THE SEROLOGICAL AND LOCAL IMMUNE RESPONSE OF CATTLE TO *BRUCELLA ABORTUS*

By

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Doctor of Veterinary Medicine

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Baton Rouge, Louisiana

1982

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
December, 1986

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Thesis Approved:

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PREFACE

This dissertation is composed of five chapters, each of which is a complete and independent manuscript, prepared in accord with the American Society for Microbiology guidelines. Chapter I provides a review of literature for Brucella serology, immunoglobulin class and subclass response to B. abortus, and vaginal tests for B. abortus. Chapter II describes a fluorometric immunoassay (FIAX) for the detection of serum antibody to B. abortus and was published in the December 1984 issue of the Journal of Clinical Microbiology. Chapter III compares another fluorometric immunoassay (TRACK XI) with other serologic tests for detection of serum antibody to B. abortus and is scheduled for publication in the February 1987 issue of the Journal of Clinical Microbiology. Chapters IV and V pertain to the class and subclass immunoglobulin response in the serum and vaginal secretions of cattle in response to B. abortus 19 vaccine, B. abortus cell surface protein vaccine, and challenge with virulent B. abortus 2308; both of these chapters are intended for publication in the immediate future.

Thanks are in order to so many colleagues and friends that have helped me in so many different ways.

This work was supported by a cooperative research agreement among the Oklahoma State University College of Veterinary Medicine, the Robert S. Kerr Foundation, and the United States Department of Agriculture - Agricultural Research Service (USDA-ARS). Drs. Bill Deyoe and Louisa

Tabatabai and Ms. Judy Patterson of the USDA-ARS all provided knowledge and services that were essential to these studies. The help of Dr. Doug Fulnecheck and Mr. Rob Smith of the Kerr Foundation is appreciated. The assistance of the State-Federal Brucellosis Laboratory in Oklahoma City is gratefully acknowledged.

Daryl Laboratories provided the TRACK IX system and reagents used in some of these studies. The help of Dr. Joe Rosebrock at Whittaker M.A. Bioproducts (FIAX) is appreciated.

The technical assistance of Mr. Chad Faulkner, Ms. Sharon Oltjen, and Ms. Rene Simons was essential to these studies, and Rene's efforts during the final stages of my work was of great help to me.

I thank Dr. W.D. Warde for elevating my interest in statistics through his enthusiastic and enjoyable teachings. I thank Ms. Serena Shubert for her encouragement and helpful editorial comments. I especially thank my good friend Ms. Betty Fruits for her hours of assistance in the bibliography search.

Mr. John Voigt, my brother-in-law and friend, is responsible for my interest in computers. His assistance was invaluable in writing the discriminate analysis program used in these studies. I also thank Ms. Sarah Voigt (Sally) for her encouragement.

The help and support of the other residents and graduate students is appreciated.

A very special thanks and appreciation to Sherl Holesko for her care and attention in typing and formatting this work.

I wish to thank Dr. Carl Fox for his interest, enthusiasm, and knowledge of the FIAX system, and Drs. Roger Panciera and Charles Qualls, Jr., for their dedication to diagnostic pathology training.

Dr. Anthony Confer is due special thanks for instructing me in research, diagnostic pathology, and scientific writing skills.

From childhood, my father has helped to develop my scientific interests and encouraged, above all else, precision and integrity in not only scientific work, but in life itself. And for this and many other things I thank him.

And to my mother, she gave me a sense of humor that has proved to be invaluable to me.

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CHAPTER I

THE SEROLOGICAL AND LOCAL IMMUNE RESPONSE TO *BRUCELLA ABORTUS* IN CATTLE: AN INTRODUCTION WITH A REVIEW OF THE LITERATURE

Introduction

In 1887 Dr. David Bruce published an article entitled "Note on the discovery of a microorganism in Malta fever" (7). After that date the literature has been almost exponentially flooded with reports, reviews, observations, and research data all dealing with various aspects of brucellosis. A book appropriately entitled *Brucellosis Bibliography* was published in May of 1980 by the Cattle Disease Staff, Emergency Programs, Veterinary Services, Animal and Plant Health Inspection Service, United States Department of Agriculture. This book contains references to more than 4000 articles or English abstracts pertaining to brucellosis. It is not my intent to duplicate their efforts. Instead, I shall discuss several review articles comparing the attributes and negative aspects of serological tests for bovine brucellosis. Selected references dealing with several newer serological tests available for detection of serum antibodies to *Brucella* are given. The two major areas of emphasis of the review will pertain to the research presented in Chapters 4 and 5. These emphases are: a review of the articles

presenting data on class and subclass immunoglobulin (Ig) response of cattle to Brucella abortus and a review of the vaginal mucus test for detection of immunoglobulin (Ig) to B. abortus in vaginal mucus.

Serological tests

One of the more frequently referenced books or articles dealing with brucellosis is Laboratory Techniques in Brucellosis (3). This book provides the single best source of not only serological tests, but methods of bacteriology and vaccine production. The techniques for performing the tests, as well as preparation of reagents for use in the tests, are well described. The USDA tube agglutination test (ST), plate agglutination test (PLATE), European tube agglutination test, numerous variations of complement fixation (CF) tests, the buffered Brucella antigen tests (CARD and Rose Bengal plate test), mercaptoethanol test (2-ME), and the Coombs antiglobulin test (Coombs) are all described in detail. There are several articles that provide reviews of immunology with an emphasis on Brucella serology (25, 43, 66).

The heat inactivation test and acidification of the PLATE are discussed as a method of differentiating different types of Brucella agglutinins (43). The heat inactivation presumably removes IgM activity (43) and acidification removes nonspecific agglutination (66). The rivanol test (RIV) (alias acridine dye test or 2-ethoxy-6,9 diamino-acridine lactate test) is discussed and compared to 2-ME. Both tests remove IgM and may be helpful in differentiating antibodies resulting from vaccination from those resulting from infection (43). Although blocking antibodies (antibodies that bind antigen but do not agglutinate) do not appear to be a problem in the diagnosis of brucellosis, a

method of determining if blocking antibodies are present is given (43). The fluorescent antibody staining, Castaneda surface fixation, and gel diffusion tests are also discussed (43).

A review of the CF test is presented by Jones (32) and concludes that cold fixation of complement yields higher titers than warm fixation especially on low titered samples. The CF is a laborious and tedious assay (25, 43, 66), but it does provide some advantages over the ST. Positive CF titers from strain (S) 19 calfhood vaccination do not persist for as long as do vaccine-induced titers measured by ST. Also, cattle that are infected retain elevated titers longer as detected by CF than with ST (23, 32, 66). One study (45) showed that of 6482 samples tested there were no sera that had significant complement-fixing antibodies that failed to react with one or more agglutination tests. Another investigator (2) stated that the CF was superior to ST by demonstrating that 11% of the culture-positive cattle (in one study) had ST titers below 100 international units (presumably seronegative). In another study (37), only one of 180 sera was found to be negative by PLATE and ST but was CF positive.

Nonspecific agglutination reactions were inhibited by lowering the pH of the PLATE test to 4.0 (59). The lowered pH and coupling Brucella antigen with Rose Bengal stain led to the development of both the CARD and Rose Bengal plate agglutination tests (46). The CARD test showed close agreement with ST (50); greater than 90% of the samples, that had high CF titers and negative ST titers, were negative to CARD. Samples that were high titered by Coombs antiglobulin tests were consistently negative by the CARD. Thus, complement fixing antibodies and incomplete (nonagglutinating) antibodies are not detected by the CARD. The lowered

pH 3.65 of the CARD is used to minimize the agglutinating properties of 19S (IgM) immunoglobulins, which are often thought to be nonspecific or due to vaccination (50). The results obtained with the CARD should be similar to those obtained using the Rose Bengal plate agglutination test, but another report (42) showed closer relationship between Rose Bengal plate agglutination and CF than with ST. In another study (46) it was shown that the CARD correctly identified all culture-positive cattle, whereas the ST failed to properly classify 89 of 184 infected cattle. One study using vaccinated and nonvaccinated cattle (29) showed that if, in nonvaccinated cattle, the CARD was negative then there was at least a 98% chance that all other tests (CF, RIV, ST, ELISA) would be negative as well. However, if the cattle had been vaccinated, there would be at least a 90% chance that all other tests would be negative. If any test was negative, there was at least a 98% chance that RIV would be negative. Of 1401 sera that were CARD positive, 68% were also CF positive and of the 1051 sera that were CF positive 90% were CARD positive. This study further showed that in adult vaccinated cattle the ST did not detect immunoglobulins that were detectable by the CARD and CF.

One problem that has been encountered with some serological tests is the prozone effect (30, 53). The prozone effect is the competition for test antigen by antibodies that will not produce the desired secondary immunological reaction (in the case of CF, they will not fix complement; for CARD and ST, they will not agglutinate). An indirect hemolysis test (52) was developed to avoid the problem of prozones. Another investigator evaluated the indirect hemolysis test and stated that it should be considered the serological test of choice in detecting

B. abortus infection (65). The hemolysis-in-gel test has also been reported not to be affected by prozone (48, 60).

A radioimmunoassay for the detection of antibodies to B. abortus was developed (16) and later modified (14). Comparison of the radioimmunoassay to other tests indicated that it was sensitive and capable of detecting low levels of serum antibody (13, 14, 15, 27).

Numerous authors have described the enzyme-linked immunosorbent assay (ELISA) for the detection of serum antibodies to B. abortus in cattle (12, 17, 18, 28, 61, 63, 64, 67, 68). One article discussed the use of monoclonal antibodies in the ELISA (16). Most discussion of the ELISA test indicated that it is a sensitive test, yet it may have false positive reactions that lower specificity. One author (69) disputed this by not finding a single ELISA positive serum among 677 animals from 9 brucellosis-free herds thus claiming excellent specificity. The problems of defining sensitivity and specificity are discussed further in Chapter III. In general, the ELISA titer appears to rise later and continue to rise longer than titers measured by most other serological tests.

A quantitative fluorescent immunoassay using B. abortus antigen coupled to sepharose beads has been described (55). Two additional quantitative fluorescent immunoassays, FIAX (Chapter II) (26) and TRACK (Chapter III) (manuscript submitted), using a fluorometer to quantitate fluorescence have been described. The fluorescent immunoassays provide excellent sensitivity in evaluating serum for antibodies to B. abortus. They are primary binding assays and, as such, are not subject to the prozone effect. The fluorescent immunoassays do not appear to be as susceptible to changes in reagents and reaction times as is the ELISA

system. The FIAX system provides a simple method for correcting background fluorescence for each serum sample. The ELISA system could be modified similarly by testing each serum sample in an uncoated microtiter well and subtracting the optical density (OD) of this well from that of the antigen coated well.

Immunoglobulin class and subclass response

A review of bovine Ig is provided by Butler (8). An overview of the serological response of cattle to S-19 and natural field exposure with emphasis on the class and subclass of Ig detected by numerous tests is given in several articles (25,43,62,66,69).

One study (56) noted that only IgG1 or both IgG1 and IgM agglutinins were present in the sera of cows that had ceased to shed B. abortus, whereas chronic shedders showed high IgG1 and lesser IgG2 and IgM antibodies. Typical agglutination was produced by IgM and IgG2, but prozone effects were noted in IgG1 after heating or fractionation. In contrast, another investigator (21) found in the Rose Bengal plate agglutination test that IgG1 was essential for activity and that the removal of IgG2 or IgM had little affect on that test. Later, Wood, et al. (70) showed a positive correlation between increased serum total protein, gamma globulin concentration (mostly IgG1), and reactions to the Rose Bengal plate test. In that study, CF activity was confined to the IgG1 fraction, but ST (dithiothreitol reduced) and Coombs activity were present in the IgG2 fraction. This correlation between CF and Rose Bengal test would support the work of Morgan (42). An interesting finding of another investigator was that dithiothreitol was not a reliable substitute for 2-mercaptoethanol (40).

It was confirmed by another investigator that IgG1 was the major and IgM the minor Igs responsible for complement fixation and that IgG2 and IgA did not fix complement (6). The study also showed that IgM and IgA lost agglutination activity when heated to 65°C for 15 minutes, yet IgG fractions did not. Further, it was shown that all Igs had lowered titers in the acid pH of the Rose Bengal plate test. In another study by the same investigator (5) using a radial immunodiffusion technique, no serum IgA was found following vaccination with S-19. IgM was found at its peak 16 days following vaccination and IgG1 peaked at 32 days. IgG2 was present in very small amounts and disappeared within 180 days. The investigator later reported that a large amount of IgG2 was nonagglutinating and was present in higher quantities than had been previously reported (4). It was still felt that first exposure to Brucella antigens produced predominantly IgG1, and on second exposure the majority of the nonagglutinating antibody was associated with IgG1, but significant amounts of IgG2 antibody were detected.

In a study using S-45/20 (rough) vaccine, animals not previously exposed to Brucella antigens responded to vaccination initially with IgM followed by IgG1 and IgG2 (20). Upon revaccination, both IgG classes responded rapidly and IgM only minimally. In animals initially vaccinated with S-19 (smooth) and revaccinated with S-45/20, the serologic response was due to IgM and IgG antibodies to both smooth and rough antigens.

Allan (1) provided a quantitative comparison of serological tests to different antibody classes. In that study, it was again confirmed that IgG2 did not fix complement. In fact, in a paper by some of the same authors (41), it was shown that high levels of IgG2 inhibited

complement fixation. On a molar basis, IgM was shown to be 10 times more efficient at fixing complement than IgG1. However at 60°C, IgM appeared to be labile in serum thus IgG1 may be more effectively measured by the complement fixation test. Their data shows that the Rose Bengal plate test detects IgM more efficiently than IgG1 and IgG2, and thus should behave more like the ST than CF. This also refuted the work of Corbel (21) stating that the Rose Bengal test only detected IgG1.

An ELISA system for the detection of Ig class and subclass Brucella specific serum response was described (35). A prozone-like effect was noted in that dilution of serum resulted in an increased binding of IgG1 and IgG2. A proposed mechanism was that at low dilutions IgM would compete with the IgG subclasses, but at higher dilutions the lower affinity IgM would not compete as effectively and IgG would bind. The authors also reported a nonspecific binding of IgM to the lipopolysaccaride (LPS) antigen that was used in the assay. It was further noted that lowering the pH to 4.2 such that specific IgM binding (as seen in agglutination) is diminished and nonspecific antibody-antigen reactions dissociate, caused an increase in binding with the anti-IgM reagent.

The interaction of specifically purified isotypes of bovine antibody to B. abortus was studied using hemolysis-in-gel and ELISA (49). IgM and IgG2 did not react well in the hemolysis-in-gel test, but IgG2 did cause limited lysis (fix complement) if fresh bovine serum was present. The hemolysis-in-gel test was good at determining IgG1, but not as good as ELISA. The ELISA also detected IgG2 well, but did not perform well in measurement of IgM. In a later study, Nielsen (47) improved the ability of ELISA to detect IgM response, but the ability to

detect IgG1 and IgG2 responses was decreased. A radioimmunoassay study using protein-A reactive antibodies (IgG2) showed that animals that were challenged and culture positive had markedly higher IgG2 titers than those that were challenged and culture negative, regardless of initial vaccination status (36).

Another investigator (51) did not show any complement fixing activity in IgG2 from infected cows. The highest CF activity was seen in the IgG1 fraction and with little CF activity in the IgM fraction. The major agglutinating activity was found to be IgG2 in vaccinated cattle and IgG1 was negligible and detectable only by buffered antigen tests.

One author (9) states that using the A-ELISA method (10, 11), IgG1 appears to provide a method of distinguishing between cattle infected with vaccine or field strains from those that were exposed, but not infected. These results represented only 10 cattle and appear inconclusive.

Vaginal mucus tests

In 1951, two Danish investigators first examined vaginal and uterine secretions from cattle for the presence of Brucella agglutinating antibodies (31). They found it was possible to have positive uterine titers in the absence of serum titers and vice versa. All culture positive animals (3/8) had uterine titers and one of those was seronegative. Using vaginal tampons, they further investigated two cattle herds and found numerous cattle with negative serum titers and positive vaginal titers; two of these animals later developed positive serum reactions and aborted. After abortion high vaginal titers were noted.

A detailed description of a vaginal tampon technique for collection of cervicovaginal mucus for "Vibrio fetus" (Campylobacter fetus) agglutinates has been described (54).

A study was done to determine if local production of antibodies to Brucella occurred in cattle (34). Uterine washings and tube collected vaginal mucus were used in this study. Subcutaneous vaccination with S-19 did not induce local immunoglobulins, but the author stated that his findings on this were not satisfactory. Following vaccinations with numerous large doses of S-19 intramuscularly, vaginal agglutinins could be detected, but the author thought that most of this was caused by leakage of serum Ig only at the time of abortion. It was pointed out that local production of Ig through local infection with S-19 vaccine should be considered. When large doses of S-19 were instilled in the uterus, local agglutinins were produced with little affect on serum titers.

The same investigator later surveyed 147 dairy herds using the vaginal mucus test (33). There was some evidence that vaginal mucus tests do not detect S-19 vaccine-induced antibodies, when the vaccine was given subcutaneously. It was shown that all culture positive fetuses came from cows with a positive vaginal mucus test. It was pointed out that, following abortion, organisms ceased to exist from the third week onwards, irrespective of level of vaginal mucus antibody titer. They concluded that the vaginal mucus test was a good indicator of field infection, and there was no evidence of false positive reactions. Coauthoring another manuscript, that investigator discussed the use of the vaginal mucus test and other tests in the eradication of brucellosis from Northern Ireland (22).

Roberts (58) compared two methods of collection of vaginal mucus and examined the presence of vaginal agglutinins after S-19 vaccination. Ten of 30 heifers were positive by the vaginal test after inadvertently being vaccinated four times prior to testing, the last dose being 10 weeks prior to testing. The vaginal antibody titers were higher using the tampon method of collection than using mucus collected with a glass tube. Most of the vaginal samples that were positive following S-19 were taken within only a few months of vaccination and secondly were from tampons that had been inserted for two hours. Tampons that were inserted for 30 minutes showed lower titers and lower total protein. The authors suggested that the tampons absorb circulatory antibody from the mucosal surfaces of the vagina. In animals that were vaccinated experimentally, it was thought that vaginal titers were related to the amount of serum agglutinin present, but the possibility of S-19 stimulating local immunity was not dismissed. Some tampons appeared to have no mucus when collected, but were strongly positive when tested.

In the study of a S-19 vaccinated Brucella-infected herd, the vaginal mucus sample collected by tampons gave numerous false positive reactions to both calfhood and adult vaccinated cattle (44). These false positives fell to a negligible level when vaginal mucus samples were collected by the tube method. They also noted 23.5% of the vaginal mucus titers from Brucella-infected cows were negative. They felt that serum agglutination titers furnished evidence of field infection before a positive mucus titer was obtained.

