SEROLOGICAL RESPONSES OF CATTLE TO *VIBRIO FETUS* VACCINE AS MEASURED BY THE COMPLEMENT FIXATION TEST AND TUBE AGGLUTINATION TEST

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By

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INTRODUCTION

The term "vibriosis" has been used to indicate the presence of infection with Vibrio fetus which as yet appears to occur naturally only in cattle and sheep (Plastridge et al., 1951). Vibrionic abortion was observed in Great Britain as early as 1913 by McFadyean and Stockman (1913) and was reported for the first time in the United States by Smith (1918). It has subsequently been reported in Denmark, Sweden, Germany, Japan, Australia, North Africa, Canada and several areas of the United States (Prier, 1952).

Because the incidence of reported abortions due to infection with Brucella sp., commonly referred to as brucellosis, has been far higher than that of abortions due to vibriosis, comparatively little interest has been directed toward the investigation of vibrionic abortion. As the diagnosis and prevention of brucellosis improved, more attention was directed to the occurrence and significance of vibriosis in cattle and sheep. In addition to abortions attributed to vibrionic infections, the failure of cows to conceive after repeated services during outbreaks of vibriosis in the herd has been observed. Therefore, the term "vibriosis" includes those vibrionic infections causing sterility as well as abortions.

Accurate figures are not available on the economic importance of vibrionic abortion and "nonbreeding" but it has been estimated that infertile cows as a result of the infection, have cost the American cattle industry approximately $500,000,000 annually in lost calves and reduced milk production (Osborne, 1952). In brucella free herds, although brucellosis and vibriosis may be present simultaneously, V. fetus has caused an estimated annual abortion rate of 4 to 20 percent and it has been suggested that many abortions occur so early in gestation that they remain undetected (Plastridge, 1949; Plastridge et al., 1951). Tunnecliffe and Marsh (1954) of the Montana Veterinary Research Laboratory reported that the number of abortions due to vibriosis in the intermountain states during the 1952 lambing season was conservatively estimated to be 100,000. This loss amounted to approximately $1,500,000 to $2,000,000. These estimates serve as only a partial indication of the losses attributable to vibriosis in cattle and sheep.

Attempts have been made to establish diagnostic procedure and to produce effective therapeutic agents for vibriosis. However, the difficulties encountered in the isolation and cultivation of the specific organisms in vitro have greatly hampered these efforts. Serological tests are in use as supplementary aids but the sole method of positive diagnosis is the isolation and identification

of *V. fetus* from the fetus and/or the placental membranes (Prier, 1952).

Antibiotics, streptomycin and penicillin, have been reported as effective agents in the treatment of cattle in which vibrionic abortion and nonbreeding have occurred (Haubrich, 1950; Chambers, 1950; Easterbrooks and Plastridge, 1952). The control of the disease by means of vaccination has also been attempted but with relatively little success (Plastridge *et al.*, 1951; Osborne, 1952). However, these attempts do not preclude the possible control of vibriosis by vaccination. In the development of a vaccine for such a purpose, serological tests may serve as an indication of the immunological responses elicited in the vaccinated animals by the vaccine.

The purpose of this thesis is to record the serological responses, as determined by the complement fixation test and tube agglutination test, of ten heifers to inoculation with *V. fetus* vaccine.

REVIEW OF LITERATURE

Apparently, vibrio-shaped organisms were first reported found in aborted ovine fetuses by McFadyean and Stockman (1913) in Great Britain. Vibrionic bovine abortion was reported by Smith (1918) in the United States and since that time, has been reported from many other regions of the world.(Prier, 1952).

However, after the initial discoveries, little attention was devoted to vibrionic abortions during the following decade. By virtue of the larger economic losses inflicted by brucellosis, efforts were largely directed toward the development of effective controls of this disease. It has been noted that, while brucellosis remains the most important single cause of abortions and nonbreeding in the cattle population as a whole despite the reduced incidence, the relative importance of vibriosis tends to increase as the incidence of brucellosis decreases (Plastridge et al., 1951).

With the increased recognition of vibrionic abortions, it was also noted that impaired breeding efficiency by the interference with conception occurred in herds of cattle where active outbreaks of vibrionic abortions were in progress (Plastridge et al., 1947; Stegenga and Terpstra, 1950; Haubrich, 1950). This relation of breeding difficulties

to vibrionic infection has not been observed to occur in sheep (Tunnecliffe and Marsh, 1954). It has been observed that the organism is also one of the causes of retained placentas in cattle (Moore, 1949, 1950). Ward (1948) reported finding the organism in minor skin infections of man but its presence as a primary etiological agent of human disease has not been demonstrated. Vibrionic abortions have been induced in previously non-infected gravid guinea pigs by various laboratory methods as well as by contact with experimentally infected females and by transmission from infected males during coitus (Ristic et al., 1953, 1954). Gravid guinea pigs are used for testing the pathogenicity of cultures and as a medium for maintaining pathogenicity (Osborne, 1952; Tunnecliffe and Marsh, 1954). However, sheep and cattle appear to be the only natural hosts of V. fetus (Prier, 1952).

The source of infection is not definitely known but cattle may become infected through the introduction of new members into the herd, by a herd sire acting as a biological or mechanical agent of transmission, or by other means yet to be determined. Pregnant ewes have been experimentally infected with V. fetus by ingestion and by vaginal, intravenous and intraperitoneal routes of inoculations (Tunnecliffe and Marsh, 1954). The same workers obtained evidence which discredits ewes as carriers, or rams as agents of

transmission. It is generally accepted that viable organisms are eliminated by infected animals before, during and after abortion. The hay, soil and manure may thus be contaminated and it has been shown that V. fetus may remain viable in such materials for periods up to ten days (Lindenstruth and Ward), 1948).

Following the introduction of infection into a herd of cattle, all sexually mature females not previously exposed appear to be equally susceptible and the greatest losses occur during the first twelve months of the disease (Plastridge et al., 1951). In older cattle, the disease appears to be self limiting but reappears when new stock is introduced into the herd. It has been suggested that a distribution of positive blood tests among all age groups of a herd indicates a recent infection, and that a low incidence of reactors, limited primarily to animals 1 to 3 years of age, indicates that the infection has been present in the herd for a year or more (Plastridge et al., 1951).

