VIBRIO FETUS INFECTION IN THE FETAL AND NEONATAL CALF

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PREFACE

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Bovine vibriosis is a disease resulting in infertility and abortion. It is caused by the bacterium, Vibrio fetus, which is gram-negative and varies from short comma-shaped rods to long spirals.

Investigations demonstrate that vibriosis is the most important single cause of bovine infertility in North America and Europe. As cited by Frank (1956), it was estimated in "Losses in Agriculture" of 1954 that bovine vibriosis was responsible for 40% of bovine infertility and cost the American cattle producer more than $137,000,000 annually. While some loss is represented by abortion and decreased milk production it is mainly related to decreased reproductivity.

Frank (1958) and Horlein (1963) pointed out that the most extensive vibriosis infections in the United States are either found in Western range herds of beef cattle or in dairy herds using natural service. "Repeat breeding" in females and a low breeding ratio in bulls are the most constant clinical signs.

The essential features of bovine vibriosis can be briefly stated. The disease caused by Vibrio fetus leads to temporary infertility by causing early death of the embryo within the first trimester of pregnancy, rarely after the second half of gestation. Hence, the main indications of infection are those of poor conception, a persisting number of unbred heifers or cows in a herd, and the detection of

numerous non-pregnant female animals if pregnancy examinations are made.

The disease is usually spread by chronically infected bulls. These bulls often remain as carriers indefinitely and treatment is difficult and hence not advisable. All females appear to be susceptible to infection. However, the infection is often self-limiting in the female and infected heifers apparently recover in 2 to 9 months. Attention is usually centered on the breeding problems in the replacement heifers of an infected herd. The control of vibriosis in range herds is difficult. Good herd management and immunization procedures have resulted in larger calf crops and are of value in reducing economic loss. However, immunization does not eliminate the infection from a herd so there is still a need for improved diagnostic methods even where control of the disease by immunization is practiced.

The plan of this work was to detect the accumulation of Vibrio fetus antigens in different tissues of infected fetal and neonatal calves. It was hoped that a comparison of the concentration of these antigens in various tissues by an immunofluorescent method at different fetal and neonatal ages would give an indication of the pathogenesis of the disease. These results could be compared with the bacteriologic, histopathologic and serologic findings which were evaluated by other investigators. It would then be possible to determine the suitability of the fluorescent antibody test for Vibrio fetus in fetal tissues.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Vibrio fetus is the etiologic agent of genital vibriosis of cattle. The infection has been considered to be limited to the uterus and appendages in the female. A chronic carrier state results after the acute stage of the infection in only a small number of female animals. The male is usually infected by exposure at breeding and the infection usually persists indefinitely.

History of Vibrio fetus

MacFadyean and Stockman of England (1913) first recognized Vibrio fetus infection as an "epizootic abortion" in ewes. A comprehensive literature review was made by Morrison (1960).

Smith (1918, 1919, 1920) described cases of vibrionic abortion in cows and pointed out that only the Vibrio fetus organisms could be isolated. He examined aborted fetuses and found Vibrio fetus in 23.8% of the cases.

Lerche as cited by Simon (1957), examined 1,565 aborted bovine fetuses and isolated Vibrio fetus from 22 aborted fetuses. Others have reported Vibrio fetus infection in cattle (Horlein and Kramer, 1963; Hughes, 1963 and Kendrick, 1963).

Bacteriological Characteristics

Price et al. (1955) and Brayner and Frank (1955) have shown that Vibrio fetus cultures are indol-negative, do not produce hydrogen sulfide and are catalase positive. These examinations are used to differentiate Vibrio fetus from the nonpathogenic vibrios isolated normally from the bovine genital tract.

Plastridge and associates developed improved cultural media and serological methods for studying Vibrio fetus and this, in part, has hastened and simplified the research on this bacterium and the disease it causes in animals and man (Plastridge et al., 1955, 1943, 1949, 1951).

Pathogenesis of Vibrio fetus

It has been shown by clinical syndromes and bacteriologic identification methods that Vibrio fetus is pathogenic for man (Eden, 1962; Hood and Todd, 1960; King and Bransky, 1961; Mondell and Ellison, 1963; Wheeler and Borcher, 1961). This adds a further interest to the disease from the standpoint of pathogens transmitted from animals to man.

The pathogenicity of Vibrio fetus for laboratory animals such as rabbit, mice and pregnant guinea pigs has been studied. Guinea pigs have been used to study the gross pathology of experimental Vibrio fetus infection (Ristic and Morse, 1953). Webster and Thorp (1953) used 12 day old embryonated hen's eggs to propagate the Vibrio fetus organism as well as to confirm its pathogenicity for this specific host. The inflammatory response of the bovine uterus to Vibrio fetus infection has been reported (Dozsa and Mitchell, 1962; Frank and Bryner, 1953; Peterson and Newsom, 1964; Borner and Oberst, 1950; Frank

and O'Berry, 1961; Estes, Bryner and O'Berry, 1965). The pathogenic changes observed in the tissues of the genital tract of infected cows has been regarded as an inflammatory process involving the endometrium which results in abortion in the pregnant cow as well as delayed conception in the bovine and ovine. It was shown by Osborne (1964) and Estes (1965) that extracts of Vibrio fetus induced a hypersensitivity reaction in the bovine. Since then the disease caused by Vibrio fetus organism has been studied from the standpoint of an interaction between antigen and host antibody which results in tissue destruction capable of inducing abortion or delayed conception.

