep erythrocytes were susceptible to lysis in high dilutions of homologous antisera and complement.

Howarth[25] also described a macroscopic tube agglutination test. He tested sera of different animals and human using killed formolized antigen. The serum antigen dilution was kept for 12 hours at 37.5 C then for 6 hours at room temperature. After this time readings were made. A positive test was indicated by the appearance of grossly visible fluffy clumps. This technique was compared with the microscopic agglutination-lysis, using sera with high or low end point dilutions, and showed a close correlation. Fifteen per cent of the sera tested by the macroscopic agglutination method had end point dilution values which were one dilution lower than with the agglutination-lysis test.

Stoenner[42] employed these following tests: complement fixation, capillary tube and agglutination-lysis, for testing sera from herds of cattle recently infected by Leptospira pomona and also having a history of serological evidence of past infection. He found that the titers were highest with the agglutination-lysis test. He also showed that both the agglutination-lysis and capillary tube tests were comparable for detecting residual antibodies from herds with previous infection; whereas, the complement fixation test was limited in this respect.

Cox[12] described the preparation of his hemolytic antigen from nonpathogenic Leptospira biflexa and described the use of the hemolytic reaction in testing leptospirosis in human sera.

Cox et al.,[13] compared the hemolytic test with the microscopic agglutination-lysis test using 455 human sera which had been stored at -40 C for several years. The results obtained from their studies indicated that the microscopic agglutination test might be replaced by the hemolytic test when diagnosing human leptospirosis.

Newberne[29] conducted experiments to compare the rapid plate test and capillary tube test of Stoenner to the agglutination-lysis test using dog and pig sera. From the results of this study the rapid plate method seemed to be the better method for screening sera. The advantages which were gained from this were twofold: (a) improved correlation of serological surveys due to standardization of the antigen; (b) enabled the practitioner to perform rapid plate screening tests in the field.

Bryan[7] tested 15,092 serum specimens from different herds for Leptospira pomona antibodies. He developed a rapid plate test using antigen prepared with Giemsa's stain. This test gave positive results with sera that showed titers of 1:100 or higher by the agglutination-lysis test.

Hirschberg[23] modified a plastic depression tray for use in dark field microscopic agglutination-lysis test. A comparison of the results by this method corresponded favorably with the ordinary test tube method incubated 2 hours at 50 C or refrigerator overnight.

Galton *et al.*,[18] recently developed a macroscopic slide test for leptospirosis diagnosis. They found this technique to compare favorably with microscopic agglutination test with regards to its sensitivity. Because of the simplicity of the test, the authors suggest that this method is well adapted for small laboratories. One of the advantages of this test is that several antigens may be combined into pools containing as many as four antigens per pool, which make possible the examination of large numbers of sera.

# CHAPTER III

## MATERIAL AND METHODS

The following sources of sera were used throughout this study: ten samples of human sera which were positive for Leptospira pomona were obtained from Col. M. B. Starnes, V.C., Walter Reed Army Institute of Research, Washington, D.C. Representative samples of sera, positive for Leptospira pomona were obtained from the following animal species: cattle, sheep, goats, dogs, guinea pigs and hamsters.

These sera had been stored at -20 C. The serum donors were animals which had been utilized by various workers in the Department of Microbiology and Public Health in their research.

Turbid sera were centrifuged at 5000 rpm for 15 minutes in the Servall angle centrifuge, Model A.

At least 5 negative serum samples from the same animal species under examination were included as controls for the serological tests.

Master dilutions for each sample were made by adding 0.05 ml serum to 0.5 ml of sterile "Chang's buffer," which is prepared by dissolving 4.0 g $Na_2HPO_4 \cdot 7H_2O$, 0.8 g $KH_2PO_4$ and 8.0 g NaCl in distilled water to make two liters. The buffer had a final pH of 7.0. The buffer consisted of the ingredients of Chang's medium[8] less the liver extract, tryptose and rabbit serum. Subsequent tenfold dilutions up to $10^{-8}$ were made from this original dilution.