Vibriosis in sheep appears to be self limiting also, and generally appears and disappears within a year. The relative susceptibility of previously infected ewes is as yet a controversial question. Sheep which had been previously infected were challenged by exposure to intravenous inoculations of V. fetus and it was found that such sheep were as susceptible as previously uninfected animals (Tunnecliffe

and Marsh, 1954). It was noted by these workers that the challenge inoculations were so severe that exposure was in no way comparable to that encountered under natural conditions. Wiggins (1955) attempted to determine the lasting effects of vibrionic abortion in sheep. No noticeable effect was detected the year subsequent to the infection. This was felt to be the result of two probable effects; the sustained permanent injuries to genital tracts or general health, and benefit from rest by not nursing lambs the previous year.

The agglutination test serves as an aid in establishing the presence of infection. However, the transitory nature of the positive reaction limits the use of this test despite the improved methods of antigen production (Huddleson, 1949; Plastridge et al., 1949). McFadyean and Stockman (1913) reported titers varying from 1:10 to 1:1000 by agglutination tests on blood obtained from 4 ewes inoculated with infectious material. Since that time the results of agglutination tests performed by numerous workers, indicate the percentage of positive reactors and the titers obtained varied from one group of animals to another, and from animal to animal. A rapid drop of titer between 14 to 60 days after abortion by infected animals was noted by Blakemore and Gledhill (1946). They also noted that titers were higher with homologous antigens than heterologous antigens. Levi (1950) found that a far higher titer was obtained with homologous antigen,

and approximately half of the infected ewes which reacted significantly with homologous antigen, failed to do so with a heterologous antigen. It was concluded by Marsh and Tunnecliffe (1955) that the agglutination test appeared to be of limited diagnostic value due to the variable amounts of agglutinins produced by infected animals, the transitory nature of the titer, and recognition of the occurrence, frequently within the same herd, of several serological types of V. fetus. The agglutination test does have value as a diagnostic aid, particularly when the antigen is prepared from a homologous strain, and Osborne (1952) has suggested that it be used as a basis for the diagnosis of a herd infection. On the basis of agglutination tests made on cattle with recent clinical evidence of vibriosis and cattle slaughtered after failing to conceive, Plastridge et al. (1952a) have recommended agglutination tests of both blood serum and vaginal-cervical mucosa in diagnosing infertility. There is little doubt that animals reacting to the test are actual carriers and potential sources of infection (Prier, 1952).

Vibrio fetus antigen and specific anti-serum (rabbit-origin) were reported capable of fixing complement (Batlin, 1949) but no published reports are available on the use of complement fixation tests as a means of diagnosis. Undoubtedly, many infected animals remain undetected and will

remain so as long as the isolation and identification of the organism from aborted fetuses, placental membranes or vaginal tracts remain the only accurate method of a positive diagnosis of infection with V. fetus.

The therapeutic value of some antibiotics has been investigated by a limited number of workers. Neomycin, chloromycetin and terramycin have exhibited a marked in vitro antivibrio activity while penicillin and streptomycin under the same conditions produced less effects (Prier, 1952). Easterbrooks and Plastridge (1951) found that streptomycin yielded promising results in vitro, and subsequent use of streptomycin as an intrauterine infusion for cows which had been bred three or more times without conceiving, indicated the value of such treatment. This confirmed work conducted by Haubrich (1950) in which streptomycin intrauterine infusions produced significant results in combating sterility associated with vibriosis. Penicillin was used effectively as intrauterine infusions by Chambers (1950) in five nonbreeding cows. As a control measure, it has been suggested that artificial insemination in which antibiotics, streptomycin and/or penicillin, are added to the semen, be employed routinely (Plastridge et al, 1951; McEntee et al., 1954).

Vaccination is a potential control measure which offers promise but is as yet little explored. There are two reports available on the use of a V. fetus vaccine as a control

measure against vibriosis. The first investigator employed a formalinized-killed suspension of V. fetus cells with a density corresponding to a McFarland nephelometer reading of 5 (Plastridge et al., 1951). Serologically negative cows heifers of breeding age were given three subcutaneous injections of 5 ml each of the vaccine at five day intervals. The average number of services required was 2.5 for 63 animals following the final inoculations; the average number of services required for a control group of 47 animals was 2.3. Of the 63 inoculated animals, 4 aborted and 2 were sold because of sterility; of the 47 controls, 3 aborted and 4 were sold because of sterility. From these observations, the authors concluded that the bacterin was of no value in the control of vibriosis since there were no significant differences in the abortion rates, number of animals sold due to sterility, and the conception rate between the treated and untreated groups.

The second investigation, made by Osborne (1952), held more promise of vaccination as a potential control of vibrionic infections. An avianized vaccine was prepared by diluting cells, grown in embryonating eggs, with physiological saline to yield 76 percent transmission on a photoelectric colorimeter. Nine virgin heifers were selected for vaccination. All had been found to be free of vibriosis as indicated by eight negative agglutination tests over a nine month period,

and free of brucellosis as determined by three negative agglutination tests, prior to the initial inoculation. Three inoculations were made subcutaneously in the lateral cervical region at seven-day intervals. The amount of inoculum varied: 1 or 2 ml at the first inoculation, 3 or 5 ml at the second inoculation, and 5 ml for all animals at the final inoculation. Ten heifers, also found to be vibriosis and brucellosis free, served as controls. On the 21st day following the final inoculation, the nineteen heifers were challenged by intrauterine (or intracervical) infusion. The challenge dose was 1 ml of a freshly isolated strain of V. fetus which was standardized to 76 percent transmission on the photoelectric colorimeter.

After the challenge dose, all heifers were observed daily, and bred by artificial insemination on all observed estrous periods during the following six months. Sixty-six percent of the vaccinated heifers conceived by the end of the six months period as compared with thirty percent of the unvaccinated group. Agglutination tests indicated that all vaccinated heifers became reactors by two weeks after the initial inoculation, and maximum titers were recorded twenty-one days after the final inoculation. It was concluded by Osborne that, although the titer proved highly transitory and not as high in all instances as should be

elicited by a potent vaccine, the increased conception rate of the vaccinated heifers appeared to justify further investigation of a vaccine to control vibrionic infections in cattle and sheep.