Methods of Diagnosis

The possibility of using an agglutination test for the diagnosis of vibriosis has been studied. Inconclusive results have been obtained. Blackemore (1946) and later Levi (1950) used strains of Vibrio fetus of ovine origin for the detection of vibrionic abortion in sheep. They concluded that the test had little diagnostic value. Roberts et al. (1950) questioned the reliability of the agglutination test for vibriosis when used for diagnosis of individual cases. Plastridge (1941) considered a serum agglutination titer of 1:200 or higher as positive indication of vibriosis in cattle, 1:100 as suspicious and 1:50 as insignificant. Plastridge, as cited by Roberts (1950) indicated the value of serum titers in evaluating experimental infections when serum values were determined prior to and after infection.

The vaginal mucous of cows has also been used with a high degree of success in the diagnosis of Vibrio fetus infection in heifers. The procedure is based on the presence of vibrio agglutinins in the vaginal

secretions of clinically infected cows. These agglutinins can best be measured 30-60 days after service.

Cultural and direct microscopic examinations of fetal fluids are of great value in making a positive diagnosis of vibrionic abortion in cattle (Moore, 1950). The best material for examination, in order of preference for bacteriologic evaluations are abomasum, liver, spleen, lung and brain. Bacteriological cultures of Vibrio fetus from other sources as preputial washings or semen from chronically infected bulls has been difficult or impossible due to the overgrowth of contaminating bacteria. Osburn (1967) described histologic lesions in aborted fetuses which are helpful in diagnosing the disease.

The demonstration of antigenic substances in tissues with fluorescent antibodies was reported by Coon et al. (1941). This method made available a rapid tool for the identification of bacterial antigens (Cherry et al., 1960; Coons and Kaplen, 1950). In recent years this method was found to have many practical applications and has been used in the identification of a variety of microorganisms, including Vibrio fetus (Mellick and MacEntee, 1965; LeRoy and Kletchner, 1968; Dunn et al., 1965; Belden and Roberstad, 1965; O'Berry, 1964; Manclark, 1965).

CHAPTER III

EXPERIMENTAL DESIGN AND METHODS

Experimental Animals

Thirteen heifers which had not been bred previously and came from a herd with excellent breeding records were evaluated in this experiment. They were mated to a two year old bull who had only been used on other vibrio related experiments and had maintained an excellent breeding record through the conclusion of this study. The heifers were assembled and maintained in a separate fenced pasture at the Pawhuska Veterinary Research Station until they were brought individually to the Veterinary Research Building at Oklahoma State University for the laparotomies and other evaluations.

At the University, the heifers were held in a small pasture until they could be transferred to the Clinical Research section of the Department of Medicine and Surgery for the surgery. They were moved to individual, screened stalls after the laparotomy until observations were completed. Commercial feeds and prairie hay from meadows at the Pawhuska Station were used as feed for the entire period of the experiment.

At the 6th, 7th and 8th month of gestation, laparotomies were performed on groups of two or more of the heifers respectively. Either 1½ ml. of a 48 hour broth culture which contained 10^9 viable V. fetus var. venerealis organisms per 1 ml. or 1½ ml. of sterile water

(noninfected control) was injected into the fetal membranes (Table I). Aborted fetuses were examined promptly at the time of abortion or as soon as they were found on unobserved abortions.

One normal calf (Table I) was injected at one day of age with 1½ ml. of a 48 hour broth culture of 10^9 viable V. fetus var. venerealis organisms per 1 ml. into the jugular vein. A second calf of this same age group serving as control received 1½ ml. of sterile water which was injected into the jugular vein. Two other calves at 30 days of age were injected with 10^9 suspension of organisms in the same manner as the one day old calf (Table I). Injected and control calves were not allowed to nurse the dams. They were fed a commercial milk preparation.

On the day selected for the tissue evaluation, the calves were transported to the Pathology Necropsy Laboratory and electrocuted. A detailed necropsy was conducted on each calf. Similar procedures were used in examining the fetuses. Care was taken to collect adequate and satisfactory samples for bacteriologic and fluorescent antibody studies.

Blood samples for serological determinations were collected from all the experimental animals just prior to the start of this study, at approximately three month intervals during the study and at the time of abortion. Blood samples were collected from the calves at the time of necropsy.

Bacteriological Methods

The original strain of Vibrio fetus var. venerealis was originally obtained from Dr. Paul B. Barto, Parasitology and Public Health, College of Veterinary Medicine, Oklahoma State University. This strain was used to prepare inoculums for the experimental reproduction of the

infection as well as for the preparation of hyperimmune serum.