The antigen used was the "Johnson" strain of Leptospira pomona prepared from 4-7 day old cultures which had been grown at 30 C in

"Antigen vials" (500 x 1000 mm) containing approximately 50 ml of Chang's fluid medium[8] enriched with 0.01 per cent hemoglobin (Difco) and sterile rabbit serum to give a final concentration of 10 per cent.

Antigen density was estimated with a dark field microscope and adjusted to approximately 250 cells per field at 590 x magnification or approximately $10^8$ leptospirae per ml. Appropriate dilutions were made with sterile Chang's medium and then centrifuged in an International Clinical Centrifuge Model C.L. for 15 minutes at 1500 rpm.

The formalinized antigen was prepared by the addition of a 0.1 per cent formaldehyde solution to the diluted culture. This culture was centrifuged in the same manner as was done with the live antigen. The formalinized antigen was used within one hour after preparation.

A modified darkfield type illumination was used by inserting a star diaphragm into the Abbe condenser and employing 100 x optical magnification.[28]

The agglutination-lysis tests, using living antigens, were incubated in a thermostatically controlled water bath at 37 C for 2 hours. A similar test was also conducted in parallel but was incubated at room temperature, approximately 22 C for 16-18 hours. The formalinized antigen test was also incubated at room temperature for 16-18 hours.

In the comparison of these three methods of preparing the reagents for agglutination lysis, the modified microscopic agglutination-lysis tube test was used.[28] The highest dilution which gave any evidence of agglutination or lysis or both was noted as end point for the tests.

In all of the tests employed in this work the amount of antigen added to the dilution of serum was 0.1 ml. The dilution factor created by adding the antigen was not included in the serum dilutions as expressed in this thesis.

# CHAPTER IV

## RESULTS

The data shown in Tables I through VIII indicated that regardless of incubation time or temperature, formalinization of leptospirae had a deleterious effect on agglutination with leptospiral antiserum.

When living antigen was used, however, the agglutination or lysis reaction occurred with higher dilutions of serum than when formalinized antigen was used. It can be noted from the tables that time and temperature of incubation were important factors in determining the end point titers of the sera.

The salient feature of these results was that agglutination or lysis end points were always higher when living antigen was incubated with the sera at room temperature for 16-18 hours than when allowed to react at 37 C for 2 hours.

In some cases agglutination was not seen in the lower dilution (usually 1:10) of antiserum. However, the remaining dilutions lysed the leptospirae to varying degrees to the end point titer.

In general, when formalinized antigen was used, lysis never occurred. In the lower dilutions of antiserum the cells were adherent in the form of clumps consisting of a loose network. This agglutination resembled the "H" type seen in Salmonella.

When living antigen was used with lower dilutions of the antiserum, the phenomenon of agglutination always took place and preceded the formation of "lysis balls" or "degenerative granules" although the

number of leptospirae was greatly diminished in comparison with the control. Loose floccular clumps, as seen, with formalinized antigen, were never found because the leptospirae had become lysed.

EXPLANATION OF SYMBOLS USED IN EXPRESSING DEGREE OF AGGLUTINATION, LYSIS OR BOTH IN TABLES I THROUGH VIII

(+) Indicates 100 per cent agglutination, lysis or both.

(+P) Indicates 75 per cent agglutination, lysis or both.

(P) Indicates 50 per cent agglutination, lysis or both.

($\overset{+}{-}$) Indicates 25 per cent or less agglutination, lysis or both.

(-) Indicates no agglutination, lysis in all dilutions above $10^{-1}$.