MATERIALS AND METHODS

Preparation of Stock Antigen

The antigen employed for the inoculation of the test animals and for use in the serological tests was provided by Dr. J. J. Stockton of the Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan. The strain of *V. fetus* used was designated as Strain 1236. It was isolated from a swab from the cervical mucosa of heifer No. 1236 from the Experimental Dairy Herd at the Larro Research Farm, Detroit, Michigan, in May, 1950.

Until ready for use in the preparation of antigen, the culture was lyophilized following the method described by Stockton and Newman (1950). The strain was grown in thiol medium (Difco), and the upper layer containing the growth was removed from five tubes of four-day-old cultures and suspended in sterile skim milk. After thorough mixing, 0.25 ml of this suspension was dispensed to vials made from Pyrex tubing (7 mm outside diameter). The vials were placed in a dry-ice-alcohol bath (-70 C) and after freezing, the contents were dried for 48 hours as a pressure of approximately 100 μ. After drying, the cultures were sealed *in vacuo* and stored at 4 C.

Seed cultures for the preparation of stock antigen were made by reconstituting lyophilized specimens in 10 ml quantities of sterile Difco Experimental Broth #73 or #75. These were incubated 48 hours at approximately 35 C and under approximately 10 percent CO_2 tension. Organisms were then subcultured in sterile broth. Following incubation, microscopic examinations were made employing a phase contrast microscope. These specimens were examined for purity and motility. Only pure, actively motile cultures were used for inoculation of stock antigen medium.

Twenty ml of seed culture was placed into 1 liter of sterile Difco Experimental Broth #73 or #75 contained in a 3 liter compressed Erlenmeyer flask. These were incubated in the same manner as the seed cultures. Following incubation, the organisms were concentrated by centrifugation employing an IEC Model PR-1 refrigerated centrifuge. Specimens were subjected to a relative centrifugal force of approximately 3000 x G for 30 minutes. The sedimented cells were washed thrice with 0.3 percent formalin neutral 0.85 percent NaCl solution. The washed cells, which constituted the stock antigen, were suspended in a suitable volume of 0.3 percent formalin neutral 0.85 percent NaCl solution and stored at 4 C.

This stock antigen was used for preparation of the vaccine, and also for the tube agglutination and complement fixation tests.

Preparation of Vaccine and Inoculation of Test Animals

For preparation of the vaccine, cell counts were made of the stock antigen using a Petroff Hauser Bacterial Counting Chamber and the phase contrast microscope. As determined from this count, the suspension was concentrated or diluted to yield a cell count of approximately 50 x 10^9 cells per ml.

Ten heifers, ranging in age from 8 1/2 to 16 3/4 months, were selected from the dairy herd at the Larro Research Farm, Detroit, Michigan. These animals were brucella-negative as determined by the plate agglutination test. Eight of the heifers had been vaccinated with Brucella M vaccine. The vibrio vaccine was administered subcutaneously in either the right or left cervical region. The vaccine was administered to the heifers at the intervals and in the quantities indicated in Table 1.

Blood samples were collected from all animals at the time of the initial inoculation and periodically thereafter for approximately nine months. The samples were obtained to determine the serological responses of the vaccinated heifers by the complement fixation and tube agglutination tests. The samples were taken on the dates indicated in the tabulated results of the serological tests (Table 8).

The serum of each blood sample collected was separated from the clot by centrifugation. The sera were decanted into

TABLE 1

INOCULATION DATA

Animal Number	Date of Birth	Age at 1st Inoc. (mo.)	Date Bruc. M. Vacc.	Date V. fet. Vacc. (amounts in ml)		
				Oct. 8 1954	Nov 5 1954	Dec 21 1954
1655	5/15/53	16 3/4	12/29/53	1.0	2.0	2.0
1656	5/21/53	16 1/2	12/29/53	1.0	2.0	2.0
1663	6/26/53	15 1/2	12/29/53	1.0	2.0	2.0
1666	7/22/53	14 1/2	12/29/53	1.0	2.0	2.0
1668	7/31/53	14 1/4	12/30/53	1.0	2.0	2.0
1682	10/5/53	12	7/16/54	1.0	2.0	2.0
1685	10/15/53	12 1/4	7/16/54	1.0	2.0	2.0
1688	11/3/53	11	7/16/54	1.0	2.0	2.0
1702	1/3/54	9 1/4		1.0	2.0	2.0
1707	1/24/54	8 1/2		1.0	2.0	2.0

individual sterile tubes, plugged and centrifuged to insure complete separation from red blood cells. After the second centrifugation, the sera were transferred to individual sterile milk vials, stoppered and stored at -40 C.

Procedure for the Tube Agglutination Test

Density determinations of the antigens used in the serological tests were made with a Cenco Industrial Photelometer using a red filter (650 mμ). The instrument was adjusted to read 100 percent transmission through a tube containing 5 ml of 0.3 percent formalinized saline. Stock antigen was added in 0.01 ml increments to 5 ml formalinized saline in a tube corresponding in light transmission to the tube used to standardize the instrument. After each addition, the suspension was thoroughly agitated and the density determined photometrically. The process was repeated until the transmission through the saline suspension was reduced to 76 percent. The dilution of antigen thus determined was used to test each serum sample as indicated in Table 2. All quantities are given in ml, and a separate tube of 1 ml of antigen only served as control.

Serum-antigen mixtures were incubated 48 hours at 37 C and were read over a concave mirror using artificial, reflected light. The serum titer was recorded as the highest dilution of serum in which there was perceptible agglutination which persisted during slight manual agitation.

TABLE 2

TUBE AGGLUTINATION TEST

Tube	1	2	3	4	5	6	7	Control
Antigen	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Serum	.08	0	0	0	0	0	0	0
Transfer to right	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0
Final Dilution*	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	-

*Serial dilutions continued for higher titers.