The venereal strain of V. fetus used to prepare the suspension for the infection of cows had been isolated originally from an aborted fetal calf and carried on an artificial medium for some time. This strain was grown in flasks containing a medium of 28 gm brucella broth*, 2 gm agar, 12.5 gm glutathion** and 37.5 mg of sodium thioglycollate†† per liter. The inoculum for the flasks was made by carrying the vibrio strain for several passages on agar plates. After 24 hours, the plates were carefully observed for bacterial growth and several smooth type colonies were selected. Gram stained smears were made and, if microscopically pure, the colonies were used to inoculate broth media. A Gram stained preparation from the broth was examined, and if pure, the broth culture was used for seeding of the flasks. The seeded flasks were incubated at 37° C in a controlled environment chamber$^{\Delta}$ containing 81.25% nitrogen, 12.5% oxygen and 6.5% carbon dioxide. After 72 hours, a blood agar medium which contained 51 grams cystine heart agar†† , 5 grams of agar ††, and 5% citrated bovine blood was streaked with growth from each flask, and the cells from these flasks were collected. The collection of cells was made by flooding the flasks with saline and rocking them back and forth until the organisms were washed from the agar. This cell suspension was poured through a sterile funnel containing a small amount of sterile glass wool into a sterile bottle, after examination of the plates revealed that the growth from each flask produced only colonies with smooth characteristics.

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* Albimi Laboratories, Flushing, New York
** Eastman Organic Chemicals, Inc.
†† Difco Laboratories, Detroit, Michigan
$^{\Delta}$ National Appliance Company, Portland, Oregon

The first criterion used to identify the pathogenic characteristics of Vibrio fetus colonies was the smoothness of three day old colonies on blood agar which were observed at 40X magnification, using the indirect lighting method of Henry (1933).

The quantitative catalase test as described by Brynner and Frank (1955) was used as a second criterion of pathogenicity. This was determined by measuring gas displacement in a 16 mm tube that contained a subculture of Vibrio fetus var. venerealis cells to which an equal amount of 30% hydrogen peroxide had been added.

A third criterion of pathogenicity was the production of hydrogen sulfide in Triple Sugar Iron medium. Sixty-five gm of Triple Sugar Iron (TSI)* medium with agar was dissolved in a liter of distilled water, tubed, autoclaved and slanted. Growth from a subculture was used for stab inoculations of TSI tubes. Hydrogen sulfide production was determined by the blackening of the TSI medium.

Intravenous suspensions were prepared by diluting the cell suspension of viable Vibrio fetus var. venerealis until a density of 10^9 organisms was obtained when compared against a set of #3 MacFarland standards. Amounts of 5 ml were dispersed in serum vials and stored at 4° C. These preparations constituted the live organism used for infection of experimental animals and the antigen for the tube agglutination test.

For the production of hyperimmune serum, it was necessary to produce antigen of the same colonial type, hence the same procedure described for the production of the infective culture was used to

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* Difco Laboratories, Detroit, Michigan

prepare the antigen. The harvested cells were killed by diluting them with physiological saline that contained 0.3 percent formalin. This suspension was adjusted to an optical density of 0.37 at 525 mu in a Spectronic 20*. A suspension of five times the above concentration was emulsified with an equal volume of Freund's complete adjuvant**.

Rabbits of mixed breed and weighing approximately eight pounds were immunized. Five rabbits were used for the production of rabbit antiserum by giving them an intravenous injection of the formalinized antigen with adjunant via the marginal ear vein at 3-4 day intervals. Each animal was given four injections increasing in volume from 0.5 ml, 1.0 ml, 1.5 ml to 2.0 ml.

One week after the last injection a maximum amount of blood was collected from all rabbits by means of intracardiac puncture using a 10 ml glass syringe and an 18 gauge, 2½ inch needle. It was possible to collect 20 to 30 ml of blood by this method. The serum was separated and agglutination tests were performed on each of these sera. Serum having a titer of 1:200 or higher was considered adequate. The sera were stored at -70° C.

At the time of evaluation, liver, lung, abomasum, brain and spleen were taken from aborted fetuses and calves for bacteriological culture. Blood agar plates were streaked and incubated for three days in the same artificial atmosphere as previously described and the colonies tested in the same manner as mentioned for the preparation of the infective culture.

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* Bausch and Lomb, Rochester, New York
** Difco Laboratories, Detroit, Michigan

Serological Methods

The tube agglutination test for titration of the sera was performed as follows:

An initial 1:25 dilution of serum was made by adding 0.1 ml of serum to 2.4 ml of antigen in the 1st of 6 tubes.

1.0 ml of antigen was placed in each of the remaining tubes and serial dilutions were made by transferring 1.0 ml from the first tube to the next resulting in dilutions of 1:25, 1:50, 1:100, 1:200, 1:400, etc.

The test tubes were held at 37°C. in H_2O bath for 24 hours.

Readings were made after slight agitation of the tubes and the results recorded.