While studying a method to more successfully culture B. abortus from vaginal samples, it was reported that the vaginal mucus agglutination test was the most consistent of the tests employed in their study

(39). Later, Caughey (38) showed only 4 of 20 culture positive animals were positive by the vaginal mucus agglutination test, yet a fluorescent antibody staining test and modified Ziehl-Nielsen stain of the vaginal mucus appeared more successful at identifying culture-positive animals. The results of 1140 vaginal mucus agglutination tests from 663 cows in seven herds infected with B. abortus were presented (57); 97 of these samples were positive, but all were positive by one or more serological tests. The authors stated that the vaginal mucus agglutination test appears to be specific for field infection and thus useful in individual animals, but in a herd, it provided little additional information beyond that from the serological tests.

References to the class and subclass antibody response in the vaginal secretions of cattle exposed to B. abortus were not found. Butler (8) and Duncan (24) provided general information on bovine class and subclass Igs in both sera and vaginal secretions. Secretory IgA seems to be the major Ig present in vaginal secretions. The quantity of IgA in vaginal secretions exceeds that observed in the serum and thus indicates local synthesis or a selective transport mechanism as is found with IgG1 in mammary secretions. Vaginal IgM is detected only occasionally and usually coincides with an increase of other serum proteins. Such a response is seen postpartum and also has been associated with estrus. IgG1 and IgG2 are seen in similar concentrations in vaginal secretions with IgG1 being slightly more abundant. The ratio of IgG1 to IgG2 is similar in vaginal secretions and serum indicating that vaginal IgG may be present predominantly as serum transudates rather than as local secretions.

In two separate studies, Corbeil (18, 19) described class and subclass vaginal Ig response of cattle to Campylobacter (Vibrio) fetus. Two cattle that were parenterally immunized had only IgG1 and IgG2 Campylobacter-specific vaginal antibodies 10 - 17 weeks after immunization, whereas three cattle that were locally immunized had only an IgA response 7 - 9 months after immunization. IgM was shown to be present early, in the course of infection, but usually diminished earlier than the other Igs.

While many questions about Brucella serology still remain unanswered, and other questions remain in constant dispute. One point remains clear, that the ultimate test in Brucella serology has not yet been developed. Primary binding serological assays appear to be superior in sensitivity to other assays that require secondary antibody traits for their action. Although prozone-like phenomena are described in primary binding assays, the prozone effect seldom results in a negative test result, only a confusing increase in titer when the serum is diluted. Simpler and more economical tests, such as the CARD, may be more applicable for large scale screening of populations, but in pathogenicity studies or in the scrutiny of a problem herd, primary binding assays are more appropriate.

Our goals for the studies described herein were to develop a test that could accurately, consistently, and easily measure the serological response of cattle to B. abortus. A fluorometric immunoassay (FIAX) was developed and met those needs. The test was compared to other serological tests and in many respects was superior. A commercial fluorometric immunoassay test (TRACK) was evaluated and it also surpassed the standard serological tests. The TRACK was even more sensitive than

FIAX. The major advantage of the FIAX over the TRACK was the ability of the FIAX to correct for nonspecific binding of serum. For this reason, FIAX was selected as the best test system to use in the evaluation of the class and subclass immune response in the serum and vaginal secretions of cattle exposed to S-19 vaccine, B. abortus cell extracts, and virulent S-2308.

Both the serum and vaginal Ig responses were followed throughout the course of vaccination and challenge to determine if any associations in the Ig response and immunity could be determined. Immunity was assessed on protection from abortion and clearing of Brucella infection.

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CHAPTER II

DETECTION OF SERUM ANTIBODY TO *BRUCELLA ABORTUS* IN CATTLE BY USE OF A QUANTITATIVE FLUOROMETRIC IMMUNOASSAY

INTRODUCTION

Bovine brucellosis caused by *Brucella abortus* is an economically important disease associated with abortions and infertility. Despite an active vaccination program with the live bacteria *B. abortus* 19, infections and disease are still prevalent in the United States (8, 12).

Several serological methods are currently used to indicate infection with *B. abortus* in live cattle (1, 11, 14). These include complement fixation (CF), Rivanol precipitation (RIV) and standard tube agglutination (ST) tests. These serological tests rely on secondary reactions, including the ability of antibody to bind complement or to cause agglutination. And, because they require subjective determinations, they are prone to variation among laboratories. There are several factors related to the host-parasite interaction that cause these serological tests to be less than optimal. First, animals in the early stages of infection may not have a detectable serum antibody titer (12). Second, cattle that are chronic carriers of the organism may not have detectable antibody titers to the organism. Other chronic carriers frequently will have a decline in antibody titer before abortion, and

this titer subsequently may remain diminished (4,12). Third, cattle that have received strain 19 vaccine may have titers that are indistinguishable from those of cattle with virulent field strain infection (11 12, 14).

Recently, a semiautomated quantitative fluorometric immunoassay (FIAX; Whittaker M.A. Bioproducts, Walkersville, MD) has been described as a means of detecting serum antibody to viruses, bacteria, fungi, and parasites in humans and animals (3,6,7,16). In cattle, the FIAX system has been reported to quantitate the antibody response to Pasteurella haemolytica, Anaplasma marginale (3, 7), and Pasteurella multocida (R. J. Panciera, R.E. Corstvet, A. W. Confer, and J. A.Rummage, Am. J. Vet. Res., in press). Because the FIAX system is a primary binding assay and is rapid, simple, and inexpensive, adaptation of it as a diagnostic test for bovine brucellosis should be considered.

The purpose of this manuscript is to describe an adaptation of the FIAX test for the detection of antibodies to B. abortus in cattle. Results obtained by the FIAX test are compared with those obtained by three conventional serological tests.

MATERIALS AND METHODS

FIAX TEST. The basic protocol for detecting antibody with the FIAX system has been previously described (3, 6, 7). In general, FIAX is an indirect immunofluorescence test, in which specific fluorescence due to binding of a fluorescein isothiocyanate-conjugated antiglobulin is quantitated as a fluorescent signal unit (FSU) by a fluorometer. Preliminary studies showed optimal conditions for this particular test to be a 1:51 working dilution of unknown serum and a 1:800 dilution of

fluorescein isothiocyanate-conjugated rabbit antibovine immunoglobulin G (IgG) (heavy and light chain specific) (Cappell Laboratories, Cochranville, Pa.).

A linear regression curve was used to convert serum sample FSU obtained from the FIAX fluorometer into nanograms of immunoglobulin binding per StiQ sampler. This curve was calculated by using four known concentrations of purified bovine IgG (Cappell Laboratories), as determined by the Bio-Rad method (Bio-Rad Laboratories, Richmond, Calif.) with bovine albumin as a standard. For each FIAX test, 25 ul of four concentrations (5, 10, 20, and 40 ug/ml) of immunoglobulin, five replicates per concentration, were applied directly to the StiQ sampler and allowed to dry overnight at 37°C. These StiQs were washed in phosphate-buffered saline containing 0.15% Tween 20 for 10 min, followed by incubation in the conjugated antibovine IgG for 20 min, and again washed in phosphate-buffered saline-0.15% Tween 20 for 10 min. An FSU was obtained on all replicates, and the arithmetic mean was calculated for each of the four concentrations of immunoglobulin G. The natural logarithm of the protein concentration (in micrograms) was then plotted against the logarithm of the FSU to obtain a linear regression curve. The Pearson product moment correlation was calculated for the regression ($r = 0.993$), and a significant correlation was found ($P < 0.01$). A mean FSU was calculated from duplicate or triplicate samples for each test serum and plotted on the regression curve to determine micrograms of immunoglobulin binding for each serum sample. These values were multiplied by 1,000 (to convert micrograms to nanograms) and designated as FIAX titers. For the purpose of evaluation, a FIAX titer was defined as

positive when more than 51.5 ng of immunoglobulin was bound per StiQ (highest value obtained for the negative controls).

Antigen. The antigen used for the FIAX test was derived from a soluble B. abortus 1119 antigen (BASA) (2) obtained from the National Veterinary Services Laboratories, U.S. Dept. of Agriculture, Ames, Iowa. The original preparation was obtained from autoclaved cells suspended in distilled water. BASA was modified to BASA-protein (BASA-P) by extensive dialysis, centrifugation, and ammonium sulfate precipitation (L.B. Tabatabai, and B. L. Deyoe, Dev. Biol. Stand., in press). BASA-p contained 0.289 mg of carbohydrate and 2.42 ug of 2-keto-3-deoxyoctulosonic acid per mg of protein (5, 15). BASA-P as suspended in phosphate-buffered saline (0.01 M, pH 7.4) at a concentration of 25 ug of protein per ml as determined by the Lowry method (9) with bovine serum albumin as a standard. Twenty-five microliters of this suspension (0.625 ug) was applied to one side of the StiQ samplers (Whittaker) and allowed to dry overnight at 37°C before use in the FIAX test.

Conventional Tests. The standard serological tests (CF, RIV, and ST) were performed on all serum samples, using standard protocols (1,11). A positive CF test was defined by a 3+ or greater reaction at a serum dilution of 1:10. A positive RIV test was defined as precipitation at a serum dilution of 1:25 or greater. A positive ST test reaction was defined as agglutination at a serum dilution of 1:100 or greater.

Sera. A total of 285 serum samples were used in this study. Of these sera, 90 were taken from 90 cattle 10 to 12 weeks after challenge with ca. 10^7 CFU of virulent B abortus 2308. Of these 90 challenged cattle, 63 had received strain 19 vaccine 8 months before challenge. Eighty-eight sera were from cattle receiving strain 19 vaccine alone: 24

received 10^9 CFU, and 22 received 10^{10} CFU. Sera were collected from these cattle at 1 and 6 months, postvaccination only (22 samples from the low dose and 20 samples from the high dose were available for the 1-month sampling). One hundred and seven sera represented negative controls. These were from cattle that had received neither strain 2308 nor strain 19 and were from certified brucellosis-free herds.

Culture techniques. Standard culture techniques were used to detect B. abortus in the 90 challenged animals, beginning at 14 weeks after challenge (1). The following tissues were collected and cultured for B. abortus: spleen, uterine washings, each quarter of the udder, and parotid, mandibular, retropharyngeal, bronchial, hepatic, prescapular, prefemoral, popliteal, internal iliac, and supramammary lymph nodes. All B. abortus isolated were examined for characteristics of B abortus 19 and 2308. Animals were considered negative when cultures were negative for isolation of B. abortus.

Statistical analyses. Mean antibody titers were compared by multiple t tests. A t test for equal and unequal variances was calculated for the mean titers for each of the comparisons. An F statistic was calculated to determine whether unequal variances were present. If the probability of F was less than 0.05, unequal variances were used in calculating t test values. Cross-tabulation comparisons of positive and negative classifications of sera by the four tests were performed by chi-square analysis. A correlation of serological tests was determined by the Pearson product-moment correlation. Correlations of serological and culture results were also compared by chi-square analysis. All analyses were calculated by using the Statistical Analyses System (SAS) (13).

The sensitivity and specificity of each serological test was calculated: sensitivity = ([number of true positives]/[number of true positives + number of false negatives]) X 100%; specificity = ([number of true negatives]/[number of true negatives + number of false positives] X 100% (10). For the purpose of comparison, culture results were considered absolute in that true positives referred to all cattle that were culture positive and true negatives referred to all cattle that were culture negative. False-negative referred to cattle that were serologically negative but culture positive. False-positive were cattle that were serologically positive but culture negative.

RESULTS

Comparison of mean titers. The mean antibody titers as determined by all four serological tests were significantly different between challenged cattle and negative controls ($P < 0.001$) (Table 1). For all four serological tests, a significant difference ($P < 0.001$) also was observed when the mean antibody titers for challenged cattle were compared with the mean titer for strain 19 vaccinates. There was a significant difference ($P < 0.05$) between mean antibody titers determined by the four tests for strain 19 vaccinates and control cattle.

In the cattle receiving strain 19 only, at 1 month after vaccination there was no significant difference ($P > 0.10$), by any of the four tests, between mean antibody titers for cattle vaccinated with 10^9 CFU and those for cattle vaccinated with 10^{10} CFU (Table 1). In these vaccinated cattle, antibody titers were significantly higher ($P < 0.001$) at 1 month than at 6 months after vaccination.

For analysis, challenged cattle (Table 2) were subgrouped according

to culture status and whether they were vaccinated or nonvaccinated. Analysis of data from all four serological tests for these subgroups of challenged cattle revealed that mean antibody titers for culture-positive cattle were significantly higher ($P < 0.001$) than mean antibody titers for culture-negative cattle. For all tests except FIAX there was a significant difference ($P < 0.05$) between mean antibody titers of vaccinated and challenged versus nonvaccinated and challenged cattle. All four serological tests showed significantly higher ($P < 0.05$) mean antibody titers in animals that were vaccinated and culture-positive than in those that were vaccinated and culture-negative. All four serological tests also showed a significantly higher ($P < 0.05$) mean antibody titers in animals that were nonvaccinated and culture positive than in those that were nonvaccinated and culture negative. No significant difference ($P > 0.05$) was observed in any of the four tests when vaccinated mean titers and nonvaccinate mean titers of culture-negative animals were examined. Of animals that were culture positive, mean titers were significantly higher ($P < 0.05$) in the nonvaccinates as compared with the vaccinates by all tests except FIAX.

Comparison of serological and culture results. Results of culture status versus serological classification for challenged cattle are presented (Table 3). There was a significant association ($P < 0.01$) for contingency comparisons of the FIAX, ST, RIV,and CF tests with culture results. There was a significant association ($P < 0.001$) between the percentage of positive and negative sera detected by each of the four tests in a contingency table analysis. Results of linear regression analysis indicated that there was a significant linear association ($P < 0.0001$) among the four serological tests (Table 4).

Sensitivity and specificity. The FIAX test had the highest sensitivity and the lowest specificity of the four tests examined (Table 5). Specificity was best for the CF test.

DISCUSSION

The results of these studies indicate that the FIAX test is readily adaptable for the detection of antibodies to B. abortus in cattle. The FIAX test readily detected titers due to vaccination or challenge. The FIAX demonstrated low nonspecific binding of antibody as indicated by animals without detectable levels of immunoglobulin binding to the antigen. This may be inherent in the antigen used (BASA-P) and not due to the FIAX test itself. Preliminary results in this laboratory with heat-killed, phenol-preserved B. abortus (standard tube agglutination test antigen) as an antigen source, indicated a higher nonspecific binding of immunoglobulin than was observed with BASA-P. BASA-P has been shown to be adaptable to the enzyme-linked immunosorbent assay as well (L.B. Tabatabai and B. L. Deyoe, personal communication). Therefore, the choice of antigen may be critical.

The sensitivity of the FIAX test was greater than that of the ST, RIV, or CF tests. The specificity of the FIAX test appeared less than the specificity of the other tests. A FIAX titer of more than 51.5 was defined as positive. This value represented the highest titer obtained for negative controls. Allowing a margin between the highest negative control value and the lowest designated positive response would increase the specificity but decrease the sensitivity of the test. The apparent lower specificity of the FIAX test should be examined with care. All 90 cattle used in calculating specificity were challenged with virulent B.

abortus 2308, and the greater number of false-positive (FIAX-positive culture-negative) cattle detected may indicate the ability of the FIAX test to detect cattle harboring low numbers of the organism. The greater number also may have been due to the more sensitive FIAX test identifying residual vaccine titers. Five of the six sera that were false-positive by the FIAX test were from cattle that had been vaccinated, but because of the small sample size, statistical significance ($0.05 < P < 0.06$) could not be demonstrated. Further evidence incriminating residual titers as the cause of FIAX false-positive reactions were seen. When the mean titers of vaccinated and challenged cattle were compared with mean titers of nonvaccinated and challenged cattle, only the FIAX test did not show a significantly lower mean titer in the vaccination group.

The FIAX, ST, RIV, and CF tests all demonstrated a significant ability to differentiate on the basis of mean titer, challenged from vaccinated challenged from control cattle, and vaccinated from control cattle. But because of the overlap of titers among these groups, the FIAX test did not appear any more advantageous than the ST, RIV, or CF test in determining serologically whether an individual animal was infected with virulent strain 2308.

Agreement among the FIAX, ST, RIV and CF tests on classification of an animal as positive or negative was significant on all 285 serum samples. Based on correlation coefficients obtained, there may have been a slightly higher agreement between the FIAX and RIV tests than between FIAX and each of the other two assays. This would be logical because the FIAX and RIV tests primarily detect an IgG response to B. abortus, whereas the ST and CF tests would detect IgM responses as well (1, 12).

From these studies, it can be concluded that there are several advantages to the FIAX test as used in brucellosis serology. A nonlogarithmic endpoint titer can be achieved with one working dilution of serum. The test is rapid and relatively simple to perform. Consistency is maintained in the evaluation of results as no subjective measurements are required. With alterations in reagents, measurement of other classes or subclasses of immunoglobulin would be possible. The FIAX test appears to have greater sensitivity than the ST, RIV, and CF tests.

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TABLE 1. Means and ranges of antibody titers to B. abortus

Status of animal	No. of sera	Test	Antibody titers[a] Range	Mean	SEM
Controls	107	FIAX	0-51.5	3.5	0.8
		RIV	0	0.0	0.0
		CF	0-20.0	0.7	0.4
		ST	0-100.0	3.3	1.2
Challenged (strain 2308)	90	FIAX	0-891.0	163.5	19.4
		RIV	0-400	87.2	13.2
		CF	0-640	97.8	22.4
		ST	0-3,200	474.2	88.2
Vaccination (strain 19)[b]	88	FIAX	0-318.4	34.7	6.5
		RIV	0-200.0	21.3	5.0
		CF	0-80.0	6.1	1.6
		ST	0-400.0	54.8	8.9
10^9 CFU (1-month postvaccinate)	22	FIAX	0-318.4	56.7	17.4
		RIV	0-200.0	33.0	12.7
		CF	0-80.0	10.0	3.9
		ST	0-400.0	62.5	19.9
10^9 CFU (6-month postvaccinate	24	FIAX	0-33.1	6.9	2.5
		RIV	0	0.0	0.0
		CF	0	0.0	0.0
		ST	0-50.0	11.5	3.4
10^9 CFU (1-month postvaccinate)	20	FIAX	0-236.6	76.7	2.8
		RIV	0-200.0	57.5	13.6
		CF	0-80.0	16.0	4.7
		ST	0-400.0	113.8	25.7
10^{10} CFU (6-month postvaccinate)	22	FIAX	0-56.4	4.7	2.8
		RIV	0	0.0	0.0
		CF	0	0.0	0.0
		ST	0-200.0	41.0	9.4

[a]FIAX titers are expressed as nanograms of immunoglobulin binding per StiQ.
[b]No subsequent challenge.