Procedure for the Complement Fixation Test

Preparation of the Diluent

Eighty-five hundredths percent buffered saline solution was used in all complement fixation tests and for the dilution and titration of all reagents used in this test. The concentration of the sodium chloride was maintained above 0.80 percent and below 0.90 percent to avoid laking of red blood cells employed in the indicator system.

A stock solution of buffered saline (pH 7.3) was prepared according to the following formula:

NaCl	170.00 gm
KH_2PO_4	2.78 gm
Na_2HPO_4	11.30 gm
H_2O q.s.ad.	1000.00 ml

For use, dilute 1:20 in distilled water (Kent et al., 1946).

Preparation of Sheep Red Blood Cell Suspension

Modified Alsever's solution was used to preserve sheep red blood cells (Kent et al., 1946) to be used in the complement fixation test. This solution contained the following:

Dextrose	2.05 gm
Na citrate	0.80 gm
NaCl	0.42 gm
Citric acid	0.055 gm
H_2O q.s.ad.	100 ml

After thorough mixing, the solution was dispensed in 25 ml quantities into 100 ml Florence flasks. Sterilization was accomplished by autoclaving at 121 C for 10 min. The solution had a final pH of 6.1 and was stored at 4 C.

Whole blood was drawn aseptically from sheep and added immediately to an equal volume of modified Alsever's solution. The contents were briskly rotated 15 minutes to insure thorough mixing and to avoid clotting. The Alsever's-whole-blood mixture (hereafter refered to as Alsever-WBM) was stored at 4 C and allowed to stand 3 to 4 days to permit the susceptibility of the cells to lysis by complement and hemolysin to become constant (Kabat and Mayer, 1948). The level of susceptibility remained unchanged 8 to 12 weeks.

A two percent suspension of red blood cells was used in the complement fixation tests and in the titrations of the reagents. When the total amount of the two percent suspension required for one day's use was estimated, the amount of Alsever-WBM needed to yield that quantity was calculated to insure sufficient suspension and avoid waste. An approximate relation was found between the desired volume of two percent suspension of red blood cells and the required amount of Alsever-WBM. If the final volume of the two percent suspension was referred to as x, then x times 0.02 indicated the amount of packed cells to be diluted 1:50. This amount of packed cells was present in that quantity of Alsever-WBM

found by dividing the amount of packed cells required by the percentage of red blood cells present in the Alsever-WBM. Since the whole blood was added to an equal volume of Alsever's solution, the concentration of the red cells was reduced approximately from 45 percent to 22.5 percent. Therefore, (x) (.02) / (.225) equalled the amount of Alsever-WBM required. Since (.02) / (.225) was constant and equalled 0.089, the amount of Alsever-WBM required for a day's use was calculated simply as 0.089 times the amount of two percent suspension of the red blood cells needed. For example, if 80 ml of two percent suspension were wanted, 0.089 times 80 ml indicated that 7.12 ml of Alsever-WBM would yield the needed quantity.

The quantity of Alsever-WBM thus determined was placed in 15 ml graduated conical centrifuge tubes. The red blood cells contained in the centrifuge tubes were washed thrice by the addition of buffered saline, centrifugation and removal of the supernatant. Each centrifugation lasted for a period of ten minutes in an International Clinical centrifuge, Model CI. In the event the supernatant was red, the cells were discarded as too fragile.

The packed volume of red blood cells obtained in the graduated centrifuge tubes was diluted 1:50 with buffered saline after the removal of the supernatant, to yield a two percent suspension. This suspension was prepared daily

to avoid any increase in the susceptibility of the red cells to hemolysis.

Titration of Hemolysin

The minimal hemolytic dose, hereafter referred to as M.H.D., of hemolysin is the smallest quantity of a given dilution of hemolysin which will yield complete hemolysis of a given amount of red blood cells in the presence of an excess of complement at a given temperature in a specified length of time. This is also referred to as one unit of hemolysin and is used interchangeably with M.H.D.

To determine the M.H.D. of hemolysin or complement, one must be titered against the other. The titer of a particular lot of hemolysin is relatively stable and once titrated, need not be determined daily. This is the standard against which the complement, much less stable than hemolysin, can be titrated daily immediately prior to use.

Commercially prepared hemolysin obtained from Sharpe and Dohme (Antisheep Hemolysin Glycerinized Rabbit) was used for the complement fixation tests and titration of the other reagents. To determine the M.H.D. of hemolysin, the commercial preparation was diluted 1:100 and titrated against complement from a pool of fresh guinea pig sera which was diluted 1:10. The titration was accomplished as shown in Table 3. All quantities are given in ml.

TABLE 3

TITRATION OF HEMOLYSIN

Tube	1	2	3	4	5	6	7	8
Saline	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Comp.	0.1	0.1	0.1	0.1	0.1	0.1	0	0.1
Incubated at 37 C for 30 min. in water bath								
Hemol.	.01	.02	.03	.05	.08	0.1	0.1	0
RBC 2 percent	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubated at 37 C for 30 min. in water bath								

The complement fixation tests relies on an indicator system comprised of hemolysin and sheep red blood cells. Under the proper conditions, if there is no interference by other factors, complement lyses red blood cells which have been sensitized by hemolysin. Some specific antigen-antibody combinations are capable of 'fixing' complement so the complement is no longer free to lyse the sensitized red blood cells. Therefore, in the titration of hemolysin (Table 3), if hemolysis occurs in either tubes 7 or 8, hemolysin or complement respectively, is capable of lysing

the red blood cells. Any tests employing such hemolysin or complement are of no value.

In reference to Table 3, the smallest amount of hemolysin, contained in the 1:100 dilution, which caused complete lysis of the RBC, was designated as the M.H.D.

For the titration of other reagents and for complement fixation tests, commercially prepared hemolysin was diluted with buffered saline to yield three M.H.D. per 0.1 ml.

Preparation and Titration of Complement

Complement was obtained by aseptically drawing five to eight ml of blood from each of five to eight unanaesthetized guinea pigs by cardiac puncture. After the blood was drawn, it was placed in sterile test tubes, the tubes plugged and the blood allowed to clot in a slanted position. After clotting, the tubes were placed upright in a rack and allowed to stand at room temperature for 1 hour. They were then refrigerated at 8 C for 2 hours. After reaming the clots, the tubes were centrifuged for 20 minutes. The serum was decanted into sterile tubes, the tubes plugged and again centrifuged for 20 minutes. The supernatant of all tubes in which there was not more than slight hemolysis of the red cells, was poured into sterile flasks and stoppered. Care was exercised to avoid the transfer of any remaining cells.