Immunofluorescent Methods

Antisera were fractionated by precipitating the globulins twice with a concentration of 3.52 M ammonium sulfate (Lewis and Cherry, 1964). The globulin solution obtained was then dialyzed against frequent changes of 0.85% sodium chloride solution until sulfate was no longer detected in the saline solution after overnight use. The presence of sulfate was determined by adding saturated barium chloride solution to an equal volume of the saline. When no precipitate resulted, the globulin solution was considered free of sulfate and ready for the determination of protein concentration.

The protein content of the globulin fraction was determined by the modified biuret method described by Gornall et al. (1949).

The dye employed was crystalline fluorescein isothyocianate

(FITC)*. The amount of globulin fraction used for all the direct conjugation procedures was 5 ml containing 1.5 gm% of protein. Two methods of conjugation were used and identified as follows:

Conjugate "A" was performed according to the method of McKinney, Spillane and Pearce (1964). The amount of FITC used was 0.025 mgm/mgm globulin dissolved in an aqueous solution of Na_2HPO_4. Normal saline was added to the globulin solution and the FITC solution to bring the final volume to twice the starting volume of globulin. The reaction was allowed to proceed for 60 minutes at 25° C. Unreacted FITC was removed by gel filtration on Sephadex G-25 using phosphate buffer solution (PBS) pH 7.5 (0.01 M phosphate buffer; 0.85% NaCl) as the eluting buffer. The characteristics of the method were the use of a glass column (20 mm x 200 mm) and 8 gm of dry Sephadex which was ample for 5 ml of conjugate.

Conjugate "B" was prepared using the method of dialysis labeling by Clark and Shepard (1963). Data obtained by the author indicated that the conjugate prepared by this method exhibits half the level of nonspecific staining of Conjugate "A" when both are compared at equal FITC concentration. In order to attain approximately equal concentrations of FITC in the two conjugates, it was necessary in the preparation of conjugate "B" to dissolve 1.25 mg of FITC in 50 ml of an aqueous solution of 0.1 M Na_2HPO_4 adjusted to pH 9.5 with an aqueous solution of 0.1 M Na_3PO_4. A dialysis sac was filled with 5.0 ml of globulin and tied so that air was excluded. The sac was completely immersed in the FITC solution at 25°C for four hours to permit conjugation of the dye with the globulin. The sac was then transferred into

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*Baltimore Biological Laboratories, Baltimore, Maryland

a container of PBS pH 7.5 (0.1M phosphate buffer, 0.85% NaCl) at 5°C. Frequent changes of PBS were made until the PBS showed no fluorescence under ultraviolet light.

All conjugates were adsorbed with tissue homogenate before staining the tissue sections. The homogenates were prepared by the method of Nairn (1964). Fresh liver samples were obtained from heifers slaughtered at the meat packing plant at Oklahoma State University. The liver tissue was cleaned of blood by dicing it with scissors and washing several times with an aqueous solution of 0.85% sodium chloride. The product was mixed with cold physiological saline and homogenized in a blender. The product was frozen at -76° C for four hours then thawed and centrifuged at 10,000 rpm for 10 minutes. The sediment was washed twice with PBS pH 7.5 (0.01M phosphate buffer, 0.85% sodium chloride). After the final centrifugation the sediment was stored at -20° C. At the time of adsorption, the homogenated tissues and conjugate were thawed and mixed at room temperature. For a more complete explanation of reagents and formulas, as well as steps in the preparation of the chromatographic columns the reader is referred to the Appendix at the end of this work.

Because preliminary experiments showed that specimens stained with the conjugate "A" and "B" did not detect Vibrio fetus var. venerealis antigens in tissues, the indirect fluorescent antibody technique was used for the detection of antigen in tissue.

The indirect fluorescent antibody method consisted of exposing the tissue section first to unlabeled specific antibody and second to fluorescent isothiocyanate labeled antiglobulin serum.

Antibovine globulin, prepared in rabbits, was supplied by Dr. L. B.

Belli of the Department of Parasitology and Public Health, College of Veterinary Medicine, Oklahoma State University. The conjugation of the rabbit-antibovine globulin was carried out using the method of Corstvet (1964). The unlabeled specific antibody used was that from the convalescent serum of infected cows.

Tissue sections, bacterial smears and colony imprints were used to determine the staining efficiency of the various conjugated antisera. The conjugates "A" and "B" were used to stain various bacterial colonies such as Vibrio fetus var. venerealis, Mycoplasma gallisepticum and Erysipelothrix insidiosa. Bacterial smears were made from Vibrio fetus colonies grown on blood agar plates. The colonies were washed off plates with normal saline (0.85% NaCl). A drop of this suspension was spread on the surface of a coverglass. The smear was allowed to air dry and fixation was accomplished by gentle heat. The colony imprints were prepared by toughing a coverglass to the colonies growing on the surface of blood agar and allowed to air dry. The bacterial impressions were treated thereafter in the same fashion as bacterial smears.

In order to stain the slides one drop of conjugate was added to the preparations. The slides held in a sealed moist chamber were then placed in an incubator at 37°C. for 30 minutes. Excess conjugate was poured off and the coverglasses rinsed in PBS pH 7.5 (0.01 M phosphate buffer; 0.85% NaCl) for 15 minutes. After completing a second rinse for 10 minutes, and final rinse in distilled H_2O the excess of buffer was poured off and coverglasses were mounted on slides using 10% glycerol in PBS.