TABLE 2. Antibody titers to B. abortus in challenged cattle

Status of challenged animals	No. of sera	Test	Antibody titers[a] Range	Mean	SEM
Challenged (strain 2308)	90	FIAX	0-891.0	163.5	19.4
		RIV	0-400.0	87.2	13.2
		CF	0-640.0	97.8	22.4
		ST	0-3,200.0	474.2	88.2
Culture (positive)	57	FIAX	0-891.0	235.1	25.5
		RIV	0-400.0	136.8	17.9
		CF	0-640.0	154.4	33.2
		ST	0-3,200.0	736.4	127.2
Vaccination[b]	36	FIAX	0-819.0	277.7	36.6
		RIV	0-400.0	93.1	20.2
		CF	0-640.0	71.7	30.3
		ST	0-1,600.0	313.2	77.7
Nonvaccinated	21	FIAX	5.3-444.5	247.8	30.5
		RIV	0-400.0	211.9	27.6
		CF	0-640.0	296.2	63.3
		ST	0-3,200.0	1461.9	125.3
Culture (negative)	33	FIAX	0-307.8	39.7	11.6
		RIV	0-50.0	1.5	1.5
		CF	0	0.0	0.0
		ST	0-100.0	21.2	3.8
Vaccinated	27	FIAX	0-307.8	40.7	14.1
		RIV	0-50.0	1.9	1.9
		CF	0	0.0	0.0
		ST	0-100.0	20.4	4.4
Nonvaccinated	6	FIAX	23.5-55.5	35.5	5.5
		RIV	0	0.0	0.0
		CF	0	0.0	0.0
		ST	0-50.0	25.0	6.5
Cumulatively vaccinated[b]	63	FIAX	0-891.0	147.6	24.6
		RIV	0-400.0	54.0	12.9
		CF	0-640.0	41.9	17.8
		ST	0-1,600.0	187.7	47.8
Cumulatively nonvaccinated	27	FIAX	5.3-444.5	200.5	29.3
		RIV	0-400.0	164.8	27.5
		CF	0-640.0	230.4	54.6
		ST	0-3,200.0	1142.6	227.0

[a]FIAX titers are expressed as nanograms of immunoglobulin binding per StiQ.

[b]Vaccinated with strain 19 before challenge.

TABLE 3. Comparison of serological and bacteriological results of 90 animals challenged with strain 2308

Test	Result	Bacteriological results[a] (%)	
		Positive	Negative
FIAX	Positive	46.7	6.7
	Negative	16.7	30.7
ST	Positive	43.3	1.1
	Negative	20.0	35.6
RIV	Positive	43.3	1.1
	Negative	20.0	35.6
CF	Positive	38.9	0.0
	Negative	24.4	36.7

[a]Numerous lymph nodes, spleen, uterine washings, and each quarter of the mammary gland were cultured. Culture results were defined as positive when B. abortus S-2308 was isolated.

TABLE 4. Comparison of linear correlations among serological tests

Test comparison	Correlation coefficient (r value)
FIAX vs CF	0.600
FIAX vs RIV	0.722
FIAX vs ST	0.601
CF vs RIV	0.456
CF vs ST	0.681
CF vs FIAX	0.600
RIV vs ST	0.762
RIV vs CF	0.456
RIV vs FIAX	0.722
ST vs FIAX	0.610
ST vs RIV	0.762
ST vs CF	0.681

TABLE 5. Comparison of relative sensitivity and specificity of four serological tests

Test	Sensitivity[a] (%)	Specificity[b] (%)
FIAX	79.2	84.6
ST	76.0	97.1
RIV	76.0	97.1
CF	72.2	100.0

[a] Sensitivity = ([number of true positives]/[number of true positives + number of false negatives]) X 100%.
[b] Specificity = ([number of true negatives]/[number of true negatives + number of false positives]) X 100%.

CHAPTER III

A COMPARISON OF THE TRACK XI™ FLUOROMETRIC IMMUNOASSAY SYSTEM WITH OTHER SEROLOGIC TESTS IN THE DETECTION OF SERUM ANTIBODY TO *BRUCELLA ABORTUS* IN CATTLE

INTRODUCTION

Bovine brucellosis caused by *Brucella abortus* is an economically important disease associated with abortion and infertility. Despite numerous methods of eradication, including vaccination and test and slaughter, the disease has remained prevalent in many areas of the world (8, 12).

Most eradication schemes involve the use of serological tests on live or slaughtered cattle to indicate the presence of *B. abortus* infection within cattle herds. Several of the more commonly used serological tests include the complement fixation (CF), Rivanol precipitation (RIV), and buffered antigen (CARD) tests (11, 12, 15). Several authors describe the use of an enzyme-linked immunosorbent assay (ELISA) for the detection of serum antibodies to *B. abortus*. (3, 5, 16). We have recently described a fluorometric immunoassay (FIAX) to detect serum antibodies to *B. abortus* in cattle (9).

The TRACK XI System (TRACK) is a commercially available

fluorometric immunoassay system (TRACK XI, Daryl Laboratories, Santa Clara, CA) that has been adapted for the detection of antibodies to several antigens in various animal species (10). This manuscript presents the results obtained using the TRACK for detection of serum antibodies to B. abortus in cattle. Antibody responses detected by the TRACK were compared to those obtained from two primary binding assays, FIAX and ELISA, as well as three standard serological tests (CF, RIV, and CARD).

MATERIALS AND METHODS

TRACK test

The TRACK system is an immunofluorescence system in which serum anti-brucella antibodies are bound to B. abortus antigens and detected with specific fluorescein isothiocyanate-conjugated (FITC) antiglobulin. Anti-brucella antibodies are quantitated using the TRACK XI fluorescence reader.

Tests were run on plastic disposable test tracks. The tracks (Daryl Laboratories) have 12 individual wells that are coated with a three-dimensional colloid-phase polymer. The antigen, applied to the wells by the manufacturer, was a soluble extract of B. abortus strain 1119 (S-1119). The antigen (Dr. Richard A. Harte, Personal Communication, Daryl Laboratories, 1986) was prepared as follows. Standard tube test antigen (S-1119, USDA, Ames, Iowa), was diluted 1:75 in 0.066 molar carbonate/bicarbonate buffer, pH 9.0, sonicated, and extracted with 1% sodium deoxycholate in phosphate buffered saline (PBS). The material was centrifuged and the pellet re-suspended in PBS, heated to 100°C for twenty minutes, re-centrifuged at 10,000 g for thirty minutes, and 30 uL

of the supernatant was applied to each track well and allowed to dry.

Tests were conducted at room temperature as follows. The tracks were soaked for 10 minutes in distilled water, then patted dry. Twenty ul of undiluted test serum was applied to a test well containing the colloid bound antigen. High, medium, and low TRACK test control sera were supplied with the test and these TRACK test control sera were run on each track. Following 10 minutes incubation at room temperature and a brisk rinse in distilled water, 20 ul of undiluted FITC-goat antibovine IgG (Daryl Laboratories) was then applied to each well and incubated for 10 minutes. The track was briskly washed in distilled water, patted dry, and read in the TRACK XI fluorometer. A microprocessor within the fluorometer utilized the TRACK test control sera from each track for calculating a linear-linear standard curve of fluorescent signal versus a known value (referred to as a TRACK titer) from the TRACK test control sera on each track. The titers of the nine remaining test samples on the track were extrapolated from the standard curve. In this laboratory, TRACK titers of <29 were determined to be negative using a discriminate analysis method (14).

The reproducibility of the TRACK system was determined using replicates of the TRACK test control sera. Seven tracks were used to test the variation among tracks. Each track used high, medium, and low TRACK test control sera to serve as controls and each track had a high, medium, and low TRACK test control sera to serve as a test sample for determining variability. The variation within a track was measured using a high, medium, and low TRACK test control sera for the standard curve on each of three tracks; the remaining nine wells on each of the

three tracks had one of the three types of TRACK test control sera (high, medium, or low).

The variability of the TRACK system using different antigen preparations and FITC-conjugates were compared using replicates of the high and low control sera. Non-antigen coated blank tracks were prepared in our laboratory with a soluble extract of B. abortus strain 1119 (S-1119) (BASA-d) (2, 17). Thirty ul of PBS (pH 7.2) containing 0.625 ug Basa-d was applied to each of the wells of the blank tracks and allowed to dry overnight at 37°C. The conjugate used was a 1:2 dilution with PBS of FITC-rabbit anti-bovine IgG (heavy and light chain specific) (Cappel, Cooper Biomedical, Malvern, PA). The assays were performed as outlined above and the results obtained compared to those obtained using manufactured antigen-coated tracks and the supplied Daryl Laboratories conjugate.

FIAX test

The FIAX (FIAX, Whittaker M.A. Bioproducts, Walkersville, MD) system, like the TRACK system, is a fluorometric immunoassay. In the FIAX test, 25 ul of PBS (pH 7.2) containing 25 ug/ml BASA-d antigen (17) were applied to a nitrocellulose disc attached to a plastic carrier (StiQ, Whittaker). All StiQs were incubated at room temperature for 30 minutes in 0.51 ml of a 1:51 dilution of test serum, washed for 10 minutes in PBS, containing 0.15% Tween-20, and then incubated with 0.5 ml of a 1:800 dilution of FITC-rabbit anti-bovine IgG (heavy and light chain specific) (Cappel). The StiQ-associated fluorescein was determined in a fluorometer; and FIAX titers, expressed in ng of immunoglobulin binding, were extrapolated from an IgG standard curve. The

mean of duplicate or triplicate samples was used in calculating FIAX titers for each test serum. For purposes of this study, FIAX titers of < 36 ng of immunoglobulin bound per StiQ, based on a discriminate analysis, were termed negative.

ELISA test

The ELISA was performed as previously described (5). One hundred ul of BASA-d in carbonate buffer (pH 9.6) were used to coat wells of a polystyrene microtiter plate (Nunc, Denmark) overnight at room temperature. Following three washes in PBS with 0.05% Tween-20, 100 ul of serum diluted 1:250 in PBS-Tween 20 buffer, containing 1.0% bovine serum albumin (BSA), were added to duplicate wells of the plate and allowed to incubate 1.5 hours at room temperature. Following three additional washes, 100 ul of a 1:400 dilution of horseradish peroxidase conjugated, affinity-purified rabbit anti-bovine IgG (Pel Freeze, Rogers, AR) were added to each well and allowed to incubate for 45 minutes at room temperature. After six washes in PBS Tween-20 buffer, 100 ul of substrate, containing o-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.04% of a 30% solution) in phosphate-citric acid buffer, were added to each well. Plates were incubated in the dark for 30 minutes at room temperature. The reaction was stopped with 40 ul of 0.25 M sulfuric acid. An average OD 490 was determined on duplicate samples and ng of IgG per well determined from a standard curve. Values of < 20 ng of immunoglobulin bound per well were considered negative.

Conventional tests

The standard serological tests (CARD, CF, and RIV) were performed

using standard protocols and reagents (NADL Diagnostic Reagents Manuals 65d and 65e), at the State-Federal Brucellosis Laboratory, Oklahoma City, OK. The standard procedures have been described (1, 11). The standard serological tests were evaluated using the criteria of Deyoe (6). The CARD test was defined as negative if no agglutination was observed. A negative CF test was defined by less than a 3+ reaction at a serum dilution of 1:10. The RIV test was considered negative if no precipitation was observed at a serum dilution of 1:25.

Serum samples

Two hundred and ninety-eight serum samples from cattle used in four separate field trial experiments were tested by each of the serological tests. Of these sera, 134 represented negative controls because they were from heifers that had been neither vaccinated with S-19 B. abortus nor challenged with a virulent field strain (S-2308) of B. abortus and were from dams that were seronegative. Forty-three serum samples were from heifers (these samples represent the same animals that had been used as controls) one month following vaccination with 10^9 or 10^{10} colony forming units (CFU) of S-19. One hundred and twenty-one serum samples (54 of these samples represent animals that had been used as vaccinates and/or controls) were taken from cattle 10-12 weeks following a mid-gestational, intraconjunctival challenge with approximately 10^7 CFU of virulent B. abortus Biotype 1 S-2308 (S-2308 is a standard challenge strain supplied by the National Animal Disease Laboratory, Ames, IA) (6).

Culture techniques

Standard culture techniques (1), were used to detect B. abortus in the 121 challenged animals, either at the time of abortion or 14-18 weeks after challenge. Tissues from the fetus (spleen, stomach contents, lung, mesenteric lymph nodes, and placenta), and dam (milk samples, mammary gland, supramammary lymph node mandibular lymph node, internal iliac lymph node, and spleen) were cultured for B. abortus. Animals were considered culture negative when B. abortus could not be isolated from any of the tissues.

Abortions

Cows were determined as abortion positive if they delivered a dead or weak, premature calf. Cows which delivered healthy calves were termed abortion negative. Abortions were classified as N/A if no calf was delivered or definitive abortion could not be determined.

Sensitivity and Specificity

The sensitivity and specificity (4) was determined using 188 serum samples; 134 were negative controls (disease negative) and 54 were samples from cattle that had been challenged, aborted, and were culture positive (disease positive). A true positive was defined as a serum sample from an animal that was disease positive and seropositive; a true negative was a serum sample from an animal that was disease negative and seronegative. A false negative was defined as a serum sample from an animal that was seronegative and disease positive; a false positive was a serum sample from an animal that was seropositive and disease negative. Sensitivity was defined as [(# of true positives)/(# of true

positives + # false negatives)] X 100%. Specificity was defined as [(# of true negatives)/(# of true negatives + # false positives)] X 100%.

Statistical analysis

The means for the TRACK reproducibility study were compared using multiple two-tailed Student's t-tests (14). A p-value of < 0.05 was considered statistically significant.

RESULTS

Variation of TRACK

The means, standard deviations of the means, and coefficients of variation (CV) of TRACK, using the TRACK test control sera as test samples, are presented (Table 1). A significant difference was observed between high and medium TRACK test control sera and medium and low TRACK test control sera for both among tracks and within tracks. When the means for the among track samples were compared to their respective within-track means, only the middle TRACK test control sera means were not significantly different. The highest CV observed was 16.1% among tracks for the low TRACK test control sera.

The mean titers and standard deviations are presented (Table 2) for replicates of high and low test sera using different antigen preparation and a different conjugate than those supplied by the manufacturer. When the mean titers within the high sera group were compared, significant differences were not observed for values obtained using the Daryl conjugate or the Cappel conjugate. Significant differences were not observed in comparing the mean titers of the commercial-prepared antigen-coated tracks to those of the BASA-d antigen coated tracks

prepared in our laboratory. Within the low serum group, only one combination (TRACK antigen and Daryl conjugate) was significantly different than the other comparisons of antigen and conjugate.

Mean Titers

Means, standard errors of the means (SEM), and ranges of titers are presented by classification groupings of the sera (Table 3) for the TRACK, FIAX, ELISA, CF, and RIV tests. In all five tests, the lowest antibody values were in the control group. The S-19 vaccinated group had mean titers higher than the control group and lower than the challenged group by all five serological tests. Within the challenged group, sera from culture-positive or abortion-positive animals had a higher mean titer than sera from culture negative or abortion negative, respectively.

The percentage of serum samples that were positive by the six serological tests is presented (Table 4). The TRACK, RIV, and CARD tests all had no false positive reactions in the control group. The ELISA had the highest number of positive reactions (11.2%) in the control group. In the vaccinated group, all tests had a similar number of positive sera; the ELISA had the highest (62.8%), and the CF the lowest (44.2%). The TRACK detected the highest number (76.9%) of the sera in the challenged group as positive, whereas the CF and RIV detected the lowest (49.6%). Of the sera from animals that were challenged and subsequently determined to be culture positive, TRACK detected the highest (90.3%); the other five tests were similar with CF (72.4%) being slightly lower than the rest. The RIV (8.9%) detected the least number of positive sera for the group that was challenged and determined to be culture

negative; TRACK (53.3%) detected the most positives in that group. All tests detected greater than 80% of the sera as positive from the group that was challenged and aborted; TRACK (96.4%) had the most positives and ELISA (82.1%) the least. Of the sera from the cattle that were challenged, but did not abort, CF (9.1%) detected the least number of positives and TRACK (54.5%) the most. Of the animals that were challenged, 42 were both culture negative and abortion negative. Twenty-seven of the 42 (Table 5) were seropositive by at least one serological test. The TRACK detected the highest number of positives from this group (21/42), whereas the CF and RIV detected the lowest number of seropositives (2/42). Of the 121 animals that were challenged, 54 were both culture positive and abortion positive. Fourteen of the 54 (Table 6) were seronegative by at least one serological test. The TRACK detected the lowest number of seronegatives (2/14), whereas the ELISA detected the highest number of seronegatives (10/14). The results of the serologic tests for 13 animals that were challenged, culture positive, but did not abort are presented in Table 7. There were two animals that were challenged, were abortion positive, but were culture negative, both of these animals were positive by all five serologic tests.

Sensitivity and Specificity

Sensitivity and specificity of the six serological tests are presented (Table 8). The TRACK had the highest sensitivity (96.3%) and specificity (100%). The ELISA had the lowest sensitivity (81.5%) and specificity (88.8%).

DISCUSSION

The TRACK system is reproducible and has a variance small enough to allow for clear and repeatable distinctions between the high, middle, and low range TRACK test control sera. A statistical difference was observed in both the high and low TRACK test control sera when among track means were compared to within track means, but it is questionable as to its practical significance (less than 15% difference for the high TRACK test control sera). The CV's are all below 17% which appear to compare favorably to results obtained for other serological assays within this laboratory. The variation among antigen preparations or conjugates is not thought to be a major problem in use of the TRACK system. The various combinations of antigen and conjugate appear to perform in a similar manner. This is most likely due to the standard curve which is calculated each time the test is performed. This fitting of a standard curve has been used in the FIAX and ELISA systems and has proven to be a reliable method of correcting for small differences in antigen or conjugate preparations. A preliminary study showed a 1:2 dilution of the Cappel conjugate was required to give results similar to those of the Daryl Laboratories conjugate. It should be noted that one of the low sera was significantly lower than the other three. These lowered values would still be classified as negative and would not affect the interpretation of the test.

In this study, the mean titers were lower in the vaccinate group than in the challenged group. However, on the basis of percentage of positive sera, the TRACK was not any more advantageous than the other assays in discriminating between vaccinal titers and those resulting from field strain infection.