The sera were pooled in one flask to assure uniformity of the hemolytic activity of the complement obtained.

The pooled sera were dispensed into sterile pyrex tubes in the approximate amounts required for daily use. The tubes were hermetically sealed with a cross-fire oxygen torch, exercising caution to avoid any heat deterioration of the complement. The tubes were rotated in a dry-ice-alcohol bath and when the contents were frozen, were removed to a CO_2 chest (-40 C) for storage.

The hemolytic activity of the complement remained relatively stable throughout a three-to-four-week period when prepared and stored in the above manner. However, it is perhaps the most critical reagent employed in the complement fixation test and was therefore titrated daily. An occasional tube of complement was titrated which had undergone sufficient deterioration to necessitate its disposal. This was believed to have resulted from faulty sealing of the tube and the subsequent deleterious effect of CO_2 on the complement.

The M.H.D. of complement is the smallest quantity of a given dilution of complement which causes complete hemolysis of a given amount of red blood cells in the presence of an excess of hemolysin (3 units) at a specified temperature in a definite period of time. Immediately prior to titration of the complement to determine the M.H.D., the complement was

allowed to thaw and the tube was opened. One tenth ml was removed and diluted 1:10. The remainder was refrigerated to avoid any loss of hemolytic activity. The diluted complement was titered as indicated in Table 4. All quantities are given in ml.

TABLE 4

TITRATION OF COMPLEMENT

Tube	1	2	3	4	5	6	7
Saline	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Comp.	.01	.03	.05	.07	0.1	0.1	0
Incubated at 37 C for 30 min. in water bath							
Hemol.	0.1	0.1	0.1	0.1	0.1	0	0.1
RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubated at 37 C for 30 min. in water bath							

Tubes 6 and 7 (Table 4) should show no hemolysis as explained previously in describing the titration of hemolysin. If no hemolysis occurred in the other tubes, or if hemolysis occurred only in tube 5, the complement, from which the 0.1 ml was taken for titration, was discarded as having too little hemolytic activity. In addition, it was

found to be advisable to include 2 units (Table 5) of antigen in the daily titrations of complement. A few lots of antigen which exhibited no anticomplementary action when titrated with one pool of complement, were found to be slightly anti-complementary with another pool of complement.

The M.H.D. of complement was that quantity in the tube (Table 4) containing the smallest amount of complement in which there was complete hemolysis of the red blood cells. The remaining complement was diluted with buffered saline to yield 2 M.H.D. per 0.1 ml for the titration of antigen and complement fixation tests.

Titration of Antigen

Stock antigen was diluted with buffered saline to correspond to a light transmission equal to that of tube 3 of a McFarland Nephelometer. The adjustment was made using a Cenco photelometer with a red filter (650 mμ). It should be noted that formalinized saline which was used as a diluent for the antigen in the agglutination tests, was not a satisfactory diluent for the antigen used in the complement fixation tests. The dilution required for the different lots of antigen varied and it was believed that the formalinized saline was directly anticomplementary or acted on the antigen in such a manner that the anticomplementary activity of the antigen was increased.

An undiluted, known-positive antiserum of bovine origin was inactivated by heating in a water bath at 56 C for 30 minutes and used in the titration of the antigen. Only those lots of antigen which yielded the specified results in Table 5 were used in complement fixation tests. All quantities are given in ml.

TABLE 5

TITRATION OF ANTIGEN

Tube	1	2	3	4	5	6	7	8
Saline	1.0	1.0	1.0	.1.0	.1.0	.1.0	1.0	1.0
Antigen	.01	.03	.05	0.1	0.1	0	0	0
Comp.	0.1	0.1	0.1	0.1	0	0.1	0	0.1
Antiser.	.02	.02	.02	0	0	0.1	0.1	0
Incubated at 37 C for 30 min. in water bath								
Hemol.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
RBC 2 percent	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubated at 37 C for 30 min. in water bath								
Required Results*	H	PH	NH	H	NH	H	NH	H

* H - hemolysis PH - partial hemolysis NH - no hemolysis

The minimal fixing dose, or unit, of the antigen was contained in .05 ml (Table 5). This yielded two units per 0.1 ml of diluted antigen and this was the amount used in the complement fixation tests. Tubes 4 through 8 were controls as follows: 4-antigen not anticomplementary; 5-antigen not hemolytic; 6-antiserum not anticomplementary; 7-antiserum inactivated; and 8-hemolytic system properly adjusted.

The procedure outlined in Table 5 was repeated using a known negative serum. The same results were obtained with the exception of tubes 2 and 3. Complete hemolysis was necessary in both of these tubes to indicate the inability of negative serum-antigen mixture to fix complement.

The Complement Fixation Test

To test the individual serum samples obtained from the inoculated heifers, complement was diluted to yield 2 units per 0.1 ml after daily titration (Table 4), hemolysin was diluted to yield 3 units per 0.1 ml (Table 3), stock antigen was diluted to yield 2 units per 0.1 ml (Table 5), and a 2 percent suspension of red blood cells was prepared. It is to be noted that all reagents were refrigerated when not in use and all glassware used was meticulously clean. Serum samples were allowed to thaw, inactivated at 56 C for 30 minutes in a water bath and tested as shown in Table 6. All quantities given are in ml.

TABLE 6

THE INITIAL COMPLEMENT FIXATION TEST

Tube	1	2	3	4	5	6
Saline	1.0	1.0	1.0	1.0	1.0	1.0
Antigen	0.1	0.1	0.1	0.1	0	0
Comp.	0.1	0.1	0.1	0.1	0.1	0
Serum	.01	.02	.04	0	0.1	0.1
Incubated at 37 C for 30 min. in water bath						
Hemol.	0.1	0.1	0.1	0.1	0.1	0.1
RBD	0.5	0.5	0.5	0.5	0.5	0.5
Incubated at 37 C for 30 min. in water bath						

Tubes 4, 5, and 6 served as controls: 4-antigen not anticomplementary; 5-serum not anticomplementary; 6-serum inactivated.