The bacterial preparations of Mycoplasma gallisepticum and Erysipelothrix insidiosa were obtained from Dr. R. E. Corstvet of the

Department of Parasitology and Public Health, College of Veterinary Medicine, Oklahoma State University. The smears were treated in the same fashion as that used for staining of Vibrio fetus preparations.

The staining of tissue sections by the indirect fluorescent method was made by exposing the tissue sections for 30 minutes, first to unlabeled bovine serum and second to labeled antibovine globulin. The stained preparations were mounted in a solution of buffered glycerol, pH 7.2.

The tissue sections were also stained with the procedure of Taylor (1968) for the study of Gram positive and Gram negative bacteria.

Tissue blocks were cut from liver, abomasum, lungs, spleen, brain, mediastinal lymph nodes and thymus of infected and control animals. The tissues were frozen by placing them on aluminum foil over a slab of dry ice. Slices of tissues 4-6 mm thick were mounted on object discs. These object discs were kept on the quick freezing stage of a cryotome* until sectioned. After the section was cut, a coverglass at room temperature, held by a suction device, was placed against the section which adhered to the coverglass on contact. The section was dried at 37°C. for 15 minutes and fixed by treatment in acetone** for 10 minutes.

The slides containing the tissue sections were identified on one edge by writing the number of the cow or calf as well as the type of tissue contained. The sections were wrapped and placed in sealed containers. They were stored at -70° C.until used.

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* International Equipment Company, Massachusetts
** Mallinbrodt Chemical Works, St. Louis

CHAPTER IV

RESULTS

Morphologic Appearance of Fetuses

The results of Vibrio fetus inoculations into the fetal membranes of six heifers and the jugular veins of three calves are listed in Table I. Only the gross lesions were described in these results as the microscopic lesions are being evaluated and reported separately (Osburn, 1968).

Two cows inoculated during the second trimester (6th month) of pregnancy showed, when examined per rectum, that evidence of fetal movements ceased approximately two to four days after the inoculations and the fetuses were aborted within a maximum of six days (see Table I). Both fetuses were born dead. Gross placental lesions consisted of small gray-white discoloration in the cotyledonary areas. These areas were only 1-2 mm in their greatest diameter and were later identified as areas of necrosis. No gross pathologic alterations were observed in the fetuses.

The four cows inoculated within the third trimester of gestation (7th, 8th months), aborted fetuses within four to thirty-one days. All aborted fetuses were alive, but that from cow 02 was dead when recovered. Fetuses born alive died in approximately thirty minutes. Gross lesions were observed only in the fetal membranes. These consisted of edematous yellow foci of necrosis up to 3-5 mm in diameter

that sometimes coalesced to form large plaques in the cotyledons and chronic surface.

There were no gross lesions observed in the control calves or their placentas.

Morphologic Appearance of Calves

No gross detectable changes were observed at the time of necropsy on postnatally infected calves. However, calf 423 was observed to develop a sudden paralysis of rear limbs, difficult breathing and drooling of saliva at fifteen days after being infected. This calf recovered in a few hours and appeared normal. Symptoms of bloating appeared the following day but were never marked and disappeared within a week.

Serologic Results

Serum obtained from the fetuses and subjected to the tube agglutination test showed a negative titer of agglutinins to Vibrio fetus var. venereaalis. Postnatally infected calves reacted to the same agglutination test with low titers at the dilutions of 1:25 for the calf no. 999 and 1:50 for no. 423 (Table I).

Bacteriologic Results

Bacterial recoveries revealed that Vibrio fetus could be recovered consistently from the abomasum, liver, lung, spleen and brain of aborted fetuses at the 6th, 7th and 8th months of pregnancy. Vibrio fetus was recovered only from spleen, liver and brain of the one day old calf (no. 999). One of the two calves (no. 118) at 30 days of

age, yielded a culture of Vibrio fetus from spleen, liver and brain; the other calf (no. 423) was negative. The abomasums of postnatally infected calves were not cultured. Abomasum of calves that live for a very short time, the stomach content is apt to be contaminated because of the ingestion of other bacteria. It is then unsuitable for cultural purposes. Lungs shown to be negative on media were overgrown by contaminants. Calves used as controls were negative to bacterial recovery.

Immunofluorescent Results

Tissue sections from organs such as abomasum, liver, spleen, lungs, brain, thymus, mediastinal lymph nodes and placenta of nine animals that had been experimentally inoculated with Vibrio fetus var. venerealis gave specific fluorescence when they were evaluated by the indirect FAT (Table I). No specific fluorescence was observed with the same conjugate when it was applied to tissue preparations of the same organs obtained from Cesarean delivered fetuses which had been inoculated with sterile distilled water.