TABLE I

RESULTS OF VARIOUS SEROLOGICAL TESTS ON HUMAN SERA*

| No. serum | 37 C - 2 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Formalinized Antigen, SERUM DILUTIONS** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | P | +P | P | - | | | | | P | +P | P | ± | - | | | | ± | +P | P | - | | | | |
| 2 | +P | P | ± | - | | | | | +P | + | P | - | | | | | ± | +P | - | | | | | |
| 3 | + | +P | P | - | | | | | +P | +P | P | ± | - | | | | P | + | P | - | | | | |
| 4 | +P | ± | - | | | | | | +P | P | - | | | | | | +P | - | | | | | | |
| 5 | + | P | - | | | | | | + | +P | P | - | | | | | +P | P | - | | | | | |
| 6 | + | +P | P | - | | | | | + | +P | +P | P | - | | | | + | P | ± | - | | | | |
| 7 | + | P | ± | - | | | | | + | P | ± | - | | | | | + | P | - | | | | | |
| 8 | + | P | - | | | | | | + | P | ± | - | | | | | +P | P | - | | | | | |
| 9 | +P | P | ± | - | | | | | + | +P | P | - | | | | | + | P | - | | | | | |
| 10 | P | ± | - | | | | | | +P | P | - | | | | | | P | - | | | | | | |

*Results obtained with 10 normal sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

TABLE II

RESULTS OF VARIOUS SEROLOGICAL TESTS ON CATTLE SERA*

| No. serum | 37 C - 2 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Formalinized Antigen, SERUM DILUTIONS** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | + | + | P | - | | | | | + | + | +P | P | - | | | | + | +P | P | - | | | | |
| 2 | +P | P | ± | - | | | | | + | +P | P | ± | - | | | | + | P | - | | | | | |
| 3 | ± | + | + | +P | P | ± | - | | + | + | + | +P | P | P | - | | + | +P | P | ± | - | | | |
| 4 | + | + | +P | P | - | | | | - | + | + | +P | ± | - | | | + | +P | P | - | | | | |
| 5 | +P | P | +P | - | | | | | + | +P | +P | P | - | | | | P | P | ± | - | | | | |
| 6 | + | + | +P | P | ± | - | | | + | + | + | +P | P | - | | | ± | + | +P | ± | - | | | |
| 7 | ± | + | + | P | ± | - | | | + | + | +P | +P | P | ± | - | | + | +P | P | ± | - | | | |
| 8 | + | + | + | P | - | | | | ± | + | + | +P | P | - | | | + | +P | P | - | | | | |
| 9 | ± | + | + | +P | P | - | | | P | + | + | + | P | - | | | - | +P | + | P | - | | | |
| 10 | +P | + | + | P | ± | - | | | +P | + | + | P | P | ± | - | | + | + | P | - | | | | |
| 11 | +P | + | + | P | - | | | | + | + | + | +P | P | ± | - | | + | +P | P | - | | | | |
| 12 | +P | ± | - | | | | | | +P | P | ± | - | | | | | P | - | | | | | | |
| 13 | ± | +P | P | - | | | | | + | +P | P | ± | - | | | | +P | + | ± | - | | | | |
| 14 | +P | P | - | | | | | | + | +P | P | - | | | | | +P | + | + | - | | | | |
| 15 | P | - | | | | | | | P | P | ± | - | | | | | P | - | | | | | | |

*Results obtained with 10 normal cattle sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

TABLE III

RESULTS OF VARIOUS SEROLOGICAL TESTS ON SHEEP SERA*

| No. serum | 37 C - 2 hours, Living Antigen, SERUM DILUTIONS** 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Room Temperature 16 - 18 hours, Living Antigen, SERUM DILUTIONS** 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Room Temperature 16 - 18 hours, Formalinized Antigen, SERUM DILUTIONS** 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | +P | P | - | | | | | + | +P | P | ± | - | | | | + | +P | P | - | | | | |
| 2 | + | +P | P | ± | - | | | | + | + | + | P | - | | | | - | + | +P | P | - | | | |
| 3 | ± | +P | - | | | | | | +P | P | - | | | | | | P | ± | - | | | | | |
| 4 | + | + | +P | P | - | | | | P | + | + | P | - | | | | ± | + | P | ± | - | | | |
| 5 | + | + | +P | P | - | | | | + | + | + | +P | P | - | | | P | + | +P | P | - | | | |
| 6 | ± | +P | P | - | | | | | + | + | +P | ± | - | | | | + | P | - | | | | | |
| 7 | + | +P | P | - | | | | | + | + | P | - | | | | | + | +P | - | | | | | |
| 8 | P | +P | +P | P | - | | | | + | + | P | ± | - | | | | + | + | P | ± | - | | | |
| 9 | ± | + | +P | P | - | | | | + | + | + | +P | P | - | | | + | + | P | P | - | | | |
| 10 | P | + | +P | P | - | | | | + | + | + | +P | P | - | | | + | + | P | ± | - | | | |
| 11 | ± | + | +P | P | - | | | | + | + | +P | P | ± | - | | | + | +P | P | - | | | | |
| 12 | + | +P | P | - | | | | | ± | +P | P | ± | - | | | | + | +P | ± | - | | | | |
| 13 | ± | + | P | - | | | | | + | +P | P | ± | - | | | | + | P | ± | - | | | | |
| 14 | ± | + | P | ± | - | | | | + | + | +P | P | - | | | | + | +P | P | - | | | | |
| 15 | P | + | P | - | | | | | + | + | P | ± | - | | | | + | +P | ± | - | | | | |