The initial serum samples, and subsequent samples, were tested as shown in Table 6, until complement fixation was obtained. As the response of the heifers developed, as indicated by a positive fixation test, i.e., a lack of hemolysis in tubes 2 and 3 (Table 6), it was necessary to make serial dilutions with buffered saline of the serum

samples. Serial two-fold dilutions were made from a dilution of 1:5 through a dilution of 1:1280. Thereafter, the following dilutions were made: 1:1920, 1:2560, 1:3200, 1:3840, 1:4480, 1:5120, 1:5760, and 1:6400. The range of dilution was anticipated on the basis of the results obtained from the previous samples and were tested as shown in Table 7, if, for example, a previous titer had been 1:40. Quantities are given in ml.

TABLE 7

THE COMPLEMENT FIXATION TEST OF DILUTED SERUM

Tube	1	2	3	4	5	6	7
Saline	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Antigen	0.1	0.1	0.1	0.1	0.1	0	0
Comp.	0.1	0.1	0.1	0.1	0.1	0.1	0
.02 ml Ser. dil.	1:20	1:40	1:80	1:160	1:320	1:1	1:1
Incubated at 37 C for 30 min. in water bath							
Hemo.	0.1	0.1	0.1	0.1	0.1	0.1	0.1
RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubated at 37 C for 30 min. in water bath							

The highest dilution of the serum in which there was complete fixation of the complement employing 0.02 ml of that dilution, was recorded as the titer of the serum. If the serum proved to be improperly inactivated as indicated by hemolysis in tube 7, or if the serum appeared anti-complementary, as indicated by no hemolysis in tube 6, the results were not recorded.

RESULTS AND DISCUSSION

The complement fixation and tube agglutination titers obtained from the test animals are recorded in Table 8. The complement fixation (hereafter referred to as CF) titer was the highest dilution of serum which completely fixed complement (Tables 6 and 7). The tube agglutination titer was the highest dilution of serum which produced a perceptible agglutination (Table 2).

Average CF and agglutination titers were determined for all serum samples obtained on the same day. These averages are recorded in Table 8, and are graphically illustrated in Figure I.

In general, both tests indicated a definite response by all test animals following the initial injection of *V. fetus* vaccine, the highest individual agglutination titer being 1:800, and the highest individual CF titer being 1:640 on the same date. While the CF titers continued to increase slightly, the agglutination titers exhibited a gradual decline until the time of the second inoculation. Following the second inoculation, administered four weeks after the initial injection, a rapid increase of titer was found by both tests at the end of the subsequent week; the highest individual agglutination titer was

TABLE 8

RESULTANT TITERS AND AVERAGES OF TUBE AGGLUTINATION (AGG) AND COMPLEMENT FIXATION (CF) TESTS

Date	Test	Number of Heifer										Average
		1655	1656	1663	1666	1668	1682	1685	1688	1702	1707	
10/8	AGG	0	0	0	0	200	100	0	25	0	0	32.5
54	CF	0	0	0	0	0	0	0	0	0	0	0
10/15	AGG	400	200	400	200	800	400	800	200	400	400	420
54	CF	10	5	5	1	10	10	10	1	20	40	11.2
10/22	AGG	800	800	800	800	400	800	800	200	400	400	620
54	CF	160	40	80	160	80	160	160	80	320	640	188
10/29	AGG	400	400	400	800	400	800	400	200	400	200	440
54	CF	320	80	160	320	160	640	320	160	320	1280	376
11/5	AGG	200	400	200	400	400	400	200	200	400	200	300
54	CF	320	160	640	1280	320	1280	640	160	320	1280	640
11/12	AGG	3200	1600	800	1600	800	800	800	800	1600	3200	1520
54	CF	1920	1280	1920	2560	1280	3200	1920	1920	2560	3840	2240
11/19	AGG	3200	800	1600	800	1600	1600	800	400	800	800	1240
54	CF	2560	1280	1280	1920	1280	3200	1920	1280	2560	3840	2112
11/26	AGG	1600	*	400	800	800	400	200	*	400	800	675
54	CF	2560	*	1280	1920	640	2560	1920	*	2560	3200	2080
12/3	AGG	800	400	400	400	200	400	400	400	200	800	440
54	CF	2560	640	1280	1280	640	2560	1920	640	1920	3200	1664
12/10	AGG	800	200	400	400	200	800	200	200	50	400	365
54	CF	2560	640	1280	640	640	2560	1280	640	1920	2560	1472
12/21	AGG	400	400	400	400	400	800	400	400	100	400	410
54	CF	3200	640	640	1280	640	1920	1280	640	1920	2560	1472
12/31	AGG	3200	3200	1600	3200	1600	800	800	800	3200	6400	2480
54	CF	5120	3840	3200	3840	3840	5760	3200	2560	5120	3840	4012
1/14	AGG	200	800	400	1600	1600	400	400	800	1600	1600	940
55	CF	3200	1920	1920	2560	1280	3840	1280	640	3200	1280	2112
1/28	AGG	200	400	400	1600	800	200	400	1600	1600	800	820
55	CF	640	160	320	640	160	1280	160	80	1280	320	504
2/10	AGG	100	400	400	400	200	200	200	400	400	800	350
55	CF	160	80	80	160	80	320	40	40	160	80	120
2/28	AGG	200	200	200	200	400	100	100	200	400	400	240
55	CF	80	20	40	80	40	80	20	40	40	20	46
3/11	AGG	100	*	200	100	200	100	100	200	200	100	144
55	CF	40	*	40	20	10	20	5	10	20	5	15
3/25	AGG	100	*	200	200	200	100	200	200	200	200	177
55	CF	10	*	5	1	1	5	1	1	1	5	3
4/22	AGG	100	*	200	*	200	100	100	100	100	200	125
55	CF	5	*	1	*	**	1	1	**	1	5	2
6/24	AGG	25	*	*	50	100	25	100	50	50	*	71
55	CF	+	*	*	+	+	+	+	+	+	+	+