When tissue sections from the same organs of infected animals were stained with the direct method of conjugates "A" and "B", negative results were obtained. Sometimes specific yellow-green fluorescence was observed and it was evaluated as suspicious when compared against the fluorescence seen in the tissue control specimen. However, this suspicious fluorescence in the parenchymal endothelial spaces of liver and spleen was not easily differentiated from small, disseminated tiny clusters of brilliance which appeared to be on the surface of the cellular cytoplasm. The confusing clusters of tiny foci of bright material were reduced by absorption of the conjugate with homologous tissue

specie preparates, but this did not entirely eliminate the problem.

The colony imprints and bacterial smears of Vibrio fetus colonies were fixed by rapid heating and stained with conjugates "A" and "B". Wide differences in the degree of brilliance was observed between imprints and bacterial smears. Colony imprints for Vibrio fetus strains when stained with homologous conjugated rabbit antiserum exhibited excellent degree of brightness versus the weak brilliancy exhibited by smears. Frequently a small amount of media was adhered to the surface of bacterial preparations and this would cause non-staining areas to appear. Bacterial colonies from Mycoplasma gallinarum and Erysipilothrix insidiosa were easily differentiated when stained with rabbit antivibrio conjugate because they did not react specifically as did Vibrio fetus colonies.

It was therefore not possible to use the direct FAT to stain colonies of Vibrio fetus in fetal tissues. Nevertheless, a high degree of specific staining was obtained when the homologous conjugate was applied to bacterial preparations.

The distribution of fluorescence in tissues as determined by the indirect fluorescent test is tabulated in Table I. In the lymphoid areas of the body such as lymph nodes and thymus fluorescence was seen as tiny granules confined within the cytoplasm and around the nucleus of cells rather than in sinusoidal areas. These cells were concentrated in the medullary area of lymph nodes, but were less prominent in the cortical zones. The degree of fluorescence seen in the lymphoid tissue varied according with the age of the animal examined. Greater fluorescence was seen in the lymph nodes beyond the 7th month of gestation and in the postnatally infected calves. Fetuses within the

first trimester of pregnancy exhibited lower concentration. Unfortunately, the control sections revealed identical fluorescence. It was concluded that no specificity could be attached to the lymph node. The same nonspecific reaction was observed in the central tissues of the thymus.

In the spleen two types of fluorescent material were evaluated. The first type appeared as numerous fluorescent granules in the cytoplasm of the cell. In the control animal, the same approximate degree of fluorescence was present in the spleen and the reaction, as in the lymph node, was regarded as a nonspecific fluorescence. The second type of fluorescent material appeared as small masses of fluorescence in the red pulp and endothelial cells. This reaction was regarded as specific when compared with the control tissue section. No difference in the concentration of specific fluorescence was seen in reference to the ages of fetuses. A low but consistent concentration was found in spleens of postnatally infected calves.

Localization of specific fluorescence was consistently seen in the liver. The antigen appeared as diffuse clumps in the sinusoidal spaces and endothelial cells of portal tracts. No difference in the degree of specific fluorescence was seen between the ages of fetuses. No specific fluorescence was found in the livers of postnatally infected calves.

Low or non-recognizable concentrations of fluorescent antigen were found in tissue sections from brain and abomasum. The pattern of fluorescence in brain was seen as isolated clumps of material close to the endothelial walls of vascular structures in the gray matter. Sections of abomasum were difficult to evaluate because the light-green

background of tissue was intense and not cut down by absorption. The fluorescence appeared to be diffuse smooth plaques along the edges of epithelial cells lining the mucosa. Negative results were obtained in sections from postnatally infected calves.

Lung sections from all infected fetuses gave moderate tissue concentration of fluorescence. The appearance of the clumps was similar to that observed in other organs and they were confined to the edges of parenchymal cells lining the alveolar spaces. No specific fluorescence was demonstrated in lungs of the one day old calf (from Cow 999) and the infected thirty day old calf (Calf 423).

Placental tissue was consistent in its exhibit of bacterial fluorescence. Greatest concentrations were seen in the epithelium of cells lining the intercotyledonary spaces and in endothelial cells of the numerous and prominent blood vessels. Placenta from Cow No. 7 had a low concentration of the fluorescence. Highest concentrations were seen in placentas from 8th month infected heifers.

The staining of tissues with Taylor's stain for the demonstration of Vibrio fetus was doubtful. There were small rod-shaped structures either isolated or in clumps in the sinusoidal spaces of the liver, the red pulp of the spleen and the alveoli of the lungs, but they looked somewhat blue rather than red as expected for Gram-negative bacteria. Furthermore, the degree of cellular artifacts due to the precipitated stain was considerable, making positive identification doubtful.