*Results obtained with 10 normal sheep sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

TABLE IV

RESULTS OF VARIOUS SEROLOGICAL TESTS ON GOAT SERA*

| No. serum | 37 C - 2 hours, Living Antigen | | | | | | | | Room Temperature 16 - 18 hours, Living Antigen | | | | | | | | Room Temperature 16 - 18 hours, Formalinized Antigen | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | - | + | P | - | | | | | + | +P | P | ± | - | | | | + | P | ± | - | | | | |
| 2 | ± | + | P | ± | - | | | | + | + | +P | P | - | | | | + | + | P | - | | | | |
| 3 | +P | +P | ± | - | | | | | - | + | P | ± | - | | | | +P | P | ± | - | | | | |
| 4 | + | P | P | - | | | | | + | + | P | ± | - | | | | + | +P | P | - | | | | |
| 5 | + | + | +P | P | ± | - | | | + | + | +P | P | P | ± | - | | + | +P | P | ± | - | | | |
| 6 | +P | P | ± | - | | | | | + | P | P | ± | - | | | | + | P | - | | | | | |
| 7 | + | +P | P | ± | - | | | | + | + | +P | P | - | | | | + | +P | P | - | | | | |
| 8 | P | ± | - | | | | | | P | P | ± | - | | | | | P | - | | | | | | |
| 9 | ± | + | P | P | ± | - | | | +P | + | +P | +P | P | ± | - | | P | + | +P | ± | - | | | |
| 10 | - | +P | P | - | | | | | +P | +P | P | ± | - | | | | + | +P | ± | - | | | | |
| 11 | +P | +P | P | - | | | | | +P | + | +P | P | - | | | | P | + | P | - | | | | |
| 12 | ± | + | +P | P | - | | | | +P | + | +P | P | - | | | | + | + | P | - | | | | |
| 13 | ± | + | +P | P | ± | - | | | +P | + | + | P | ± | - | | | + | +P | P | ± | - | | | |
| 14 | P | + | + | +P | ± | - | | | +P | +P | + | + | P | ± | - | | + | +P | +P | P | - | | | |
| 15 | ± | + | +P | P | - | | | | +P | + | + | P | ± | - | | | +P | + | P | - | | | | |

*Results obtained with 10 normal goats sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