*Test not performed **Sample anti-complementary
+Complement fixed only with undiluted serum

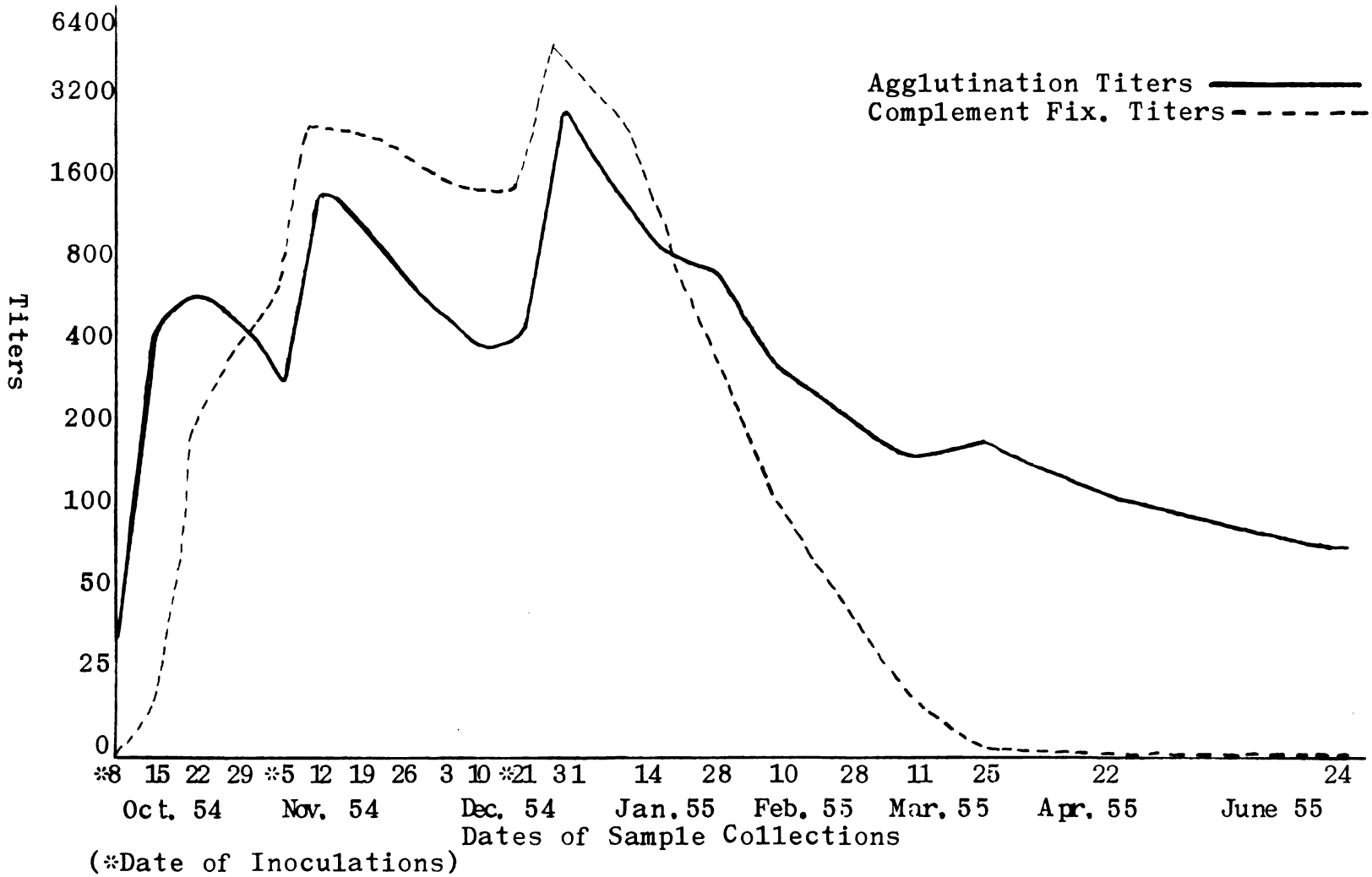

Figure I. Average Titers

1:3200, the highest individual CF titer was 1:3840. During the next five weeks, a slight decline in CF titers occurred and the agglutination titers fell to approximately the same level as that found prior to the second inoculation.

The third and final inoculation, given six weeks after the second injection, was again followed by a rapid increase in titers; the highest individual agglutination titer was 1:6400, the highest individual CF titer was 1:5760. This peak was reached ten days after the final inoculation after which the titers again decreased. It should be noted that while a peak occurred seven days after the second inoculation and the highest ten days after the third inoculation, the difference in days was a result of collection dates of samples rather than a difference in days established by titers. Had titers been determined at the same interval after the second and third inoculation, the height of the peaks or the relation of the two peaks to each other would have not, in all probability, differed significantly.

Initially, CF antibodies developed more slowly than agglutinins but higher average peaks were reached by CF antibodies than by agglutinins after the second and third inoculations. The highest average CF titer, observed ten days after the third inoculation, was 1:4012; the highest average agglutination titer, also observed ten days after

the third inoculation, was 1:2480. The individual titers at that time varied from 1:2560 to 1:5760 by the CF test, and from 1:800 to 1:6400 by the agglutination test.

The transitory nature of titers following the administration of a vaccine of V. fetus has been noted by Osborne (1952) as well as natural infections (Plastridge et al., 1951; Blakemore and Gledhill, 1946). Approximately 17 weeks after the third inoculation and approximately seven months after the initial injection, the average agglutination titer had decreased to 1:120 and complement was fixed by a serum dilution of 1:2. Approximately nine months after the initial inoculation and 26 weeks after the final, the average agglutination titer was 1:71 and complement was fixed only with undiluted serum.