The serological results from challenged cattle were examined by subgroups. By all five serological tests, the mean titers for the challenged, culture-negative cattle were similar to the mean titers for the challenged, abortion-negative cattle. These titers do not represent exact duplication of the same serum samples as 13 of 55 cattle that did not abort were culture positive, and 2 of 56 cattle that aborted were culture negative. Both of these cattle were positive by all five serological tests. By all five serological tests, the culture-positive cattle had lower mean titers than the abortion-positive cattle. Also, there was a lower percentage of seropositive animals, by all five tests, in the culture-positive group than in the abortion positive group. Therefore, the abortion-negative animals lowered the percent positive and mean titer of the culture positive group. Isolation of B. abortus from a heifer should, in most cases, represent infection, but the above serological data may indicate that cattle may asymptomatically or latently carry B. abortus without pathological or serological reactions as previously described (7, 12), and this possibility should be considered when evaluating serological data.

In Brucella abortus infections, sensitivity and specificity are difficult to define in a manner that is meaningful and beyond reproach. Because of the possibility of animals asymptomatically carrying B. abortus rather than having a pathological infection, the disease positives used in calculating sensitivity were strictly defined to be the sera from cattle that had been challenged, were culture positive, and had aborted. When evaluating sensitivity and specificity we are always defining it in terms of another test or tests. Some tests are generally regarded as incorrigible evidence, such as a bacteriological or

histopathological diagnosis (13), but even these are not infallible, especially in the case of negative results. It would seem that animals that aborted and were culture positive could well be classified as disease positive. It is also possible for an animal to be infected and even diseased and yet for numerous reasons the organism not be isolated. It is for these reason that challenged animals were excluded from the group of disease negative group regardless of culture or abortion status. Disease negatives were strictly defined as cattle that had been neither challenged nor vaccinated. The values obtained for sensitivity and specificity should be used for accurately comparing the relative sensitivity and specificity among the various tests. It is questionable whether the absolute values obtained from such strictly defined populations would extrapolate to the population(s) of cattle at large. Using these criteria, the TRACK had good sensitivity and excellent specificity. Only two serum samples from cattle that were challenged, culture positive, and abortion positive were seronegative by the TRACK test (Table 6), and both of these were negative by all other serological tests. There would appear to be a loss of specificity in the TRACK (Table 4) if the percent positives for the culture-negative (53.3%) and abortion-negative (54.5%) cattle were considered. The specificity of the TRACK is excellent for the 134 control samples that had been neither challenged nor vaccinated, thus the apparent loss of specificity may well be explained by high sensitivity of the TRACK. The TRACK probably is detecting residual titers from the initial challenge or possibly titers resulting from levels of infection below the threshold of detection by standard serological or culture techniques.

The TRACK XI is a rapid, simple, and relatively economical system

that is available commercially for use in the detection and quantitation of serum antibodies to several diseases. Although the test for Brucellosis is not currently available commercially, tracks can be supplied by the manufacturer without antigen, and the TRACK XI system can be adapted to using other B. abortus antigen preparations and conjugates. The results indicate that the TRACK XI is both reproducible and accurate. The results compare favorably with other serological methods. The TRACK XI system is faster than either the ELISA or FIAX systems. The TRACK XI system is simple because dilution of serum is not needed. The TRACK XI appears to be more sensitive than the FIAX, ELISA, CF, CARD, and RIV tests, and more specific than the ELISA and CF tests.

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TABLE 1. Variation of TRACK test control sera within the same track and among different tracks

		Mean[a]	SD[b]	CV[c]
	Within tracks			
High	TRACK test control sera $_{n=9}$	225.0	24.8	11.0
Middle	TRACK test control sera $_{n=9}$	37.4	4.3	11.5
Low	TRACK test control sera $_{n=9}$	8.2	0.7	8.1
	Among tracks			
High	TRACK test control sera $_{n=7}$	263.4	18.2	6.9
Middle	TRACK test control sera $_{n=7}$	39.7	4.3	10.8
Low	TRACK test control sera $_{n=7}$	11.7	1.9	16.1

[a]Mean TRACK titer
[b]Standard deviation of the mean
[c]Coefficient of variation

TABLE 2. Variation of TRACK titers using different conjugates and antigen preparations

Antigen	Conjugate Source	Sera	Titer	SD
BASA-d[a]	CAPPEL[c]	HIGH n=5	193.6	14.3
BASA-d	DARYL	HIGH n=8	199.7	6.9
TRACK[b]	CAPPEL[d]	HIGH n=8	208.3	14.6
TRACK	DARYL	HIGH n=5	198.8	12.0
BASA-d	CAPPEL	LOW n=5	17.5	7.0
BASA-d	DARYL	LOW n=8	17.1	2.2
TRACK	CAPPEL	LOW n=8	11.6	6.3
TRACK	DARYL	LOW n=5	2.1	4.2

[a]BASA-d - Brucella abortus soluble antigen as used in FIAX and ELISA 0.625ug per track well.

[b]TRACK - Commercially prepared and applied antigen.

[c]CAPPEL - a 1:2 dilution with PBS of Cappel rabbit anti-bovine IgG (heavy and light chain).

[d]DARYL - supplied with kit, non-diluted goat anti-bovine IgG (heavy and light chain).

Table 3. Means, standard errors of the means, and ranges of antibody to B. abortus

Group		TRACK[a] Mean	TRACK Sem	TRACK Range	FIAX[b] Mean	FIAX Sem	FIAX Range	ELISA[b] Mean	ELISA Sem	ELISA Range	CF[c] Mean	CF Sem	CF Range	RIV[c] Mean	RIV Sem	RIV Range
Controls n=134		2.2	0.4	25.0	1.4	0.5	51.5	12.9	0.6	43.7	1.0	0.3	20.0	0.0	0.0	0.0
Vaccinated S-19 n=43		39.7	4.1	125.0	65.9	11.5	318.4	47.0	6.2	190.7	13.5	3.0	80.0	44.8	9.1	200.0
Challenged S-2308 n=121		105.5	6.6	289.0	170.4	17.4	891.0	92.1	7.7	361.0	92.8	15.4	640.0	91.5	9.9	400.0
Challenged S-2308 n=121	Chal. 2308 Culture (+) n=76	141.1	7.0	288.0	241.1	22.9	891.0	118.9	9.4	347.0	144.6	22.5	640.0	137.2	12.4	400.0
	Chal.2308 Culture (-) n=45	45.4	7.0	229.0	51.0	14.0	389.4	46.9	10.5	360.0	5.3	2.7	80.0	14.4	7.6	200.0
Challenged S-2308 n=121	Chal. 2308 Abortion (+) n=56	162.7	6.3	284.0	305.1	25.7	891.0	131.0	10.4	345.0	192.0	27.9	640.0	180.8	12.2	400.0
	Chal. 2308 Abortion (-) n=55	47.0	6.2	202.0	45.5	11.0	342.5	54.9	10.5	361.0	6.2	3.4	160.0	13.6	6.3	200.0
	Chal. 2308 Abortion N/A[d] n=10	106.9	17.8	164.0	103.0	29.7	237.7	79.3	23.1	201.5	14.0	8.5	80.0	20.0	11.1	100.0

[a]TRACK titer.
[b]ng immunoglobulin bound.
[c]Titer 1/serum dilution.
[d]Abortion status not confirmed.

TABLE 4. Percent positive serum samples in each classification group by six serological tests

	TRACK	FIAX	ELISA	CF	RIVANOL	CARD
Controls n=134	0.0	0.7	11.2	6.0	0.0	0.0
Vaccinated S-19 n=43	62.8	55.8	67.4	44.2	58.1	58.1
Challenged S-2308 n=121	76.9	59.5	62.8	49.6	49.6	53.7
Culture (+) n=76	90.8	77.6	76.3	72.4	73.7	77.6
Culture (-) n=45	53.3	28.9	40.0	11.1	8.9	13.3
Abortion[a] (+) n=56	96.4	89.3	82.1	92.9	91.1	91.1
Abortion[b] (-) n=55	54.5	27.3	40.0	9.1	10.9	14.5
Abortion[c] N/A n=10	90.0	70.0	80.0	30.0	30.0	60.0

[a]54/56 Abortion (+) were culture positive.
[b]13/55 Abortion (-) were culture positive.
[c]Abortion status not confirmed.

TABLE 5. Serological reation of twenty-seven[a] challenged, culture-negative, abortion-negative cattle

	TRACK	FIAX	ELISA	CF	RIVANOL	CARD
	+	+	+	-	-	-
	-	+	+	-	-	-
	-	-	+	-	-	-
	-	-	+	-	-	-
	+	-	-	-	-	-
	-	-	+	-	-	-
	+	-	-	-	-	-
	+	-	-	-	-	-
	+	-	-	-	-	-
	+	-	+	-	-	-
	+	-	+	-	-	-
	+	-	-	-	-	-
	+	-	+	-	-	-
	+	-	-	-	-	-
	+	-	-	-	-	-
	+	+	+	-	-	-
	+	+	-	-	-	-
	+	+	+	-	-	+
	+	+	+	-	-	+
	+	+	+	-	+	+
	+	-	-	-	-	-
	+	-	+	-	-	-
	+	+	+	+	+	+
	-	+	-	-	-	-
	+	-	+	+	-	-
	-	+	-	-	-	-
	+	+	-	-	-	-
Total Positive	21	11	15	2	2	4

[a]27/42 Challenged, culture-negative, abortion-negative cattle were positive by one or more serological test(s).

+Indicates a positive serological reaction.

-Indicates a negative serological reaction.

TABLE 6. Serological reation of fourteen[a] challenged, culture-positive, abortion-positive cattle

	TRACK	FIAX	ELISA	CF	RIVANOL	CARD
	+	+	-	+	+	+
	+	+	-	+	+	+
	+	+	-	+	+	+
	+	+	-	+	+	+
	+	+	-	+	+	+
	+	-	+	-	-	-
	+	+	-	+	+	+
	+	+	-	-	+	+
	+	+	-	+	+	+
	-	-	-	-	-	-
	+	-	+	+	-	-
	+	-	+	+	-	-
	+	-	+	+	+	+
	-	-	-	-	-	-
Total Negative	2	6	10	4	5	5

[a]14/54 Challenged, culture-positive, abortion-positive cattle were negative by one or more serological test(s).

+ Indicates a positive serological reation.

- Indicates a negative serological reaction.

TABLE 7. Serological reation of thirteen challenged, culture-positive, abortion-negative cattle

	TRACK	FIAX	ELISA	CF	RIVANOL	CARD
	+	-	+	-	-	-
	+	+	+	-	+	-
	-	-	-	-	-	-
	-	-	+	-	-	-
	+	-	-	-	-	-
	+	-	+	-	-	-
	-	-	-	-	-	-
	+	-	+	-	-	+
	+	+	-	+	+	+
	+	-	+	+	+	+
	-	+	-	-	-	-
	+	+	+	+	+	+
	+	-	-	-	-	-
Total Positive	9	4	7	3	4	4

+ Indicates a positive serological reation.
- Indicates a negative serological reaction.

TABLE 8. Sensitivity and specificity of six serological tests

	Sensitivity[a] (%)	Specificity[b] (%)
TRACK	96.3	100.0
FIAX	88.9	99.3
ELISA	81.5	88.8
CF	92.6	94.0
RIVANOL	90.7	100.0
CARD	90.7	100.0

Sera n=188 : 54 Cattle were challenged, culture positive, and aborted, 134 heifers were controls.

[a]Sensitivity = ([# of true positives]/[# of true positives + # of false negatives]) X 100%.

[b]Specificity = ([# of true negatives]/[# of true negatives + # of false positives]) X 100%.

CHAPTER IV

THE IMMUNOGLOBULIN CLASS AND SUBCLASS OF ANTIBODIES TO B. ABORTUS FOLLOWING S-19 VACCINATION AND CHALLENGE

INTRODUCTION

Bovine brucellosis caused by B. abortus is a disease causing abortions in cattle and substantial economic loss to cattle producers. Despite numerous methods of eradication, including vaccination, and test and slaughter, the disease has remained prevalent in many areas of the world (11, 20, 27).

The use of B. abortus strain 19 (S-19) vaccine in cattle does provide protection from abortion, but its use can cause cattle to become seropositive or even develop persisting infections with S-19 (7, 9). Most eradication schemes use serological tests to identify cattle infected with B. abortus, and many authors have reviewed the merits of various tests (2, 20, 27). Another method of identifying infected cows that has been used is the vaginal mucus agglutination test (13, 14, 18, 24, 25).

It has been reported that different serum antibody classes and subclasses (IgG1, IgG2, IgM, IgA) may react in some serological tests, but may inhibit the reaction of other tests (1, 3, 4, 19, 21, 22, 23, 30). Several authors reported that identification of the class or

subclass of immunoglobulin present may be of some predictive value in determining if an animal is infected with virulent B. abortus or has an antibody titer induced by vaccination (6, 16).

A fluorometric immunoassay system (FIAX) for the detection of serum antibodies to B. abortus in cattle has been previously described (12). This manuscript describes a modification of that test for the detection and semiquantification of the Brucella-specific class and subclasses of serum immunoglobulin (Ig) produced by cattle in response to S-19 vaccine and subsequent challenge with virulent organisms. The FIAX was used also in semiquantitating the vaginal Brucella-specific class and subclass Ig response following challenge with virulent B. abortus strain 2308 (S-2308). The results are compared with two primary binding assays (FIAX and ELISA, both using nonclass specific conjugates) and three secondary binding assays rivanol (RIV), complement fixation (CF), and a buffered Brucella antigen test (CARD).

MATERIALS AND METHODS

Immunologic Reagents

Rabbit anti-bovine IgA serum was obtained from the National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, IA. This antiserum was obtained from rabbits immunized with bovine IgA heavy chain. The whole rabbit serum was lyophilized and stored at 4°C until reconstituted. The working dilution was 1:200 with 0.10 m phosphate buffered saline (pH 7.2) containing 0.15% v/v Tween-20 (PBS-Tween). When diluted 1:200 the antiserum contained 157.2 ug protein/ml as determined by A_{280}/A_{260} (17) using the formula: protein (mg/ml) = 1.45 X A_{280} - 0.74 X A_{260}.

Rabbit anti-bovine IgM (Mu chain specific) serum (Cooper Biomedical, Inc., Malvern, PA) was reconstituted with distilled water. To isolate the Ig, an equal volume of saturated ammonium sulfate (4°C) was slowly mixed into the antisera, and the antisera was centrifuged (Beckman J-6B, 2000 x g) for 20 minutes until the supernatant was clarified. The supernatant was discarded and double the initial antiserum volume of a 50% saturated ammonium sulfate solution was thoroughly mixed with the protein pellet. The mixture was centrifuged as before and the supernatant discarded. The protein pellet was then dissolved into a final volume of distilled water equal to the initial antiserum volume and placed in 12-14K M.W. dialysis tubing (Spectrapor membrane tubing, Spectrum Medical Industries, Los Angeles, CA). The Ig was dialyzed against four changes of a 0.2 M carbonate/bicarbonate buffer (pH 9.5). The total protein content was determined using A_{280}/A_{260} and 0.05 mg of fluorescein isothiocyanate isomer I (FITC) (Sigma Chemical Co., St. Louis, MO) was added for each mg of protein. After a 1 hour incubation at room temperature, the pH was lowered to 7.3 with 1 N HCL. The conjugate was then dialyzed against four changes of PBS (pH 7.3) and placed on a Sephadex G-50,80 (Sigma Chemical Co., St. Louis, MO) column measuring 20 mm x 40 cm. Fractions containing greater than 0.2 mg of protein/ml were pooled in 12-14 k dialysis tubing and concentrated with Aquacide II-A (Calbiochem, San Diego, CA). Total protein and molar fluorescein/protein ratio (F/P) were calculated using A_{495}/A_{280} (29). The conjugate, containing 1.64 mg protein/ml at a F/P of 8.64, was aliquoted and frozen at -70°C until use. Just prior to use, the conjugate was diluted with PBS-Tween to a working concentration of 80 ug protein/ml.

Rabbit anti-bovine IgG1 (Miles Laboratory, Elkhart, IN) Ig was precipitated from whole serum with ammonium sulfate as described above and the final pellet redissolved in PBS to equal its initial serum volume. An affinity column was prepared by dissolving 94 mg of serum IgG2 (supplied by National Animal Disease Center) in 5 ml of PBS and allowing it to react with 5 ml of Affi-gel 15 (Bio-Rad Laboratories, Richmond, CA) active ester agarose beads for 4 hours at 4°C. Twenty-three mg of protein (IgG2) was recovered in three PBS washings of the agarose beads for a binding efficiency of 75%. The agarose beads were packed in a K 10/10 column (Pharmacia, Uppsala, Sweden) and the column equilibrated with PBS. The rabbit anti-bovine IgG1 was placed on the column (to remove IgG2 activity) and run in continual reflux at a rate of 6 ml/hour for 24 hours. The unbound anti-IgG1 antibodies were collected, using PBS as an eluent, until the A_{280} returned to baseline. The column was later cleared of bound Ig using distilled water, 50 mm acetic acid, and 6 M guanidine HCL; this fraction was discarded as it was not IgG2 specific as determine in further studies. The affinity purified anti-IgG1 immunoglobulin was then dialyzed into carbonate/bicarbonate buffer (pH 9.5) and conjugated with FITC as described above. The conjugate, containing 1.95 mg protein/ml at a F/P of 5.31, was aliquoted and frozen at -70°C until use. Prior to use, the conjugate was diluted with PBS-Tween, to a working concentration of 40 ug protein/ml.

Rabbit anti-bovine IgG2 serum was obtained from the National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, IA. This antiserum was obtained from rabbits immunized with bovine serum IgG2 heavy chain. The immunoglobulins were precipitated with ammonium sulfate and conjugated with FITC as described

for the rabbit antibovine IgM antisera. The conjugate, containing 2.29 mg protein/ml at an F/P of 2.18, was aliquoted and frozen at -70°C until use. Prior to use, the conjugate was diluted with PBS-Tween, to a working concentration of 20 ug protein/ml.

FITC-sheep anti-rabbit IgG (heavy and light chain specific) (Cooper Biomedical Inc., Malvern, PA) was diluted with PBS-Tween to a working dilution of 1:400. At that dilution, it had a F/P of 3.13 and contained 38 ug protein/ml.

FITC-rabbit anti-bovine IgG (heavy and light chain specific) (Cooper Biomedical Inc., Malvern, PA) was used to quantitate total Ig in vaginal samples. It was diluted with PBS-Tween to a working dilution of 1:400. At that dilution, it had a F/P of 2.60 and contained 17.59 ug protein/ml.