TABLE V

RESULTS OF VARIOUS SEROLOGICAL TESTS ON PIG SERA*

| No. serum | 37 C - 2 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Formalinized Antigen, SERUM DILUTIONS** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | + | + | P | ± | - | | | | + | + | +P | P | - | | | | + | +P | ± | - | | | | |
| 2 | + | +P | P | - | | | | | + | + | P | ± | - | | | | + | P | ± | - | | | | |
| 3 | - | +P | + | +P | P | ± | - | | +P | + | + | +P | P | ± | - | | + | + | +P | P | - | | | |
| 4 | + | + | + | P | ± | - | | | + | + | + | +P | P | ± | - | | ± | + | +P | ± | - | | | |
| 5 | + | +P | P | ± | - | | | | + | +P | + | P | ± | - | | | + | +P | P | - | | | | |
| 6 | + | + | + | +P | P | - | | | P | + | + | +P | P | ± | - | | - | + | +P | P | - | | | |
| 7 | + | + | +P | P | - | | | | +P | + | + | P | P | ± | - | | + | +P | +P | P | - | | | |
| 8 | + | +P | +P | P | ± | - | | | +P | + | + | P | P | - | | | + | +P | P | ± | - | | | |
| 9 | + | +P | P | - | | | | | + | + | +P | P | ± | - | | | ± | + | P | - | | | | |
| 10 | - | + | +P | P | ± | - | | | P | + | + | +P | P | ± | - | | + | +P | P | - | | | | |
| 11 | +P | +P | + | P | - | | | | +P | + | + | +P | P | ± | - | | + | + | P | ± | - | | | |
| 12 | + | + | +P | P | - | | | | + | + | +P | +P | P | - | | | + | + | P | ± | - | | | |
| 13 | +P | + | P | ± | - | | | | +P | + | +P | P | ± | - | | | + | +P | P | ± | - | | | |
| 14 | +P | + | + | +P | +P | P | ± | - | P | + | + | + | + | +P | P | - | + | +P | +P | P | ± | - | | |
| 15 | P | + | + | + | +P | P | ± | - | +P | + | + | + | +P | +P | P | ± | + | + | +P | +P | P | ± | - | |

*Results obtained with 10 normal pig sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

TABLE VI

RESULTS OF VARIOUS SEROLOGICAL TESTS ON DOG SERA*

| No. serum | 37 C - 2 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Formalinized Antigen, SERUM DILUTIONS** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | + | + | + | +P | P | ± | - | | +P | + | + | + | P | P | ± | - | + | + | +P | P | - | | | |
| 2 | - | + | +P | +P | P | ± | - | | + | + | + | + | +P | P | ± | - | + | + | + | P | ± | - | | |
| 3 | +P | + | P | - | | | | | +P | + | P | ± | - | | | | + | P | ± | - | | | | |
| 4 | + | + | +P | P | - | | | | + | + | + | +P | P | - | | | ± | + | +P | - | | | | |
| 5 | + | + | + | +P | P | ± | - | | - | + | + | +P | P | ± | - | | + | + | P | ± | - | | | |
| 6 | + | + | +P | P | ± | - | | | + | + | + | +P | P | ± | - | | + | + | +P | P | - | | | |
| 7 | + | + | + | +P | P | ± | - | | + | + | +P | +P | P | P | - | | +P | + | +P | P | - | | | |
| 8 | + | + | +P | P | P | - | | | + | + | +P | +P | P | ± | - | | + | +P | +P | P | - | | | |
| 9 | + | + | +P | P | P | - | | | ± | + | + | + | +P | P | - | | + | + | +P | P | ± | - | | |
| 10 | ± | + | +P | P | ± | - | | | ± | +P | + | +P | P | ± | - | | + | +P | P | ± | - | | | |
| 11 | + | +P | P | - | | | | | + | +P | P | ± | - | | | | + | P | ± | - | | | | |
| 12 | + | P | P | ± | - | | | | + | +P | P | ± | - | | | | + | +P | P | - | | | | |
| 13 | P | + | + | P | - | | | | + | + | + | +P | ± | - | | | + | +P | P | - | | | | |
| 14 | + | + | P | + | - | | | | P | + | +P | P | - | | | | + | +P | P | - | | | | |
| 15 | + | P | ± | - | | | | | + | +P | P | - | | | | | +P | P | ± | - | | | | |

*Results obtained with 10 normal dog sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