Three heifers, 1668, 1682, and 1688, yielded serum agglutination titers of 1:200, 1:100 and 1:25 respectively immediately prior to the original inoculation. These animals did not differ significantly in their responses to V. fetus vaccine although following the second and third inoculations when the highest responses were obtained from all test animals, the agglutination titers of the three animals did not reach the average titers on these peak dates. The presence of agglutinins at the time of the initial inoculation might be attributed to previous natural experience with V. fetus. There have been reports of cross

agglutination reactions with some strains of V. fetus and Brucella abortus anti serum of rabbit origin (Kiggins et al., 1955). Agglutinin-adsorption testing indicated the antigens to be related but not identical (Kiggins et al., 1955). However, all test animals had been found to be brucella negative to the plate agglutination tests prior to inoculations with V. fetus vaccine. In addition, complement fixation tests in which B. abortus antigen was substituted for V. fetus antigen (Table 6 and/or 7) at the time of the initial inoculations and four weeks thereafter, indicated no fixation.

No relation between the observations made following the V. fetus vaccine as outlined in this thesis and that of the bacterin administered by Plastridge et al., (1951) can be made since no serological testing was reported by the latter. Osborne (1952) however, recorded serum agglutination titers obtained from nine heifers following the administration of an avianized V. fetus vaccine. As previously mentioned, three inoculations were made which varied from 2 to 5 ml per inoculation at seven day intervals. Maximum titers of 1:640 for two heifers were recorded on the twenty-first day after the final inoculation, incomplete agglutination at 1:640 for five heifers, and no agglutination for two heifers at 1:640.

On the basis of the titers obtained, the formalinized vaccine as administered (Table 1) appears to have elicited more agglutinins than the avianized vaccine. Osborne did not report titers at intervals prior to twenty one days after the final inoculation at which time maximum titers were obtained. Had tests been performed seven or ten days following the final inoculation, a higher production of agglutinins might have been indicated. However, twenty four days after the final inoculation of the formalinized vaccine, the average titer was 1:940, 1:200 for one heifer, 1:400 for three heifers, 1:800 for two heifers and 1:1600 for four heifers. These would seem to indicate a greater response by the test animals to the formalinized vaccine as administered and as the responses were tested.

The significant reaction of all test animals by the development of agglutinins and complement fixing antibodies might indicate the value of further investigation of serological responses of animals. It is recognized that results of serological tests of vaccinated animals do not necessarily measure the degree of immunity produced by a vaccine. However, a relation may be established and the serological responses elicited by the vaccine implies the need of further investigation of vaccination as a future control of vibriosis.

SUMMARY

Ten heifers were selected at the Larro Research Farm, Detroit, Michigan, to receive a Vibrio fetus vaccine. The test animals, ranging in age from eight and one-half to sixteen and three-fourths months, were negative to the brucella plate agglutination test.

The formalinized suspension of V. fetus (50×10^9 cells per ml) was administered subcutaneously into the right or left cervical region of the test animals. Three injections were made, the primary injection of 1.0 ml was followed by two injections of 2.0 ml each at four and ten week intervals.

Blood samples were collected from the test animals periodically for approximately nine months after the initial inoculation. Serum titers for antibodies vs. V. fetus were determined by means of complement fixation and tube agglutination tests. A significant acceleration of antibody production was indicated by a rapid rise in serum titers following vaccine inoculations.

The serological responses of the test animals to the vaccine suggest the merit of attempting to determine any possible standardization of serological responses as an

aid, perhaps, in detecting the presence of infection, and that further investigation of vaccination as a potential measure against vibriosis may be highly worthwhile.

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SEROLOGICAL RESPONSES OF CATTLE TO *VIBRIO FETUS* VACCINE AS MEASURED BY THE COMPLEMENT FIXATION TEST AND TUBE AGGLUTINATION TEST

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AN ABSTRACT

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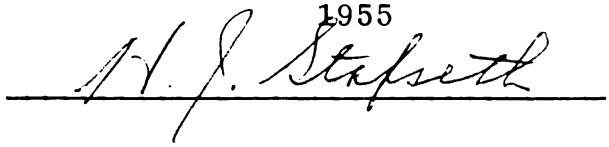
Approved by 

Infection with Vibrio fetus, clinically manifested in sexually mature female cattle as infertility or abortion of fetuses, has been estimated to have cost the American cattle industry approximately 500 billion dollars annually. Ovine vibrionic abortion, during one lambing season in the mid-western intermountain states, inflicted a loss of approximately two billion dollars in lost lambs. The greater incidence of brucellosis, now declining as a result of reliable diagnostic procedures and effective immunization, has apparently delayed extensive investigation necessary to determine adequate means for the prevention of vibriosis and a rapid and accurate diagnosis of the infection. Determining the presence of the infection by means of serological tests has thus far been delayed or hampered by the difficulty encountered originally in cultivation, the existence of heterologous strains of the organism, and lack of standardization of antigen production. As yet, the only reliable diagnosis is the isolation of V. fetus from aborted fetuses, placental membranes and/or vaginal mucosa.

To obtain an indication of the possible value of a vaccine, as determined by serological responses, complement fixation and tube agglutination, tests were performed on the sera of ten heifers inoculated with V. fetus vaccine. Three inoculations per animal were made; 1 ml was given in the

primary injection, and 2 ml in each of two subsequent injections at four and ten week intervals.

The stock antigen, from which the vaccine was prepared, was produced from cultures maintained in lyophile. The organisms had originally been isolated from the cervical mucosa of a Vibrio-infected animal. The lyophilized cultures were reconstituted and grown in Difco Experimental Broth #73 and/or #75 under approximately 10 percent CO_2 tension. Subcultures of actively motile organisms were harvested and suspended in formalinized saline. This suspension constituted the stock antigen from which the vaccine and the antigen for complement fixation and agglutination tests were prepared. The vaccine was standardized to contain approximately 50×10^9 cells per ml. The titer of the serum obtained from inoculated test animals, as determined by the tube agglutination test, was the highest dilution with which there was perceptible agglutination after 48 hour incubation. The titer determined by complement fixation, was the highest dilution of serum with which .02 ml of the serum dilution completely fixed 2 units of complement.

Significant responses elicited from the test animals by the V. fetus vaccine were clearly indicated by titers obtained by both tests employed. It was not assumed that these responses measured the degree of immunity conferred by

the vaccine. From the observed production of agglutinins and complement fixing antibodies, it seems that significant serological responses might be expected from the administration of a suitable vaccine. The protection, if any, afforded by this serological response, has yet to be evaluated.

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