TABLE I

COMPARISON OF BACTERIOLOGIC, SEROLOGIC AND FLUORESCENT ANTIBODY TECHNIQUES FOR THE DETECTION OF V. FETUS VAR. VENEREALIS IN FETUSES AND CALVES

Age When Inoculated	Animal Numbers	Death - Days After Inoculation	Bacteriologic Isolations*					Agglutination**	Fluorescent Antibody Evaluation†						Comments
			Abo.	Lung	Spleen	Liver	Brain	Titers	Abo.	Lung	Spleen	Liver	Brain	Placenta	
6 Months Prenatal	07	6	P	P	P	P	P	0	1+	2+	3+	3+	1+	1+	Aborted
	013	4	P	P	P	P	P	0	-	2+	3+	3+	-	1+	Aborted
	014∞	14	-	-	-	-	-	0	-	-	-	-	-	-	Euthanized Control
7 Months Prenatal	02	4	P	P	P	P	P	0	1+	2+	3+	3+	1+	1+	Aborted
	09	77	-	-	-	-	-	0	-	-	-	-	-	-	Euthanized Control
8 Months Prenatal	08	19	∇	∇	∇	∇	∇	No Test	1+	-	3+	3+	1+	2+	Born Alive
	05	31	P	P	-	-	-	0	1+	2+	3+	3+	1+	2+	Born Alive
	015	26	P	P	P	P	P	0	-	2+	3+	3+	1+	2+	Born Alive
	014∞	22	-	-	-	-	-	0	-	-	-	-	-	-	Euthanized Control
1 Day Postnatal	999	31	Δ	-	P	P	P	25	-	-	1+	-	-		Euthanized
	006	41	Δ	-	-	-	-	0	-	-	-	-	-		Euthanized Control
30 Days Postnatal	423	35	Δ	∇	-	-	-	50	-	-	1+	-	-		Euthanized
	118	20	Δ	-	P	P	P	0	-	-	1+	-	-		Euthanized

*P = Vibrio fetus var. venerealis isolated.

**Number - Reciprocal of the highest serum dilution showing agglutination.

Δ = Not cultured.

∇ = Media overgrown with contaminant.

∞ = Cows in two groups had identical numbers.

† 3+ = > 50% of the area of the tissue section showed specific fluorescence.
2+ = < 50% but > 15% of the area of the tissue section showed specific fluorescence.
1+ = 15% or < of the area of the tissue section showed specific fluorescence.

- = Test conducted and no positive findings observed.

CHAPTER V

DISCUSSION

This investigation was divided into two phases. Phase I concerned the development of a simple, accurate and rapid fluorescent antibody test (FAT) for detection of Vibrio fetus var. venerealis in fetal and neonatal tissues. Phase II involved the determination and evaluation of Vibrio fetus var. venerealis infection in various organs of the fetal and neonatal calf using the FAT and comparing it with the recovery of organisms by cultural methods.

The direct fluorescent antibody test was not found suitable for evaluation of specific fluorescence in reciprocal tissues. Failure of this direct fluorescent antibody technique when applied to tissue may be due to blocking or univalent antibody masking antigenic sites.

The correlation, however, between recovery of Vibrio fetus var. venerealis by cultural methods and the results of the indirect FAT for this same organism is aborted fetuses and calves dying at 35 days of age is encouraging (Table I).

In some organs or tissues, it would appear that this indirect fluorescent antibody evaluation is not specific or gives only a limited reaction under the conditions employed in these tests. In spleen, liver and lung removed from aborted fetuses, there were excellent comparisons between the results of the FAT and the isolation of the organism. However, due to the questionable location of some of the

fluorescence within cells in the spleen and its appearance in control sections, it was felt that only the liver and lung would be suitable tissues for a specific test. It was believed that typical clumped masses of fluorescent material in the red pulp were identical to the specific antigen seen in the liver and lung. However, the presence of two fluorescent materials made it a less suitable organ for testing even though the specific fluorescence is identifiable and appears in large areas of the spleen.

Osburn (1968) described specific histologic fetal lesions in the liver and lung as well as kidney, placenta, brain and lymph nodes in similarly infected animals and this would support the findings of the indirect fluorescent antibody test. The exact mechanism of the death or debility of the fetuses is unimportant in this study if it is established that organisms actually localize in the tissues.

It is unfortunate that liver and lung of fetuses aborting before the sixth month of pregnancy were not included in the present study. As additional tests are conducted and further refinements of the test are made, it will be of interest to compare tissues from younger fetuses. After birth, calves infected by injection into the jugular vein did not develop a significant degree of specific fluorescence in the three organs (Table I).

It is of interest that, while the calves injected with cultures into the jugular veins (Table I) showed elevation in agglutination titers similar to adult cattle (Osburn, 1967), the organisms did not appear to localize in appreciable numbers in the internal organs. Cultural isolations were made in some of the organs but this does not mean that great numbers of organisms were present. If the FAT with the

thymus and lymph nodes is indicative of the concentration of organisms or the presence of cells capable of beginning production of immunoglobulins, it would be of considerable value in our understanding of the pathogenesis of the disease. This concentration of either immunoglobulins or organisms is suggestive of the disease in mature cows where the organisms show great affinity for the genital tract and lymphoid tissues (Frank, 1962). More sensitive or accurate tests must be devised before this can be evaluated.