TABLE VII

RESULTS OF VARIOUS SEROLOGICAL TESTS ON GUINEA PIG SERA*

| No. serum | 37 C - 2 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Formalinized Antigen, SERUM DILUTIONS** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | ± | + | + | + | P | ± | - | | P | + | + | +P | P | P | ± | - | + | + | +P | P | ± | - | | |
| 2 | - | + | + | + | P | ± | - | | + | + | + | +P | P | ± | - | | + | + | +P | P | ± | - | | |
| 3 | P | + | + | +P | P | ± | - | | + | + | + | +P | P | P | ± | - | P | + | +P | P | ± | - | | |
| 4 | + | + | +P | P | ± | - | | | + | + | +P | +P | P | ± | - | | + | + | P | ± | - | | | |
| 5 | + | + | +P | P | - | | | | + | P | + | P | ± | - | | | + | +P | P | ± | - | | | |
| 6 | + | + | +P | P | ± | - | | | + | + | + | +P | P | ± | - | | + | + | +P | P | - | | | |
| 7 | + | + | +P | P | - | | | | + | + | +P | +P | P | ± | - | | P | +P | +P | P | - | | | |
| 8 | + | + | + | +P | P | - | | | + | + | + | +P | P | ± | - | | + | + | P | ± | - | | | |
| 9 | - | + | +P | P | ± | - | | | P | + | +P | +P | P | - | | | + | +P | +P | P | ± | - | | |
| 10 | + | + | +P | P | P | - | | | + | + | + | +P | P | ± | - | | + | + | +P | P | - | | | |
| 11 | + | + | +P | P | - | | | | + | + | +P | P | ± | - | | | + | + | P | ± | - | | | |
| 12 | - | +P | + | +P | P | - | | | P | + | +P | +P | P | ± | - | | + | +P | P | - | | | | |
| 13 | +P | + | + | P | ± | | | | + | + | + | +P | +P | P | - | | + | + | +P | P | ± | | | |
| 14 | + | + | +P | P | - | | | | + | + | + | +P | P | ± | - | | + | + | +P | P | - | | | |
| 15 | + | + | +P | P | - | | | | + | + | +P | +P | P | - | | | + | + | P | ± | - | | | |

*Results obtained with 10 normal guinea pig sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

TABLE VIII

RESULTS OF VARIOUS SEROLOGICAL TESTS ON HAMSTER SERA*

| No. serum | 37 C - 2 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Living Antigen, SERUM DILUTIONS** | | | | | | | | Room Temperature 16 - 18 hours, Formalinized Antigen, SERUM DILUTIONS** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | + | + | P | - | | | | | +P | + | P | ± | - | | | | +P | P | ± | - | | | | |
| 2 | P | + | +P | P | - | | | | +P | + | +P | P | ± | - | | | +P | + | P | ± | - | | | |
| 3 | +P | + | +P | P | - | | | | + | + | P | P | ± | - | | | + | + | P | ± | | | | |
| 4 | P | + | +P | P | - | | | | P | + | + | +P | P | - | | | ± | + | P | - | | | | |
| 5 | ± | + | +P | ± | - | | | | - | + | +P | P | - | - | | | P | + | P | - | | | | |
| 6 | ± | +P | +P | P | - | | | | +P | + | +P | P | ± | - | | | P | + | +P | ± | - | | | |
| 7 | - | +P | + | P | - | | | | P | + | + | P | - | | | | ± | + | P | - | | | | |
| 8 | ± | + | +P | P | - | | | | P | + | +P | P | ± | - | | | ± | + | P | ± | - | | | |
| 9 | +P | + | P | ± | - | | | | + | + | P | P | ± | - | | | P | + | P | ± | - | | | |
| 10 | P | + | +P | P | - | | | | +P | + | +P | P | ± | - | | | P | + | P | - | | | | |
| 11 | ± | + | P | ± | - | | | | P | + | P | P | ± | - | | | ± | + | P | ± | - | | | |
| 12 | +P | + | +P | P | - | | | | + | + | +P | P | P | - | | | + | +P | P | ± | - | | | |
| 13 | + | + | +P | +P | P | - | | | + | + | + | +P | P | ± | - | | + | + | P | ± | - | | | |
| 14 | P | + | + | P | - | | | | ± | + | + | P | P | - | | | P | + | P | ± | - | | | |
| 15 | +P | + | P | P | - | | | | +P | + | +P | P | - | | | | ± | +P | P | - | | | | |

*Results obtained with 10 normal Hamster sera (controls) were negative at all dilutions.