FIAX Tests

In general, FIAX is an indirect immunofluorescence test, in which specific fluorescence, due to binding of a FITC-antiglobulin, is quantitated by a fluorometer as fluorescence single units (FSU). The protocol for detecting B. abortus-specific antibody with the FIAX system has been previously described (12). Modifications of the FIAX test were done so that serum and vaginal class and subclass Ig could be semiquantitated. The antigen used in all FIAX tests for the detection of B. abortus specific antibody was a soluble extract of B. abortus strain 1119 (S-1119) (BASA-d) (5, 28). Twenty-five ul of PBS (pH 7.2) containing 25 ug/ml BASA-d antigen were applied to one side of a nitrocellulose disc attached to a plastic carrier (StiQ, Whitaker Biomedical), the other side of the StiQ served to measure nonspecific binding (background).

The StiQs were dried overnight at 37°C. All subsequent reactions with the FIAX system were at room temperature. The StiQs were incubated for 30 minutes with 0.51 ml of a 1:51 dilution (PBS-Tween) of test serum or vaginal mucus extract. In the case of vaginal IgA and vaginal IgM, 0.55 ml of a 1:11 dilution of vaginal mucus extract was used. The StiQs were then washed for 10 minutes in 0.6 ml of PBS-Tween, and then incubated for 20 minutes with 0.5 ml of appropriate antiglobulin. For the standard (not Ig subclass specific) FIAX, a 1:800 dilution of FITC-rabbit anti-bovine immunoglobulin G (IgG) (heavy and light chain specific) (Cappel, Cooper Biomedical Inc., Malvern, PA) was used. Following a 10 minute wash in 0.6 ml PBS-Tween, the FSU standard FIAX was determined. The Ig class specific reagent concentrations are described under their respective headings. The class specific FIAX tests were incubated 20 minutes in 0.5 ml of FITC-sheep anti-rabbit IgG, and then washed 10 minutes in 0.6 ml of PBS-Tween. The StiQ associated fluorescence was then determined using the fluorometer, and FSU calculated by subtracting the background fluorescence from the antigen specific fluorescence.

Test control sera and control vaginal mucus extracts were assigned nanogram equivalents of Ig binding values based on a linear regression of FSU of Ig vs. nanograms of Ig present on the StiQ. Each class or subclass test was based on commercially-prepared purified bovine Ig. A linear regression was then performed for the control samples vs. test samples allowing a standardized nanogram equivalents of Ig binding (FIAX titer) to be calculated for each of the test samples.

Total Vaginal Ig

The total quantity of Ig present in the vaginal mucus extract was

approximated using the FIAX system. Twenty-five ul of extract from each sample was placed on a StiQ and allowed to dry overnight at 37°C. The StiQs were then washed for 10 minutes in 0.6 ml PBS-Tween to remove any unbound material. The StiQs were allowed to react with 0.5 ml of rabbit anti-bovine IgG (heavy and light chain) for 20 minutes. Following another 10 minute wash in 0.6 ml of PBS-Tween, the FSU were measured using the fluorometer and compared to an IgG standard curve.

Antiglobulin Class or Subclass Specificity

Bovine IgG1 whole molecule (Pel-Freez, Rogers, AR), ChromPure bovine IgG, whole molecule Gamma 2 subclass (Jackson Immunoresearch Laboratories, Inc., Avondale, PA), bovine IgM (Pel-Freez), and bovine IgA radial immunodiffusion reference standard (Miles Laboratories, Elkhart, IN) were used in preparing the standard curve for nanogram equivalents of Ig binding as described above.

The Ig (except IgA) were also used in determining the degree of cross reaction among the conjugates and other immunoglobulin classes and subclasses. Triplicate samples of 625.0 ng of BASA-d, IgG1, IgG2, and IgM were applied to StiQs for each antiglobulin to be tested and allowed to dry overnight at 37°C. These StiQs were then tested by FIAX using the protocols outlined for each of the antiglobulins; the StiQs entered the test at the first wash step. The cross reactions of the conjugates with other immunoglobulins were expressed as a percent FSU relative to the FSU of an equal amount of immunoglobulin to which the conjugate was directed.

ELISA test

The ELISA was performed as previously described (7). One hundred ul of BASA-d in carbonate buffer (pH 9.6) were used to coat wells of a polystyrene microtiter plate (Nunc, Denmark) overnight at room temperature. Following three washes in PBS with 0.05% Tween-20, 100 ul of serum diluted 1:250 in PBS-Tween 20 buffer, containing 1.0% bovine serum albumin (BSA), were added to duplicate wells of the plate and allowed to incubate 1.5 hours at room temperature. Following three additional washes, 100 ul of a 1:400 dilution of horseradish peroxidase conjugated, affinity-purified rabbit anti-bovine IgG (Pel Freeze, Rogers, AR) were added to each well and allowed to incubate for 45 minutes at room temperature. After six washes in PBS Tween-20 buffer, 100 ul of substrate, containing o-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.04% of a 30% solution) in phosphate-citric acid buffer, was added to each well. Plates were incubated in the dark for 30 minutes at room temperature. The reaction was stopped with 40 ul of 0.25 M sulfuric acid. An average A_{490} was determined on duplicate samples and ng of IgG per well determined from a standard curve.

Conventional tests

The standard serological tests (CARD, CF, and RIV) were performed using standard protocols and reagents (NADL Diagnostic Reagents Manuals 65d and 65e (2, 20, 27). These standard serological tests were evaluated using the criteria of Deyoe (9).

Serum Samples

The sera used in this study were sequential samples from 69

crossbred beef heifers used in a S-19 field trial experiment (7). Sera were taken from all cattle at the start of the experiment (Day 0). Two-thirds of the cattle were vaccinated with 10^9 or 10^{10} B. abortus S-19 and were referred to as vaccinates; the remaining one-third were unvaccinated controls. Serum samples were taken at 1 month following vaccination (1MPV) and at 6 months following vaccination (6MPV). Six to eight months after vaccination the cattle were bred by natural service. The cattle were then given a midgestational, intraconjunctival challenge with 9.4×10^6 or 5.2×10^7 CFU with virulent B. abortus S-2308. Serum samples were taken 3 months following challenge (3MPC). Serum samples were stored frozen at -20°C. At the time of this study, serum samples were no longer available on all cattle for each of the bleeding dates.

Vaginal Mucus Samples

Approximately 3 - 4 months following abortion or normal calving, vaginal samples were collected on 27 of the cattle using Tampax Superplus tampons (Tambrands Inc., Lake Success, NY). The vulva was cleaned with a mild detergent and the tampons were manually placed 10 - 12 cm anterior and ventrally within the vagina. The tampons were removed after 15 - 30 minutes. Ten ml of PBS containing 0.1% sodium azide was placed on each tampon. After one hour the tampons were squeezed in a 60 ml plastic syringe and the vaginal mucus extract was collected and frozen at -20°C until use. Corrected vaginal values were calculated for each sample by the formula (ng class or subclass specific Ig bound/ng vaginal Ig) x 100%. Vaginal samples from 11 adult, calfhood vaccinated dairy cows were used as negative controls; these cattle were

from a certified brucellosis free herd and all were seronegative by standard serological tests, ELISA, and FIAX.

Statistical analysis

Mean antibody titers were compared by multiple t tests. A t test for equal and unequal variances was calculated for the mean titers for each of the comparisons. An F statistic was calculated to determine whether unequal variances were present. If the probability of F was less than 0.05, unequal variances were used in calculating t test values. Cross-tabulation comparisons of positive and negative classifications of sera, by CARD, were performed by chi-square analysis (26).

RESULTS

Immunologic purity of reagents

A table of the relative immunologic reactivity of the antiglobulins with different antigens is presented (Table 1). Sheep anti-rabbit IgG did not appear to react with BASA, and the reactions with bovine immunoglobulin were minimal. The commercial rabbit anti-bovine IgG was capable of detecting IgG1, IgG2, and IgM; it best detected IgG2, followed by IgG1 (63% of IgG2), and IgM (14.5% of IgG2). Rabbit anti-bovine IgA cross reacted with IgG1 (7.3% of IgA), slightly more than with IgG2 or IgM. Rabbit anti-bovine IgG1 had a moderate cross reaction with IgG2 and IgM (19.8% and 8.4% of IgG1 respectively). The rabbit anti-bovine IgG1 was tested against the same lot of IgG2 that had been used in the affinity column, and a FSU of 0.0 was obtained. Rabbit anti-bovine IgG2 mildly cross reacted with IgG1 and IgM (both were $< 3.8\%$ of IgG2). Both IgG1 and IgG2 had minimal cross reaction when tested

with rabbit anti-bovine IgM (<3.0%). Reproducibility of class-specific assays were in general better with replicates of serum samples than with replicates of vaginal mucus samples (Table 2).

The samples used in gaining the fluorometer to a fluorescence signal of 160 are identified in Table 2. The difference between 160 and the mean FSU values presented represents the mean background (Table 2). The test for detecting serum anti-B. abortus IgM had the highest background (more than double any of the other serum tests). The background for the vaginal tests was slightly higher than for serum tests with the exception of IgM. In the absence of sera or vaginal mucus extracts, rabbit anti-bovine IgM (data not shown) did not have background levels that were any higher than the other conjugates.

Mean antibody titers

The mean titers or percent positive of the standard serological tests are presented (Table 3). By each standard test, the S-19 vaccinates had higher antibody responses 1MPV than the nonvaccinated controls or prevaccination samples. By 6MPV, most mean antibody titers had returned to the prevaccination titers. However, the CARD still detected 13% of the samples as positive. At 3MPC, both the vaccinates and nonvaccinates had mean antibody titers that were higher than the 1MPV S-19 vaccinates. The nonvaccinates had higher mean antibody titers than the vaccinates by all tests, even when the culture positive abortion positive subgroups were compared. Of these subgroups, the FIAX and RIV had significantly ($p < 0.05$) higher titers and CARD had significantly more positive ($p < 0.05$) samples than in the nonvaccinated subgroup.

In general, the serum titers from the culture-negative, abortion-negative animals (3MPC) were less than those observed 1MPV and slightly more than prevaccination levels.

Table 4 presents the mean ($\pm$ SEM) B. abortus-specific ng of Ig binding for the class and subclass Ig. In general, the responses observed were similar to those of the standard serological tests. All classes and subclasses of Ig had higher ($p < 0.05$) mean titers in the S-19 vaccinates 1MPV than in the nonvaccinates 1MPV or prevaccinate samples. By all serologic tests the mean titers for culture positive, abortion positive nonvaccinates (3MPC) were significantly ($p < 0.05$) higher than those of vaccinates 1MPV. The mean titers for culture positive abortion positive vaccinates (3MPC) were significantly ($p < 0.05$) higher than those of vaccinates 1MPV by all tests except IgM. When titers for vaccinates and nonvaccinates were compared for 3MPC culture-positive and abortion-positive, all tests were higher in the nonvaccinate subgroup, IgG1 and IgA were significantly ($p < 0.05$) higher.

The mean ng for the Ig class and subclasses and the total vaginal Ig for the vaginal samples and corrected vaginal samples are presented in Table 5. Significant differences ($p > 0.05$) were not observed for total vaginal Ig among the different groupings and subgroupings.

The vaginal Ig means were similar to those obtained by correcting for the total Ig measured in the vaginal samples. Vaginal IgM titers were minimal and were only slightly greater than for the negative controls. Although not statistically significant ($p > 0.05$), both vaginal IgG1 and vaginal IgG2 titers appear to be higher in the culture positive, abortion positive vaccinates than in the nonvaccinates. In

general, abortion negative animals had lower vaginal titers irrespective of culture status.

DISCUSSION

The immunologic purity of reagents is essential when testing for class and subclass of Igs. In preliminary tests, many of the commercially obtained antisera and affinity-purified antiglobulins were not class or subclass specific using the FIAX system (data not shown), yet appeared to be specific when analyzed using immunoelectrophoresis or immunodiffusion. Although the latter test systems may serve as good screening methods, reagent purity should be demonstrated using the same test system and same dilutions of reagents that are to be used in a specific test.

In the present study, the major cross reaction observed (Table 1) was rabbit anti-bovine IgG1 reacting with IgG2. After purification, that antiglobulin did not react in the FIAX system with the IgG2 that had been used in the affinity column. This cross reaction could be explained by the IgG2 preparation containing a small amount of IgG1 or by some light chain activity remaining in the antiglobulin.

A large amount of background was observed in the test for IgM antibodies in serum (Table 2). This could indicate nonspecific binding of a serum protein (probably IgM) to the nitrocellulose on the StiQ and subsequent detection by the conjugate. To be detected, the protein would then have to also bind rabbit anti-bovine IgM. Bovine IgM has been described to bind nonspecifically to lipopolysaccharide antigens (15). The extent to which IgM bound nonspecifically to the antigen (BASA) in our assays was not determined, but it was not so extensive as to inhibit

discrimination of serum antibody titers in nonvaccinates from those of S-19 vaccinates or S-2308 challenged animals.

The vaginal IgM test had a much lower background than the serum test, this could be caused by minimal vaginal IgM being present. Our findings indicate minimal vaginal B. abortus-specific IgM, even in the presence of vaginal antibodies in the IgG1, IgG1, and IgA classes (Table 5). This is consistent with other studies that showed that total vaginal IgM levels were detectable only occasionally and, when present, coincided with elevated levels of IgG and serum albumin (10).

The results obtained for the primary binding assays were expressed in ng equivalents Ig binding. The tests did not appear to be strictly quantitative. Evidence of this is seen in Table 5 where the ng titers of B. abortus specific Ig sum to larger values than the total Ig; and in Tables 4 and 5, whereby the ng titers of the subclass were greater than the standard FIAX. This is not surprising because different conjugates were used at different concentrations and standardized using different immunoglobulin preparations. It is evident from Table 1 that rabbit anti-bovine IgG detected IgG2 more efficiently than IgG1. Because of this a strict quantitative comparison of amounts of Ig detected among different Ig classes should be viewed with caution, but the data do not indicate that IgG1 predominates as the major class of Ig produced as has been previously reported (3, 4, 6). Our findings of elevated IgG2 levels in infected animals were consistent with those recently reported by Lawman (16). It should be noted that different serological techniques have been used by other investigators and differences in results may be due to differences in the techniques used as opposed to actual differences in the responses of cattle.

The difference between the serum IgA titers of the vaccinates and nonvaccinates 1MPV was minimal, but statistically significant ($p < 0.05$). The S-19 vaccine contains live organisms, and a temporary infection of mucosal surfaces or the mammary gland may occur and induce Brucella-specific IgA production. Our findings would indicate minimal IgA antibodies were produced after S-19 vaccination, as compared to IgG1, IgG2, and IgM antibodies, and marked production of IgA antibodies after challenge. However, Beh (4) did not detect Brucella-specific IgA in sera from an infected cattle herd using single radial immunodiffusion. The reason for this discrepancy could be that the FIAX system detects smaller quantities of Ig than immunodiffusion. Although too few animals were present in this study to be conclusive, it appeared that high levels of serum IgA corresponded to abortion positive cattle (3MPC) possibly due to intense infection of mucosal surfaces in these cattle.

In both vaccinates and nonvaccinates, IgM titers appeared to be quite elevated as late as 3MPC. The nonvaccinates had higher levels of IgM antibodies than vaccinates. This is not surprising in that the challenge with S 2308 was the initial exposure of the nonvaccinates to B. abortus antigens and would stimulate a primary antibody response.

Most reports of B. abortus vaginal mucus tests (13, 14, 18, 24, 25) do not quantitate the titers in terms of total Ig. In studies of the vaginal mucus from cattle infected with Campylobacter fetus (8), changes occurring during estrus were due primarily to differences in vaginal water content rather than in solid content, including antibody protein. Presumably, if the tampons are extracted into a consistent volume, the total Ig should remain relatively consistent. In the present study, the

changes in mean titers obtained by correcting for total vaginal Ig were minimal.

At 3MPC generally higher serum titers were associated with culture-positive animals without regard to abortion, and higher vaginal titers were associated with abortion-positive animals without regard to culture. The high vaginal mucus titers in abortion positive cattle certainly argue against the possibility that local antibody may be protective against abortion.

Insufficient data exists at this time to state if the Ig class and subclass response of vaginal mucus, or their corrected values, will be of major diagnostic or predictive value.

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TABLE 1. Relative immunologic reactivity measured in FSU[a] of six antiglobulins with different antigens

Antiglobulin	StiQ antigen[b]	FSU[c]
Rabbit anti-bovine IgA[d]	625 ng BASA	0.3
	625 ng IgA	132.0
	625 ng IgG_1	9.7
	625 ng IgG_2	6.0
	625 ng IgM	6.7
Rabbit anti-bovine IgG_1[d]	625 ng BASA	0.3
	625 ng IgG_1	139.3
	625 ng IgG_2	27.6
	625 ng IgM	11.7
Rabbit anti-bovine IgG_2[d]	625 ng BASA	0
	625 ng IgG_1	7.3
	625 ng IgG_2	206.0
	625 ng IgM	7.7
Rabbit anti-bovine IgM[d]	625 ng BASA	0
	625 ng IgG_1	2.0
	625 ng IgG_2	2.7
	625 ng IgM	91.7
Rabbit anti-bovine IgG[e] (heavy and light)	625 ng BASA	0
	625 ng IgG_1	112.0
	625 ng IgG_2	177.7
	625 ng IgM	25.7
Sheep anti-rabbit IgG[f]	625 ng BASA	0
	625 ng IgG_1	0.3
	625 ng IgG_2	2.7
	625 ng IgM	0

[a]Fluorescent signal units.
[b]All Ig are of bovine origin.
[c]Mean of 3 replicates.
[d]Also 0.5 ml of a 1:400 dilution of sheep antirabbit IgG used in test.
[e]Commercial conjugate used in measurement of total vaginal Ig and in standard FIAX test.
[f]Commercial conjugate used to amplify the class-specific FIAX reactions.

TABLE 2. Reproducibility of FIAX test control samples.