It is likewise of interest that the increase in fluorescence in the placenta would indicate that the organisms were multiplying in that structure in the fetuses infected for longer periods of time. This is in agreement with observations by other investigators which consider that the hematomas which form at the base of the cotyledons are the initial placental exposure locations for bacterial abortifacients, (Jensen et al., 1961). The thymus and lymph nodes gave the same non-specific fluorescence as was seen in the spleen and it would be more difficult to be certain that these tissues were positive in this preliminary evaluation. The fluorescence again appeared to be within specific cells rather than spaces lining sinusoidal channels or alveolar spaces as was true in the liver and lung. No clumped masses of materials were seen as in the mixtures observed in the spleen.

As one might expect by the frequent recovery of this specific vibrio from the digestive tract in the bacterial culture studies, the fluorescence could sometimes be demonstrated in the abomasum. However, this tissue is not considered as suitable for fluorescence evaluation of the disease because the concentration of antigen in the mucosal epithelium appeared to be very low. Two types of non-specific

fluorescence were observed. One of these was the non-specific attachment of dye conjugate to tissue components and the other was autofluorescence. The latter was the major limitation in detecting specific fluorescence in the abomasal epithelium. This is probably due to the extreme complexity of tissue and cellular elements in the wall of the gastrointestinal tract (Chennakatie et al, 1966).

CHAPTER VI

SUMMARY AND CONCLUSIONS

The principal objective in this study was to develop a rapid, simple and accurate immunofluorescent test for the detection of Vibrio fetus var. venerealis in tissues from aborted fetuses and neonatal calves. Lapine and bovine hyperimmune serums were prepared by intravenous injection of one strain of Vibrio fetus var. venerealis. These sera were fractioned by chemical methods and their percentage of protein measured using the biuret method. The eryglobulin fraction was conjugated to fluorescein isothiocyanate. Homologous and heterologous Vibrio fetus colony imprints and smears were stained with the fluorescein isothiocyanate conjugated antibody as well as the tissues from the fetuses and calves.

By correlating the specific fluorescence with the recovery of Vibrio fetus by cultural methods, the indirect method of immunofluorescence was found to be accurate in some of the tissues evaluated. The direct test proved to be reliable only for the detection of Vibrio fetus in homologous colony imprints and smears. When the indirect method was conducted on fetal tissues, tissue specimens from liver and lung were the only tissues studied which were a reliable indicator of the presence of this specific vibrio. In other tissues, nonspecific reactions led to a questionable positive fluorescence. No tissues were more suitable than cultural methods for either one of the direct

or indirect tests on specimens from infected postnatal calves but this was due in part to the apparent limited localization of Vibrio fetus in the organs tested.

The indirect test may become more reliable as further modifications and refinements are developed. In its present stage of development, and as the test could be interpreted in the study, it is valuable as an adjunct tool to confirm a diagnosis in fetuses aborted late in pregnancy. It would seem to have merit when used in conjunction with the clinical history of suspect animals, the results of bacteriologic cultures, and the serum and mucous agglutination tests in establishing the status of an individual animal. It has distinct advantages in that tissue specimens may be frozen and held for multiple or delayed tests and, like the bacteriologic cultures, that it can be conducted on fetal tissues.

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APPENDIX

REAGENT PREPARATIONS

A. Buffered Saline pH 7.5 (0.01 M Buffer; 0.85% NaCl):

1. Concentrated Stock Solution:

Na_2HPO_4 (anhydrous; reagent grade)	12.0	grams
$NaH_2PO_4.H_2O$ (reagent grade)	2.2	grams
NaCl (reagent grade)	85.0	grams
Distilled water to make final volume	1000	ml

2. Working Solution (pH 7.5; 0.01 M Buffer; 0.85% NaCl):

Concentrated Stock Solution	100	ml
Distilled water to make final volume	1000	ml

B. Na_2HPO_4

0.2M

Na_2HPO_4 (anhydrous)	28.4	grams
Distilled water	q.s. to 1000	ml

0.1M

Na_2HPO_4 (anhydrous)	14.2	grams
Distilled water	q.s. to 1000	ml

C. Na_3PO_4

0.1 M

$Na_3PO_4.12H_2O$	38.0	grams
Distilled water	q.s. to 1000	ml

D. Buffered Glycerol Saline Mounting Fluid:

Glycerol, reagent grade	90	ml
Buffered saline; pH 7.5, 0.01 M (working solution above	10	ml

E. Saline Solution (0.85% NaCl):

NaCl	85.0 grams
Distilled water	1000 ml

PREPARATION OF SEPHADEX G-25 BEDS

A. Preparation of gel.

Add PBS pH 7.5 to a beaker containing 8 grams of dry Sephadex G-25 using approximately 20 ml of buffer per gm of dry powder. Stir thoroughly allowing the Sephadex to settle. Decant and discard the supernatant PBS.

B. Packing of Column.

The glass column should have diameter - to high ratio of approximately 1:10, with the column outlet closed fill with PBS the column to half capacity. Add the Sephadex gel by means of a funnel the top of which is submerged in the PBS, when 2 cm of gel has settled, open the outlet. The Sephadex than settles faster than the PBS forming a column. The column is ready for use when the PBS has drained almost to the upper surface of the gel.

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