**Numbers express serum dilutions as the negative exponent to the log base 10.

# CHAPTER V

## DISCUSSION AND CONCLUSION

The results of this work provide further evidence to current thinking that there is a great need for standardization of the microscopic agglutination-lysis test in the diagnosis of leptospirosis. The test is widely used in various laboratories. Many variations in the time and temperature of incubation, sources of antigen and methods of antigen preparation are described by various workers. Most investigators agree that the agglutination-lysis test represents the best single serological diagnostic tool we have today. Slight variations in end point titers do occur for the same serum when tested using the 16-18 hour period at room temperature method as compared with the 2 hour period at 37 C.

These studies indicate that the use of a longer period of incubation, 16-18 hours at room temperature, more nearly approaches the true end point titer of the serum. These findings are in accord with the underlying principle that the combination of antibody with respective antigen requires adequate time to react completely even under optimal conditions. The establishment of equilibrium for combination of antigen with antibody requires at least 5 minutes at 0 C. According to Dreyer and Douglas,[14] equilibrium is not attained at room temperature even within 4 hours. The combination of the ever diminishing free residues may take several hours to reach final equilibrium. Taking this principle into consideration one might account for the

low end point titers reached when using 37 C for 2 hours.

Hartwigk and Stoebbe,[22] recently stated that the most desirable time for reading the agglutination-lysis test is after 24 hours at room temperature. During the course of this thesis study, it was observed that after 18 hours of incubation the reagents frequently became contamined with microorganisms from the environment, principally in the test in which living antigen was used. Preliminary tests, undertaken during the initial phases of this investigation, showed that 16-18 hours at room temperature gave the same end point titers as obtained with 24 hours incubation. Hence the former incubation time was used throughout the investigation.

According to Eagle[15] the rate of the agglutination reaction increases rapidly between 0-30 C. Above 30 C the increase in reaction time is much slower and may even become insignificant. This phenomenon may account for the fact that slightly higher titers were observed in the tests incubated 16-18 hours at room temperature as compared to those for 2 hours at 37 C.

The results of this study show that the living leptospiral antigen was a better indicator than the formalinized organisms for obtaining high end point titers under the conditions of the two tests. This has been the experience of many other investigators; however, Hartwigk and Stoebbe,[22] stated that there is a close correlation (78.4%) of end point titers between living and formalinized (0.5%) antigen at room temperature for 24 hours.

Many laboratories utilize formalin treated antigen without concern for sensitivity. Other laboratories have found that

preparation of reliable formalinized leptospirae suspensions is a difficult problem to solve because these antigens deteriorate in sensitivity with storage.

A salient feature of this study was that the density of antigen used was kept constant throughout all the agglutination-lysis tests. Furthermore, the leptospirae were kept at a minimum number commensurate with highest titers. This is in accord with the results of Stoenner,[41] who found that tests with antigen of low cell count gave higher titers than a similar antigen with high cell count.

The prozone phenomenon did not affect the determinations of this test conducted with live antigen incubated at room temperature for 16-18 hours. However, prozones of $1:10^{1}$ to $1:10^{3}$ were observed in the tests with living antigen incubated for the shorter period.

In view of the many different opinions as to the most suitable method for leptospirosis diagnosis, a questionnaire was prepared by the American Association of Veterinary Bacteriologists.[34] Copies of this questionnaire are being sent to bacteriologists responsible for actual performance of leptospirosis diagnosis in the various states. The questions asked were relative to the type of tests used, the interpretations and significance of the results obtained.

It is hoped that in the future, a better understanding of the problem of standardization and methods for the diagnosis of leptospirosis will be forthcoming.

# CHAPTER VI

## SUMMARY

A study was made of the effect of various factors upon the agglutination-lysis test and the agglutination test for Leptospira pomona infection. It was found that incubation at room temperature for 16-18 hours yielded higher end point titers than incubation at 37 C for 2 hours, when live antigen was used. A comparison between formalinized antigen and live antigen using the 16-18 hour method showed that higher end point titers could be obtained with live antigen.

The kinetics of antigen-antibody combination at various periods of time and temperature were discussed.

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Thesis

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Effect of various factors upon the agglutination-lysis test...for Leptospira Pomona infection.