Test[a]	Control #	N	Mean FSU[b]	SEM[c]	Mean background[e]
Serum IgA	Serum 66	11	97.00	2.30	
	Serum 67	11	144.64[d]	0.31	15.36
	Serum 68	11	63.73	1.63	
	Serum 70	11	5.91	0.56	
	Serum 75	11	110.91	2.45	
Serum IgG1	Serum 66	11	141.09[d]	0.56	18.91
	Serum 68	11	169.82	6.43	
	Serum 70	11	10.82	0.82	
	Serum 71	11	56.91	3.44	
	Serum 73	11	133.64	4.04	
Serum IgG2	Serum 66	11	184.27	5.08	
	Serum 68	11	176.55	4.32	
	Serum 70	11	17.00	1.12	
	Serum 71	11	76.00	3.22	
	Serum 73	11	147.18[d]	0.33	12.82
Serum IgM	Serum 66	11	126.91	6.67	
	Serum 67	11	188.82	8.83	
	Serum 68	11	79.45	3.85	
	Serum 70	11	15.55	1.17	
	Serum 75	11	116.55[d]	1.76	43.45
Vaginal IgA	Vag 8	5	74.00	8.85	
	Vag 13	5	105.80	7.58	
	Vag 50	5	25.60	6.62	
	Vag 63	5	15.80	5.62	
	Vag 24	4	29.00	11.34	
	Vag 42	5	142.60[d]	1.12	17.40
Vaginal IgG1	Vag 8	7	138.86[d]	1.55	21.14
	Vag 13	7	85.43	2.64	
	Vag 50	7	45.43	3.27	
	Vag 63	7	7.43	0.95	
Vaginal IgG2	Vag 8	6	137.83[d]	0.83	22.17
	Vag 13	6	74.67	4.14	
	Vag 50	6	84.00	7.45	
	Vag 63	6	9.50	1.80	
	Vag 24	6	29.83	2.27	
Vaginal IgM	Vag 13	6	11.50	1.98	
	Vag 21	5	136.40[d]	0.68	23.60
	Vag 69	6	104.00	4.31	
	Vag 70	6	4.00	1.39	
	Vag 71	6	41.33	2.36	

[a]All tests are for B. abortus specific antibodies.
[b]Mean fluorescent signal unit (FS of antigen coated surface - FS of nonantigen coated background).
[c]Standard error of mean.
[d]Fluorometer gain control samples - FS of antigen surface gained to 160.
[e]Mean background: 160 - mean FSU.

TABLE 3. Mean titers of standard serological tests

Date	Treatment	No.	FIAX[a] Mean	FIAX[a] SEM[d]	ELISA[a] Mean	ELISA[a] SEM	CF[b] Mean	CF[b] SEM	RIV[b] Mean	RIV[b] SEM	CARD[c] Mean	CARD[c] SEM
PRE	Nonvaccinate	15	2.1	1.1	11.1	3.2	0.0	0.0	0.0	0.0	0.0	0.0
	S-19	38	3.1	1.4	8.0	0.9	1.1	0.7	0.0	0.0	0.0	0.0
1MPV	Nonvaccinate	19	3.5	2.4	11.8	5.1	0.0	0.0	0.0	0.0	0.0	0.0
	S-19	39	59.8	11.7	44.2	5.6	13.3	3.3	46.2	9.8	56.4	8.0
6MPV	Nonvaccinate	23	5.6	2.3	1.6	0.6	0.0	0.0	0.0	0.0	0.0	0.0
	S-19	46	5.8	1.8	2.7	0.8	0.0	0.0	0.0	0.0	13.0	5.0
3MPC	Nonvaccinate	17	327.5	42.0	139.8	9.6	124.7	12.8	182.4	12.8	94.1	5.9
Culture	Abortion											
+	+	15	322.3	47.6	149.3	6.1	136.0	10.9	193.3	6.7	100.0	0.0
-	+	1	389.4	-	123.0	-	80.0	-	200.0	-	100.0	-
-	-	1	342.5	-	15.0	-	0.0	-	0.0	-	0.0	-
	S-19	37	158.5	30.7	81.6	12.3	69.2	12.2	89.2	16.2	48.6	8.3
Culture	Abortion											
+	+	18	255.4	49.1	131.8	14.5	117.8	16.6	136.1	22.1	72.2	10.9
+	-	5	113.2	52.9	50.0	27.3	56.0	29.9	90.0	45.8	60.0	24.5
-	+	1	62.6	-	108.0	-	80.0	-	200.0	-	100.0	-
-	-	12	53.3	29.0	26.6	14.7	5.0	3.6	16.7	16.7	8.3	8.3

[a]FIAX and ELISA titers expressed in ng Ig bound.
[b]CF and RIV titers 1/maximum positive serum dilution.
[c]CARD percentage of positive samples.
[d]Standard error of mean.
PRE=prevaccination; 1MPB=1 month postvaccination; 6MPV=6 months postvaccination; 3MPC=3 months postchallenge.

TABLE 4. Mean titers[a] of class and subclass immunoglobulins in sera

Date	Treatment	No.	IgG1 Mean	IgG1 SEM	IgG2 Mean	IgG2 SEM	IgA Mean	IgA SEM	IgM Mean	IgM SEM
PRE	Nonvaccinate	15	7.4	2.0	4.6	1.0	15.1	1.1	21.4	3.5
	S-19	38	6.5	1.1	5.8	1.7	14.2	0.7	17.5	2.2
1MPV	Nonvaccinate	19	7.1	0.9	0.5	0.3	18.8	1.1	27.5	2.7
	S-19	39	85.2	7.0	96.2	11.1	37.6	3.6	99.9	13.4
6MPV	Nonvaccinate	23	12.5	1.3	3.4	0.8	10.5	1.1	9.8	2.3
	S-19	46	20.0	1.9	10.1	1.7	10.8	0.7	24.0	3.3
3MPC	Nonvaccinate	17	260.0	16.9	316.1	31.0	313.4	43.8	170.1	22.2
Culture	Abortion									
+	+	15	280.6	5.0	335.0	27.8	347.8	41.9	185.1	21.9
-	+	1	201.5	-	337.2	-	91.2	-	104.4	-
-	-	1	9.4	-	12.0	-	20.2	-	10.4	-
	S-19	37	154.2	20.5	194.9	29.7	119.1	22.2	96.2	16.8
Culture	Abortion									
+	+	18	213.9	24.1	286.9	39.4	200.7	34.8	133.9	24.4
+	-	5	188.4	60.9	217.2	77.4	71.7	27.3	102.3	41.4
-	+	1	269.5	-	168.3	-	109.0	-	104.4	-
-	-	12	48.6	24.7	61.4	37.9	26.1	14.6	42.8	26.4

[a]Titers expressed in ng immunoglobulin bound.

PRE=prevaccination; 1MPV=1 month postvaccination; 6MPV=6 months postvaccination; 3MPC=3 months postchallenge.

TABLE 5. Mean titers[a] of vaginal class and subclass immunoglobulins 3 - 4 months following abortion or normal calving

Treatment		No.	Vaginal IgG1		Corrected Vaginal IgG1		Vaginal IgG2		Corrected Vaginal IgG2		Vaginal IgA		Corrected Vaginal IgA		Vaginal IgM		Corrected Vaginal IgM		Vaginal Ig Total	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Negative Controls[b]		11	9.1	0.5	10.4	3.0	6.4	4.1	13.2	10.7	9.2	0.5	9.9	2.5	4.8	2.8	11.2	8.4	145.5	25.4
Nonvaccinate		5	38.5	20.3	24.2	12.6	94.5	8.7	57.5	6.7	63.8	22.1	39.1	14.0	7.6	5.0	4.7	3.0	169.9	15.4
Culture	Abortion																			
+	+	3	57.8	30.2	37.2	17.8	96.3	4.7	65.9	2.2	77.2	31.2	51.0	19.4	11.6	7.9	7.3	4.6	146.7	11.0
-	+	1	9.6	-	4.7	-	65.9	-	32.1	-	79.2	-	38.5	-	3.3	-	1.6	-	205.5	-
-	-	1	9.6	-	4.7	-	117.9	-	57.8	-	8.0	-	3.9	-	0.0	-	0.0	-	203.9	-
S-19		22	44.9	17.0	46.6	21.5	72.2	21.8	64.6	30.9	36.9	8.0	32.9	8.9	3.1	1.8	4.6	2.8	158.1	17.8
+	+	5	134.8	61.4	149.8	83.6	166.7	85.6	195.1	128.0	71.0	22.0	72.4	30.2	9.3	7.3	12.0	10.3	153.3	42.4
+	-	4	25.3	7.5	16.6	9.1	35.8	7.7	20.0	6.7	18.3	3.6	10.4	3.6	0.0	0.0	0.0	0.0	207.9	37.6
-	+	1	42.0	-	18.7	-	65.9	-	29.3	-	46.1	-	20.5	-	0.0	-	0.0	-	224.8	-
-	-	12	14.2	5.7	15.9	5.0	45.5	10.3	28.0	5.9	28.2	9.3	24.9	7.5	1.8	0.8	3.5	2.8	137.9	23.6

[a]Titers expressed in ng immunoglobulin bound.
[b]Seronegative nonchallenged adult cattle.

CHAPTER V

THE IMMUNOGLOBULIN CLASS AND SUBCLASS OF ANTIBODIES IN THE SERA AND VAGINAL MUCUS OF CATTLE FOLLOWING VACCINATION WITH B. ABORTUS CELL SURFACE PROTEIN AND CHALLENGE WITH S-2308

INTRODUCTION

The serum Ig class and subclass responses of cattle to B. abortus have been examined by several investigators (2, 4, 13, 14). It has been suggested that the class or subclasses of B. abortus-specific Ig may help to distinguish antibody titers due to vaccination with S-19 from those due to infection with virulent organisms (4, 14). The vaginal Brucella-specific Ig response has been investigated (11, 12, 16, 18, 19), but not in relation to Ig class specificity or protection from disease.

We recently demonstrated the serum and vaginal Brucella-specific Ig class and subclass responses in cattle vaccinated with S-19 and challenged with B. abortus S-2308 (Chapter 4). The results reported herein are the evaluation of serum and vaginal Brucella-specific Ig class and subclass in cattle vaccinated with an ineffective experimental B. abortus cell-surface protein (CSP) vaccine (Confer, et al.,

manuscript in preparation). These responses were compared to the serum antibody responses as determined by standard serologic tests (1, 17, 21). The possibility of local Ig enhancing resistance against disease also was evaluated.

MATERIALS AND METHODS

Serum samples

The sera used in this study were sequential samples obtained from 75 crossbred beef heifers used in a B. abortus CSP vaccine experiment (Confer, et al; manuscript in preparation). Sera from cattle that failed to become pregnant or died prior to the completion of the experiment were excluded from analysis. Twelve cattle were designated as non-vaccinates [received only Freund's complete adjuvant (FCA) and saline]. Fifty cattle received CSP or a chemically-modified CSP, as described in the vaccine section below. Because there were no differences among the vaccine groups, all vaccinates were considered as one group. Sera were taken prior to vaccination (Day 0), 2 weeks postvaccination (2WkPV), 4 weeks postvaccination (4WkPV), 10 weeks postvaccination (10WkPV), and 31 weeks postvaccination (31WkPV).

Fifty-six weeks after the initial vaccination, all pregnant heifers (4.5 to 6 months gestation) were challenged intraconjunctivally with 1.9×10^7 colony forming units of virulent B. abortus S-2308 (8). Additional sera were taken 4 weeks postchallenge (4WkPC), within 24 hours of abortion or normal calving (ATA), and 13 weeks postchallenge (13WkPC).

Vaginal Mucus Samples

Vaginal samples were collected 4WkPV, 31WkPV, 4WkPC, ATA, and

13WKPC using Tampax Superplus tampons (Tambrands Inc., Lake Success, NY). The vulva was cleaned with a mild detergent and the tampons were manually placed 10 - 12 cm anteriorly and ventrally within the vagina. The tampons were removed after 15 - 30 minutes. Ten ml of PBS containing 0.1% sodium azide were placed on each tampon. After one hour, the tampons were squeezed in a 60 ml plastic syringe and the vaginal mucus extract was collected and frozen at -20°C until use. Corrected vaginal values were calculated for each sample by the formula (ng class or subclass specific Ig bound/ng total vaginal Ig) x 100%. Due to difficulty in collection of vaginal samples, it was not possible to have samples from all of the cattle on each of the collection dates. Vaginal samples from 11 adult dairy cattle, which had been calfhood vaccinated with S-19 several years previously, were used as negative controls. These cattle were from a certified brucellosis-free herd and all were seronegative by standard serological tests, ELISA, and FIAX.

Vaccines

Two CSP vaccines were tested. CSP was extracted from intact, aqueous methanol-inactivated, washed B. abortus S19 as previously described (23). Briefly, cells were suspended in 1M NaCl-0.1 M Na citrate at a rate of 0.2 gm/ml and treated with glass beads in a Mickel Tissue Disintegrator (Mickel, England). A portion of the CSP was chemically modified with dodecanoyl anhydride (dCSP) (6, 22). The protein concentrations of CSP and dCSP were determined by the method of Lowry et al. (15).

One ml of each vaccine was mixed with 1 ml of FCA and injected subcutaneously at Day 0 and repeated 6 weeks later. The 75 heifers were

equally divided among 5 groups. Group 1 (nonvaccinated) was given saline and FCA. Group 2 was vaccinated twice with 2 mg CSP in FCA. Group 3 was vaccinated twice with 2 mg of dCSP in FCA. Group 4 was vaccinated initially with 2 mg of CSP in FCA and later with 2 mg of dCSP in FCA. Group 5 was vaccinated initially with 2 mg of dCSP in FCA and later with 2 mg of CSP in FCA. Groups 2-5 were collectively examined as vaccinates in this study.

Conventional serology

The standard serological tests (CARD, CF, and RIV) were performed using standard protocols and reagents (NADL Diagnostic Reagents Manuals 65d and 65e), at the State-Federal Brucellosis Laboratory, Oklahoma City, OK. These procedures have been described (1, 17, 21). These standard serological tests were evaluated using the criteria of Deyoe (8, Chapter 2).

FIAX Tests

In general, FIAX is an indirect immunofluorescence test, in which specific fluorescence, due to binding of a FITC-antiglobulin, is quantitated as a fluorescence single unit (FSU) by a fluorometer. The protocol for detecting B. abortus specific antibody with the FIAX system has been previously described (10). Modifications of the FIAX test were done so that serum and vaginal class and subclass Ig could be semiquantitated. The antigen used in all FIAX tests for the detection of B. abortus specific antibody was a soluble extract of B. abortus strain 1119 (S-1119) (BASA-d) (3, 22). Twenty-five ul of PBS (pH 7.2) containing 25 ug/ml BASA-d antigen were applied to one side to a nitrocellulose

disc attached to a plastic carrier (StiQ, Whitaker Biomedical), the other side of the StiQ served as measure of nonspecific binding (background). The StiQs were dried overnight at 37°C. All subsequent reactions with the FIAX system were at room temperature. The StiQs were incubated for 30 minutes with 0.51 ml of a 1:51 dilution of test serum or vaginal mucus extract in phosphate buffered saline containing 0.15% Tween 20 (PBS-Tween). In the case of vaginal IgA and vaginal IgM, 0.55 ml of a 1:11 dilution of vaginal mucus extract was used. The StiQs were then washed for 10 minutes in 0.6 ml of PBS-Tween, and then incubated for 20 minutes with 0.5 ml of appropriate antiglobulin. For the standard (not Ig subclass specific) FIAX, a 1:800 dilution of fluorescein isothiocyanate-conjugated rabbit antibovine immunoglobulin G (IgG) (heavy and light chain specific) (Cappel, Cooper Biomedical Inc., Malvern, PA) was used. Following a 10 minute wash in 0.6 ml PBS-Tween, the StiQ associated fluorescent signal (FSU) of the standard FIAX was determined. The Ig class specific reagents used were: rabbit anti-bovine IgA (NADC-ARS, Ames, IA) whole serum diluted 1:200, rabbit antibovine IgM (Mu chain specific) (Cooper Biomedical, Inc., Malvern, PA) at a working dilution containing 80 ug protein/ml, rabbit anti-bovine IgG1 (Miles Laboratory, Elkhart, IN) at a working dilution containing 40 ug protein/ml, and rabbit anti-bovine IgG2 (NADC-ARS, Ames, IA) at a working dilution containing 20 ug protein/ml. All of the conjugates except rabbit anti-bovine IgA were FITC-labeled. Rabbit anti-bovine IgG1 required affinity purification to minimize cross-reaction with other Ig. The techniques used for FITC-labeling, standardization, and determination of immunological purity of conjugates were presented previously (Hall, manuscript in preparation, Chapter 4). The class-specific FIAX

tests were incubated 20 minutes in 0.5 ml of FITC-sheep anti-rabbit IgG, and then washed 10 minutes in 0.6 ml of PBS-Tween. The StiQ-associated fluorescence was then determined using the fluorometer, and FSU calculated by subtracting the background fluorescence from the antigen specific fluorescence.

Test control sera and control vaginal mucus extracts were assigned ng equivalents of Ig binding based on a linear regression of FSU of Ig versus ng of commercially-prepared, purified bovine Ig present on the StiQ. The determination of positive or negative FIAX classification was calculated using a discriminate analysis system (20, Appendix). For each serum FIAX test, the discriminate functions were calculated on the means of all sera on Day 0 and the means of sera 13WkPC from culture positive, abortion positive animals. The vaginal discriminate functions were calculated using means of the 11 negative control vaginal samples with those of 4WkPC from culture positive, abortion positive animals.

ELISA test

The ELISA was performed as previously described (5). One hundred ul of BASA-d in carbonate buffer (pH 9.6) were used to coat wells of a polystyrene microtiter plate (Nunc, Denmark) overnight at room temperature. Following three washes in PBS with 0.05% Tween-20, 100 ul of serum diluted 1:250 in PBS-Tween 20 buffer, containing 1.0% bovine serum albumin (BSA), were added to duplicate wells of the plate and allowed to incubate 1.5 hours at room temperature. Following three additional washes, 100 ul of a 1:400 dilution of horseradish peroxidase conjugated, affinity-purified rabbit anti-bovine IgG (Pel Freeze, Rogers, AR) were added to each well and allowed to incubate for 45 minutes at room

temperature. After six washes in PBS Tween-20 buffer, 100 ul of substrate, containing o-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.04% of a 30% solution) in phosphate-citric acid buffer, was added to each well. Plates were incubated in the dark for 30 minutes at room temperature. The reaction was stopped with 40 ul of 0.25 M sulfuric acid. An average A_{490} was determined on duplicate samples and ng of IgG per well determined from a standard curve. Values greater than 20 ng Ig binding were considered positive.

Total Vaginal Ig

The total Ig present in the vaginal mucus extract was approximated using the FIAX system. Twenty-five ul of extract from each sample was placed on a StiQ and allowed to dry overnight at 37°C. The StiQs were then washed for 10 minutes in 0.6 ml PBS-Tween to remove any unbound material. The StiQs were allowed to react with 0.5 ml of rabbit anti-bovine IgG (heavy and light chain) for 20 minutes. Following another 10 minute wash in 0.6 ml of PBS-Tween, the FSU was measured using the fluorometer and compared to an Ig standard curve.

Statistical analysis

Mean antibody titers were compared by multiple t tests. A t test for equal and unequal variances was calculated for the mean titers for each of the comparisons. An F statistic was calculated to determine whether unequal variances were present. If the probability of F was less than 0.05, unequal variances were used in calculating t test values (20).

RESULTS

The antibody responses for the serum class and subclass Ig tests are presented (Table 1). For all dates after vaccination and prior to challenge and by all tests, the vaccinates had significantly higher ($p < 0.05$) antibody responses than those of nonvaccinates. For vaccinates, the antibody responses in each class were higher ($p < 0.05$) after vaccination and challenge than at Day 0. The nonvaccinates also had significant ($p < 0.05$) increases in antibody responses for all weeks after Day 0 with the exception of IgG1 31WkPC, IgA 2WkPV, 10WkPV, and 31WkPV, and IgM 10WkPV and 31WkPV. With the exception of IgM, all tests were higher ($p < 0.05$) at 10WkPV (4 weeks after the second vaccination) than at 4WkPV. Following challenge, insignificant differences were seen between the vaccinates and nonvaccinates. Brucella-specific IgA was the highest ATA and at 13WkPC.

The data from Table 1 is subdivided according to final abortion and culture status (Table 2.) By all tests, in general, higher serum antibody responses prior to challenge were in vaccinates that were later culture negative and abortion negative than in those that were later culture positive and abortion positive. Following challenge, the highest antibody responses were in cattle that were determined to be culture positive and abortion positive. Also after challenge, Brucella-specific IgA was minimal in those cattle that were culture negative and abortion negative.

The vaginal and corrected vaginal class and subclass Brucella-specific Ig responses are presented in Tables 3 and 4. For both vaccinates and nonvaccinates ATA, the vaginal and corrected vaginal IgM titers were significantly higher ($p < 0.05$) than control values only ATA

(Table 3). For all classes and subclasses of Ig, the vaginal titers ATA were markedly higher than on all other dates. In general, the corrected vaginal values were lower than those obtained without correction for total vaginal Ig (Tables 3 and 4). The vaccinates generally had higher antibody ATA and 13 WkPC than those of the nonvaccinates (Table 3). After challenge, most tests measured higher antibody responses in the culture positive, abortion positive groups than in the culture negative, abortion negative groups (Table 3).

The mean titers and SEM of the standard serological tests (percent seropositive for CARD) are presented (Tables 5 and 6). The nonvaccinates showed a transient rise in antibody titer at 2WkPV and 4WkPV by all tests, and the ELISA and FIAX titers were still elevated 10WkPV (Table 5). At 31WkPV, mean antibody titers as detected by all standard tests were greater than Day 0 titers in the vaccinate group, but CF and RIV were only minimally higher. At 13WkPC (Table 6), ELISA and FIAX did not distinguish ($p > 0.05$) between mean titers in sera from culture negative, abortion negative cattle and in sera from culture positive, abortion positive cattle within the vaccinated group. The highest mean ELISA titers for the vaccinates occurred 31WkPV; the highest mean ELISA titers after challenge occurred 13WkPC.

The percentage of positive serological reactors for all of the serological tests is presented (Tables 7 and 8). The ELISA had an average of 11% positive reactors prior to vaccination (Day 0). The CF and IgM tests also had a few positive reactors on Day 0. All tests showed a transient increase in the percent seropositives in the nonvaccinated group, but most of the nonvaccinates were seronegative by 31WkPV. In the vaccinated group, 31WkPC all tests had numerous positive

titers. At that time, the IgA test detected the least number of positives (20%) (Table 7). Following challenge (Table 8), all tests frequently classified sera from culture negative, abortion negative cattle as seropositive, but overall the IgA test identified the least number of positives from this group. ATA and at 13WkPC, few culture positive, abortion positive animals were classified as seronegative by any of the tests, but CF identified the most seronegative cattle from this group.

The percentage of positive vaginal and corrected vaginal tests are presented (Tables 9 and 10). Vaginal IgM and corrected vaginal IgM are not included because the mean titers for these tests were not significantly greater than those of negative controls except ATA. In general, the percentage of positive tests was lower in the corrected vaginal tests than in the corresponding vaginal tests, and the corrected vaginal test did not identify culture positive, abortion positive animals effectively (Table 10). The vaginal IgA had the lowest percentage of positive reactions following vaccination (Table 9), however, that test compared favorably to the other tests in identifying culture positive, abortion positive animals after challenge (Table 10).

DISCUSSION

High Brucella-specific serological titers were observed by all tests and within all classes and subclasses of Ig following vaccination with CSP. The nonvaccinated group (receiving only FCA and saline) responded with a Brucella-specific titer that was less than that of the vaccinates, and subsided more rapidly. This could have been because FCA stimulated antibodies to other gram negative bacteria that cross reacted

with B. abortus or antibodies to Mycobacterium sp in FCA cross reacted with B. abortus.

Brucella-specific vaginal IgA, IgG1, and IgG2 could be detected following vaccination. Vaginal IgA was markedly less than vaginal IgG1 and IgG2, as would be expected, because IgA is predominantly produced at mucosal surfaces and presumably a nonliving antigen injected subcutaneously would stimulate little immunity at a mucosal surface. Both serum and vaginal Brucella-specific IgA were prominent following challenge with virulent B. abortus S-2308.

Duncan et al. (9) theorized that in the bovine vaginal IgG1 and IgG2 were derived from serum. The data reported herein does not prove that theory. However, there was a parallel between serum and vaginal Brucella-specific antibodies in IgG1 and IgG2 subclasses, therefore, supporting the aforementioned theory. Brucella-specific IgM appears to be neither produced locally nor pass from serum into the vaginal secretions as has been reported (9). This is evidenced by high serum IgM titers with little or no vaginal IgM titers being present. Brucella-specific vaginal IgM was measured ATA and all class and subclasses of vaginal Ig were markedly elevated. This would most likely be due to contamination of the vaginal secretions with blood containing antibodies to B. abortus due to placental separation at the time of parturition.

Serum Brucella-specific IgM titers did not increase following the second vaccination, as did titers in the other Ig classes. This is as expected because the anamnestic response would be predominantly an IgG response. A marked increase in titer of all Ig was noted after challenge, especially in the nonvaccinates. At 4WkPC, the serum IgM titers were higher for the nonvaccinates than for the vaccinates. Probably an

anamnestic response occurred to several epitopes that were present in both the CSP vaccine and in S-2308, but most likely new antigens were present in S-2308 that were not in the vaccine.

In general prior to challenge, higher mean serum titers were present in vaccinates that were subsequently determined to be culture positive and abortion negative than those that were culture negative and abortion positive. Although many of these mean titers were not significantly different, the data might imply some minimal degree of antibody-induced resistance against subsequent challenge with virulent S-2308. However, resistance against B. abortus is not considered to be antibody mediated but due to effective cell-mediated immunity (21).

All of the serological tests appeared adequate in distinguishing Day 0 titers from those titers of culture positive, abortion positive cattle. None of the tests could clearly distinguish vaccinates from challenged cattle nor could they distinguish challenged, culture positive, abortion positive cattle adequately from challenged, culture negative, abortion negative cattle. Although the mean titers may have been statistically different, considerable overlapping of titers occurred among these groups. Of all the tests, serum IgA appeared to be the most effective in distinguishing vaccinated from challenged cattle and challenged, culture positive, abortion positive cattle from challenged, culture negative, abortion negative cattle. Serum IgA titers were minimal until ATA and 13WkPC. Therefore, failure to detect an early infection with B. abortus might occur if only serum IgA responses were measured. Thus the potential for using Brucella-specific IgA titers as a diagnostic test might be limited.

Previously described vaginal mucus tests for B. abortus antibodies

did not attempt to correct for total vaginal Ig (11, 12, 16, 18, 19). The method described herein, whereby titers were corrected for total vaginal Ig, did not appear satisfactory because many culture positive, abortion positive cattle were classified as negative by the corrected vaginal Ig tests. Overall, the tampon method of collection of vaginal mucus would not be considered a viable procedure on a herd basis. The procedure was tedious and labor intensive. Variations in sample quality and quantity could not be standardized. Corbeil et al. (7) described changes in water content of vaginal mucus during the estrus cycle. Sampling variation was also noted in our studies. Therefore, the interpretation of results was imprecise. The results obtained by determining Brucella-specific antibodies in a vaginal sample from an individual cow may provide additional information about the infection status of that animal, but using this on a herd basis would provide little additional information over that provided by serum titers. This finding is consistent with those of Roberts (18). After challenge, higher vaginal antibody responses were observed in culture positive, abortion positive cattle than in culture negative, abortion negative cattle. This would imply that vaginal antibodies, like serum antibodies, are indicative of infection and do not suggest protection from disease.

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TABLE 1. Mean[a] *B. abortus* titers and SEM[b] of class and subclasses of immunoglobulins

Date	Treatment	No.	IgG1		IgG2		IgA		IgM	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Day 0	Nonvaccinate[c]	12	8.1	1.0	6.8	1.0	15.1	1.4	11.8	5.7
	Vaccinate[d]	50	8.5	0.7	9.3	0.5	14.7	0.6	6.9	1.4
2WKPV	Nonvaccinate	12	113.2	19.6	138.2	19.5	12.9	4.7	105.6	23.7
	Vaccinate	50	180.8	6.2	242.2	13.4	42.5	4.1	196.6	12.4
4WKPV	Nonvaccinate	12	84.4	16.5	158.3	23.9	21.6	2.4	52.1	15.4
	Vaccinate	50	135.9	7.8	277.2	14.2	34.8	2.9	100.0	9.9
10WKPV	Nonvaccinate	12	29.5	7.8	70.4	16.0	13.2	1.0	14.4	4.1
	Vaccinate	50	172.0	6.1	354.2	16.2	44.8	3.2	76.8	6.5
31WKPV	Nonvaccinate	12	26.8	15.7	14.2	1.8	11.4	1.3	12.8	4.4
	Vaccinate	50	98.1	6.4	172.5	13.8	22.8	1.7	30.6	3.8
4WKPC	Nonvaccinate	12	166.7	25.9	225.0	43.2	46.9	14.6	131.3	33.7
	Vaccinate	50	168.9	9.8	225.7	20.3	46.1	7.7	80.0	10.8
ATA	Nonvaccinate	12	275.3	24.3	352.6	39.5	290.1	37.3	240.4	22.1
	Vaccinate	47	260.3	12.0	356.7	17.8	185.1	13.6	227.5	14.8
13WKPC	Nonvaccinate	12	273.0	25.6	371.8	41.3	313.7	81.8	232.9	41.3
	Vaccinate	50	245.8	11.6	380.0	22.9	203.5	19.3	238.5	20.9

[a]Mean titers expressed as ng Ig binding.
[b]Standard error of the mean.
[c]Nonvaccinates received FCA and saline on Day 0.
[d]Vaccinates received *B. abortus* cell surface protein and FCA on Day 0 and at 5 weeks postvaccination.
WKPV = weeks postvaccination.
WKPC = weeks postchallenge
ATA = within 24 hours of abortion or normal calving.

TABLE 2. Mean[a] *B. abortus* titers and SEM[b] of class and subclasses of immunoglobulins with regard to final culture and abortion status

Date	Treatment	Culture	Abortion	No.	IgG1		IgG2		IgA		IgM	
					Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Day 0	Nonvaccinate[c]	+	+	10	7.8	1.0	7.1	1.1	15.4	1.6	12.3	6.4
		-	-	2	9.6	3.3	5.4	0.0	13.6	1.6	7.1	-
	Vaccinate[d]	+	+	33	8.3	0.9	9.5	0.6	14.8	0.8	7.4	1.8
		+	-	5	9.3	1.6	7.1	1.1	12.6	2.5	9.1	4.3
		-	+	2	8.5	2.2	11.0	5.7	13.6	1.6	3.6	3.6
		-	-	10	8.5	1.6	9.6	1.2	15.7	0.8	4.8	2.3
2WKPV	Nonvaccinate	+	+	10	126.1	20.8	148.6	21.1	15.3	5.4	122.6	25.0
		-	-	2	48.7	30.2	86.3	44.5	0.9	0.9	20.3	4.5
	Vaccinate	+	+	33	172.1	7.5	230.2	15.2	39.4	4.8	184.6	13.7
		+	-	5	223.2	7.5	290.7	29.8	53.2	6.6	254.4	64.6
		-	+	2	125.2	7.5	307.5	58.0	51.1	4.5	213.2	36.2
		-	-	10	199.7	11.4	249.3	39.2	45.7	12.7	209.5	31.4
4WKPV	Nonvaccinate	+	+	10	92.6	18.6	173.3	25.8	22.4	2.8	60.7	17.3
		-	-	2	43.4	20.9	83.3	34.5	17.6	3.2	8.9	2.2
	Vaccinate	+	+	33	126.7	8.0	259.1	16.0	30.9	2.5	90.4	10.0
		+	-	5	148.4	44.2	332.1	55.0	58.8	19.4	155.3	54.8
		-	+	2	124.7	37.2	324.9	63.9	30.5	16.1	84.3	53.5
		-	-	10	162.3	18.1	300.1	36.8	36.6	5.4	107.3	23.2
10WKPV	Nonvaccinate	+	+	10	32.8	9.1	81.3	17.1	13.1	1.2	13.6	4.8
		-	-	2	12.8	4.0	15.8	10.6	14.0	2.9	18.5	6.2
	Vaccinate	+	+	33	164.2	7.6	341.5	20.2	43.5	4.1	76.4	8.6
		+	-	5	197.4	14.8	352.0	50.6	47.0	6.5	70.6	13.6
		-	+	2	194.0	17.2	409.4	23.9	50.8	1.5	49.3	27.1
		-	-	10	180.6	13.6	386.0	39.0	46.7	8.8	86.8	14.1

TABLE 2 (Continued)

Date	Treatment	Culture	Abortion	No.	IgG1 Mean	IgG1 SEM	IgG2 Mean	IgG2 SEM	IgA Mean	IgA SEM	IgM Mean	IgM SEM
31WKPV	Nonvaccinate	+	+	10	30.7	18.8	15.2	2.0	10.4	1.3	13.6	5.1
		-	-	2	7.1	4.9	9.2	4.1	16.6	1.5	9.0	9.0
	Vaccinate	+	+	33	90.8	8.0	159.1	18.2	22.7	2.3	30.5	4.9
		+	-	5	120.3	11.3	198.8	21.6	23.4	3.9	34.7	3.7
		-	+	2	139.1	48.9	314.7	72.3	38.8	8.9	49.1	49.1
		-	-	10	102.7	13.8	175.1	22.0	19.9	2.5	25.3	6.9
4WKPC	Nonvaccinate	+	+	10	193.1	22.2	267.2	39.5	53.6	16.8	151.3	37.4
		-	-	2	34.5	31.2	14.2	0.0	13.1	1.5	30.9	13.6
	Vaccinate	+	+	33	192.5	11.7	276.1	25.1	59.9	10.8	100.9	15.0
		+	-	5	114.9	15.3	126.8	23.6	14.6	2.2	31.6	3.5
		-	+	2	174.0	26.3	207.9	32.8	22.2	7.6	71.7	16.7
		-	-	10	116.9	16.2	112.5	27.8	21.0	3.4	36.9	7.9
ATA	Nonvaccinate	+	+	10	293.5	9.7	387.1	32.8	310.3	32.7	262.6	14.6
		-	-	2	184.2	157.2	179.7	136.3	189.2	177.5	129.5	88.8
	Vaccinate	+	+	33	286.2	7.4	402.4	11.9	208.1	11.1	259.1	12.9
		+	-	5	290.0	30.9	400.2	44.6	217.4	59.0	257.9	42.2
		-	+	1	301.5	-	372.6	-	242.2	-	261.7	-
		-	-	8	129.6	34.6	139.0	27.4	62.9	30.9	74.0	24.2

TABLE 2 (Continued)

Date	Treatment	Culture	Abortion	No.	IgG1 Mean	IgG1 SEM	IgG2 Mean	IgG2 SEM	IgA Mean	IgA SEM	IgM Mean	IgM SEM
13WKPC	Nonvaccinate	+	+	10	297.1	13.3	421.9	24.6	366.4	89.1	272.4	38.2
		-	-	2	152.5	134.0	121.2	99.5	50.5	41.3	35.5	11.2
	Vaccinate	+	+	33	279.0	8.9	450.3	21.3	262.4	19.1	301.3	21.6
		+	-	5	239.7	40.4	334.0	65.6	158.4	57.4	153.8	58.4
		-	+	2	246.0	21.3	395.3	27.1	192.7	143.7	260.6	38.7
		-	-	10	139.1	27.0	167.8	32.6	33.9	8.5	69.1	24.4

[a]Mean titers expressed as ng Ig binding.
[b]Standard error of the mean.
[c]Nonvaccinates received FCA and saline on Day 0.
[d]Vaccinates received B. abortus cell surface protein and FCA on Day 0 and at 6 weeks postvaccination.

TABLE 3. Mean[a] titers and SEM[b] of vaginal class and subclasses of immunoglobulins

Date	Treatment	No.	Vaginal IgG1 Mean	Vaginal IgG1 SEM	Corrected[c] Vaginal IgG1 Mean	Corrected[c] Vaginal IgG1 SEM	Vaginal IgG2 Mean	Vaginal IgG2 SEM	Corrected[c] Vaginal IgG2 Mean	Corrected[c] Vaginal IgG2 SEM	Vaginal IgA Mean	Vaginal IgA SEM	Corrected[c] Vaginal IgA Mean	Corrected[c] Vaginal IgA SEM	Vaginal IgM Mean	Vaginal IgM SEM	Corrected[c] Vaginal IgM Mean	Corrected[c] Vaginal IgM SEM	Ig Total Mean	Ig Total SEM
Negative	Controls[d]	11	9.1	0.5	10.4	3.0	6.4	4.1	13.2	10.7	9.2	0.5	9.9	2.5	4.8	2.8	11.2	8.4	145.5	25.4
4WKPV	Nonvaccinate[e]	8	38.5	15	63.8	33.8	73.3	17.1	106.4	24.6	13.1	1.2	19.7	4.3	4.4	2.6	8.1	5.3	95.5	22.8
	Vaccinate[f]	40	70.0	9.2	106.8	19.8	131.9	18.6	201.2	35.9	13.4	1.9	18.6	2.7	6.8	2.2	12.2	4.0	114.9	12.3
31WKPV	Nonvaccinate	4	8.6	1.2	6.3	3.2	5.1	5.1	1.9	1.9	6.6	1.0	5.2	2.9	6.8	2.7	8.3	6.9	228.1	60.7
	Vaccinate	32	47.1	6.9	64.0	18.7	35.4	13.3	70.8	36.3	6.8	0.5	7.2	1.4	12.4	5.4	24.6	14.8	197.0	23.5
4WKPC	Nonvaccinate	12	58.8	15	63.2	21.9	52.0	15.8	52.2	21.0	19.1	3.1	16.2	3.9	0.4	0.3	0.2	0.2	169.5	28.6
	Vaccinate	50	57.5	7.0	49.8	7.1	66.9	12.2	55.7	10.0	18.0	1.9	15.1	1.9	1.8	0.7	1.5	0.6	161.6	14.6
ATA	Nonvaccinate	12	279.2	50	303.4	98.8	405.2	98.9	517.6	194.0	93.2	28.7	132.6	56.4	70.5	26.2	111.0	49.5	239.6	48.4
	Vaccinate	46	352.5	24	461.9	52.3	579.9	50.0	809.9	103.0	104.9	13.5	156.0	25.3	124.6	19.0	216.4	43.1	164.2	22.1
13WKPC	Nonvaccinate	4	53.8	20	13.5	4.4	45.8	14.1	11.5	3.1	25.5	11.3	6.4	2.6	0.0	0.0	0.0	0.0	384.5	20.5
	Vaccinate	25	99.5	14	30.6	5.5	114.5	21.3	34.3	7.3	45.9	7.3	13.0	2.1	0.3	0.2	0.1	0.1	374.4	19.5

[a]Mean titers expressed as ng Ig binding.
[b]Standard error of the mean.
[c]Corrected vaginal Ig = vaginal Ig/Ig total X 100%.
[d]Seronegative nonchallenged adult cattle.
[e]Nonvaccinates received FCA and saline on Day 0.
[f]Vaccinates received *B. abortus* cell surface protein and FCA on Day 0 and at 6 weeks postvaccination.

TABLE 4. Mean[a] titers and SEM[b] of vaginal class and subclass immunoglobulins with regard to final culture and abortion status

Date	Treatment	Culture	Abortion	No.	Vaginal IgG1 Mean	Vaginal IgG1 SEM	Corrected[c] Vaginal IgG1 Mean	Corrected[c] Vaginal IgG1 SEM	Vaginal IgG2 Mean	Vaginal IgG2 SEM	Corrected[c] Vaginal IgG2 Mean	Corrected[c] Vaginal IgG2 SEM	Vaginal IgA Mean	Vaginal IgA SEM	Corrected[c] Vaginal IgA Mean	Corrected[c] Vaginal IgA SEM	Vaginal IgM Mean	Vaginal IgM SEM	Corrected[c] Vaginal IgM Mean	Corrected[c] Vaginal IgM SEM	Ig Total Mean	Ig Total SEM
4WKPV	Nonvaccinate[d]	+	+	7	42.5	17.0	71.8	37.9	82.1	16.9	120.4	23.3	13.5	1.4	21.3	4.6	5.0	2.9	9.3	6.0	89.6	25.5
		-	-	1	10.3	-	7.5	-	11.6	-	8.5	-	10.8	-	7.9	-	0.0	-	0.0	-	136.6	-
	Vaccinate[e]	+	+	25	55.7	10.0	84.1	20.4	107.7	19.7	163.4	39.1	12.5	1.1	17.0	3.1	5.2	1.6	9.6	3.4	115.9	15.8
		+	-	4	137.9	42.0	245.4	95.3	266.1	107	459.1	196	20.4	6.1	34.3	12.7	20.7	19.3	34.6	32.5	72.8	15.7
		-	+	2	111.4	61.0	272.7	171	203.1	3.4	464.1	51.1	19.1	4.1	44.8	15.0	15.4	0.8	35.0	2.6	44.4	5.6
		-	-	9	70.4	15.0	71.3	23.9	123.6	32.2	133.2	45.4	11.4	0.6	10.2	1.9	2.7	0.9	3.3	1.1	146.6	28.5
31WKPV	Nonvaccinate	+	+	4	8.6	1.2	6.3	3.2	5.1	5.1	1.9	1.9	6.6	1.0	5.2	2.9	6.8	2.7	8.3	6.9	228.1	60.7
		-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vaccinate	+	+	17	45.5	8.5	53.0	20.1	29.9	10.6	43.2	22.6	7.3	0.6	7.0	1.8	10.2	4.1	16.4	10.1	219.6	34.9
		+	-	4	29.5	2.9	12.4	2.8	4.5	3.3	2.0	1.7	5.1	1.1	2.3	0.8	5.1	1.9	2.1	1.0	269.2	52.2
		-	+	1	177.4	-	490.8	-	400.8	-	1109.0	-	4.1	-	11.3	-	163.5	-	452.3	-	36.1	-
		-	-	10	43.7	10.0	60.6	21.5	20.4	7.7	41.5	23.3	6.9	0.9	9.0	3.1	3.8	1.2	4.8	2.5	145.8	34.1
4WKPC	Nonvaccinate	+	+	10	67.9	17.0	74.0	25.0	61.6	17.5	62.1	24.1	20.6	3.5	17.9	4.6	0.5	0.3	0.3	0.2	171.4	34.1
		-	-	2	13.3	3.0	9.3	4.2	3.6	3.4	2.9	2.8	11.5	1.0	7.8	2.6	0.0	0.0	0.0	0.0	160.2	39.4
	Vaccinate	+	+	33	73.7	9.0	59.7	9.0	92.5	16.7	74.1	13.5	21.0	2.6	17.2	2.7	2.8	1.0	2.2	0.8	158.0	16.6
		+	-	5	20.7	6.6	19.4	8.6	19.1	5.1	17.6	4.9	10.7	1.4	8.6	2.3	0.0	0.0	0.0	0.0	173.6	62.8
		-	+	2	53.0	41.0	86.9	81.1	47.5	30.4	73.8	65.7	11.0	0.5	12.8	7.8	0.0	0.0	0.0	0.0	133.6	77.8
		-	-	10	23.3	6.8	24.9	8.8	10.3	2.9	10.6	3.2	13.0	1.4	11.7	2.6	0.0	0.0	0.0	0.0	173.4	38.9
ATA	Nonvaccinate	+	+	10	330.5	43.0	362.9	109	484.4	101	620.6	220	109.5	32.0	158.5	65.0	84.5	29.5	133.2	57.2	215.7	55.1
		-	-	2	22.9	4.0	6.3	0.8	9.3	3.2	2.6	0.7	11.7	5.0	3.2	1.2	0.0	0.0	0.0	0.0	359.1	20.7
	Vaccinate	+	+	33	413.7	16.0	565.3	55.1	694.1	44.7	1000.6	111	113.3	11.4	172.9	26.2	155.7	22.3	270.0	53.0	119.7	20.1
		+	-	5	267.1	88.0	191.2	140	446.4	185	383.9	313	61.5	31.3	64.0	56.5	43.0	26.3	49.4	45.2	347.0	72.3
		-	+	1	436.0	-	972.3	-	829.7	-	1850.3	-	182.0	-	405.9	-	339.3	-	756.6	-	44.8	-
		-	-	7	113.2	59.0	95.0	57.3	101.7	69.6	66.5	34.0	85.6	68.5	106.5	98.3	5.6	2.8	5.6	3.1	260.7	63.4
13WKPC	Nonvaccinate	+	+	4	53.8	20.0	13.5	4.4	45.8	14.1	11.5	3.1	25.5	11.3	6.4	2.6	0.0	0.0	0.0	0.0	384.5	20.5
		-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vaccinate	+	+	19	105.9	16.0	32.4	6.8	129.7	26.5	38.5	9.2	48.4	7.7	13.9	2.4	0.4	0.3	0.2	0.1	375.9	18.8
		+	-	2	126.4	37.0	33.4	17.8	126.4	25.7	32.6	15.0	33.9	20.2	9.7	7.3	0.0	0.0	0.0	0.0	445.7	126
		-	+	1	171.3	-	39.9	-	66.4	-	15.5	-	129.6	-	30.2	-	0.0	-	0.0	-	428.7	-
		-	-	3	17.4	17.0	13.9	13.9	26.4	10.2	15.0	10.7	10.3	1.8	4.0	1.1	0.0	0.0	0.0	0.0	299.5	86.9

[a]Mean titers expressed as ng Ig binding.
[b]Standard error of the mean.
[c]Corrected vaginal Ig = vaginal Ig/Ig total X 100%.
[d]Nonvaccinates received FCA and saline on Day 0.
[e]Vaccinates received *B. abortus* cell surface protein and FCA on Day 0 and at 6 weeks postvaccination.

TABLE 5. Mean B. abortus titers and SEM[a] of standard serological tests

Date	Treatment	No.	FIAX[b] Mean	FIAX[b] SEM	ELISA[b] Mean	ELISA[b] SEM	CF[c] Mean	CF[c] SEM	RIV[c] Mean	RIV[c] SEM	CARD % Positive[d]
Day 0	Nonvaccinate[e]	12	0.0	0.0	15.7	1.1	0.8	0.8	0.0	0.0	0.0
	Vaccinate[f]	50	0.3	0.3	15.7	0.5	0.2	0.2	0.0	0.0	0.0
2WKPV	Nonvaccinate	12	151.8	30.4	26.7	4.2	10.0	3.0	47.9	17.5	75.0
	Vaccinate	50	314.8	18.2	56.9	4.7	37.8	4.9	167.5	8.1	100.0
4WKPV	Nonvaccinate	12	73.1	26.6	45.8	9.8	5.0	2.6	25.0	11.1	50.0
	Vaccinate	50	229.0	19.3	86.9	7.2	12.4	2.4	87.0	10.7	88.0
10WKPV	Nonvaccinate	12	10.6	5.1	34.3	9.7	0.0	0.0	0.0	0.0	0.0
	Vaccinate	50	321.6	20.2	114.6	8.3	30.4	5.2	89.5	10.2	92.0
31WKPV	Nonvaccinate	12	2.6	1.5	13.8	0.9	3.3	3.3	0.0	0.0	0.0
	Vaccinate	50	112.0	13.8	164.4	19.8	6.4	1.5	14.0	4.5	64.0
4WKPC	Nonvaccinate	12	145.4	28.4	198.9	39.3	16.7	6.8	91.7	25.3	75.0
	Vaccinate	50	145.9	15.8	310.1	24.0	28.4	5.1	59.0	11.2	68.0
ATA	Nonvaccinate	12	599.4	97.9	290.8	29.3	140.0	14.4	170.8	19.9	91.7
	Vaccinate	47	732.5	49.0	314.9	15.7	123.6	9.3	163.8	11.0	93.6
13WKPC	Nonvaccinate	12	366.4	29.6	346.4	34.1	108.3	19.1	175.0	17.9	91.7
	Vaccinate	50	355.1	24.8	333.0	13.9	89.4	9.9	156.5	11.3	90.0

[a]Standard error of the mean.
[b]Titers expressed as ng Ig binding.
[c]1/maximum positive serum dilution.
[d]Percentage of positive samples.
[e]Nonvaccinates received FCA and saline on Day 0.
[f]Vaccinates received B. abortus cell surface protein on Day 0 and 6 weeks postvaccination.

TABLE 6. Mean *B. abortus* titers and SEM[a] of standard serological tests with regard to final culture and abortion status

Date	Treatment	Culture	Abortion	No.	FIAX[b] Mean	FIAX[b] SEM	ELISA[b] Mean	ELISA[b] SEM	CF[c] Mean	CF[c] SEM	RIV[c] Mean	RIV[c] SEM	CARD % Positive[d]
Day 0	Nonvaccinate[e]	+	+	10	0.0	0.0	16.1	1.1	1.0	1.0	0.0	0.0	0.0
		-	-	2	0.0	0.0	14.2	4.5	0.0	0.0	0.0	0.0	0.0
	Vaccinate[f]	+	+	33	0.0	0.0	15.3	0.4	0.3	0.3	0.0	0.0	0.0
		+	-	5	2.8	2.8	17.4	3.8	0.0	0.0	0.0	0.0	0.0
		-	+	2	0.0	0.0	16.1	1.3	0.0	0.0	0.0	0.0	0.0
		-	-	10	0.0	0.0	16.1	1.1	0.0	0.0	0.0	0.0	0.0
2WKPV	Nonvaccinate	+	+	10	171.4	32.7	29.3	4.7	12.0	3.3	57.5	19.7	80.0
		-	-	2	53.9	35.0	13.6	1.5	0.0	0.0	0.0	0.0	50.0
	Vaccinate	+	+	33	289.7	21.3	51.0	4.4	32.4	5.3	164.4	10.6	100.0
		+	-	5	463.8	40.0	88.8	20.6	60.0	12.6	200.0	0.0	100.0
		-	+	2	296.1	25.0	74.2	12.6	60.0	20.0	200.0	0.0	100.0
		-	-	10	326.8	43.4	56.6	14.0	40.0	14.8	155.0	18.9	100.0
4WKPV	Nonvaccinate	+	+	10	87.7	29.9	52.0	10.6	6.0	3.1	30.0	12.8	60.0
		-	-	2	0.0	0.0	14.7	7.0	0.0	0.0	0.0	0.0	0.0
	Vaccinate	+	+	33	211.2	22.3	79.9	8.1	8.5	2.1	79.5	12.8	84.8
		-	-	5	241.8	68.5	123.8	38.1	34.0	12.5	120.0	33.9	100.0
		-	+	2	258.6	52.0	83.2	0.5	20.0	20.0	125.0	75.0	100.0
		-	-	10	275.6	53.1	92.6	14.8	13.0	5.2	87.5	26.2	90.0
10WKPV	Nonvaccinate	+	+	10	12.7	6.0	37.6	11.4	0.0	0.0	0.0	0.0	0.0
		-	-	2	0.0	0.0	17.6	5.3	0.0	0.0	0.0	0.0	0.0
	Vaccinate	+	+	33	313.3	25.8	117.4	10.6	29.7	6.2	76.5	11.3	90.9
		+	-	5	349.0	47.5	91.9	14.1	18.0	6.6	145.0	35.7	100.0
		-	+	2	381.9	24.6	125.0	86.9	40.0	0.0	150.0	50.0	100.0
		-	-	10	322.9	50.7	114.8	17.3	37.0	15.8	92.5	25.8	90.0
31WKPV	Nonvaccinate	+	+	10	3.0	1.7	14.2	1.0	4.0	4.0	0.0	0.0	0.0
		-	-	2	0.7	0.0	11.7	0.7	0.0	0.0	0.0	0.0	0.0
	Vaccinate	+	+	33	107.0	19.0	160.5	29.1	3.6	1.6	13.6	6.4	51.5
		+	-	5	92.7	22.4	167.6	32.9	12.0	4.9	10.0	6.1	100.0
		-	+	2	227.0	54.8	246.1	35.2	20.0	0.0	37.5	12.5	100.0
		-	-	10	115.0	20.8	159.0	19.5	10.0	4.5	12.5	6.7	80.0
4WKPC	Nonvaccinate	+	+	10	168.1	28.8	228.9	40.8	20.0	7.7	110.0	26.7	90.0
		-	-	2	31.5	19.0	49.0	3.9	0.0	0.0	0.0	0.0	0.0
	Vaccinate	+	+	33	175.3	20.6	341.5	30.2	38.8	6.9	85.6	14.9	84.8
		+	-	5	67.2	13.7	313.1	95.4	8.0	4.9	0.0	0.0	60.0
		-	+	2	139.1	68.6	250.1	9.6	30.0	10.0	25.0	25.0	50.0
		-	-	10	89.7	24.9	217.0	39.1	4.0	2.7	7.5	5.3	20.0
ATA	Nonvaccinate	+	+	10	654.6	101.7	320.3	19.4	160.0	0.0	200.0	0.0	100.0
		-	-	2	323.4	275.0	143.2	116.8	40.0	40.0	25.0	25.0	50.0
	Vaccinate	+	+	33	812.8	47.1	344.1	14.0	146.1	7.8	193.9	6.1	100.0
		+	-	5	780.1	124.1	355.8	14.4	108.0	32.0	170.0	30.0	100.0
		-	+	1	590.4	-	267.4	-	160.0	-	200.0	-	100.0
		+	-	8	389.4	155.8	174.7	47.7	36.2	20.0	31.3	24.9	62.5
13WKPC	Nonvaccinate	+	+	10	376.1	21.7	390.7	16.4	126.0	17.9	190.0	10.0	100.0
		-	-	2	318.1	183.6	124.9	76.6	20.0	20.0	100.0	100.0	50.0
	Vaccinate	+	+	33	392.6	25.5	346.5	14.9	108.8	11.4	189.4	7.5	100.0
		+	-	5	281.0	36.5	364.8	26.5	96.0	39.2	125.0	46.1	100.0
		-	+	2	527.2	93.5	365.6	6.9	100.0	60.0	200.0	0.0	100.0
		-	-	10	234.1	74.0	265.9	43.0	20.0	8.4	55.0	26.3	50.0

[a]Standard error of the mean.
[b]Titers expressed as ng Ig binding.
[c]1/maximum positive serum dilution.
[d]Percentage of positive samples.
[e]Nonvaccinates received FCA and saline on Day 0.
[f]Vaccinates received *B. abortus* cell surface protein on Day 0 and 6 weeks postvaccination.

TABLE 7: A comparison of vaccinates and nonvaccinates percent positive[a] serological reactors

Date	Treatment	No.	FIAX % POS	ELISA % POS	CF % POS	RIV % POS	CARD % POS	IgG1 % POS	IgG2 % POS	IgA % POS	IgM % POS
Day 0	Nonvaccinate[b]	12	0.0	25.0	8.3	0.0	0.0	0.0	0.0	0.0	8.3
	Vaccinate[c]	50	0.0	6.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
2WKPV	Nonvaccinate	12	83.3	58.3	50.0	58.3	75.0	83.3	91.7	16.7	66.7
	Vaccinate	50	100.0	94.0	96.0	100.0	100.0	100.0	98.0	70.0	96.0
4WKPV	Nonvaccinate	12	50.0	83.3	25.0	41.7	50.0	83.3	91.7	16.7	41.7
	Vaccinate	50	92.0	100.0	48.0	80.0	88.0	96.0	100.0	56.0	80.0
10WKPV	Nonvaccinate	12	0.0	58.3	0.0	0.0	0.0	33.3	75.0	0.0	8.3
	Vaccinate	50	100.0	98.0	72.0	90.0	92.0	100.0	100.0	80.0	76.0
31WKPV	Nonvaccinate	12	0.0	8.3	8.3	0.0	0.0	16.7	0.0	0.0	8.3
	Vaccinate	50	72.0	98.0	30.0	32.0	64.0	96.0	98.0	20.0	26.0
4WKPC	Nonvaccinate	12	91.7	100.0	58.3	66.7	75.0	91.7	83.3	58.3	83.3
	Vaccinate	50	76.0	100.0	70.0	58.0	68.0	100.0	98.0	50.0	64.0
ATA	Nonvaccinate	12	91.7	100.0	91.7	91.7	91.7	91.7	100.0	91.7	100.0
	Vaccinate	47	100.0	97.9	89.4	85.1	93.6	100.0	100.0	87.2	93.6
13WKPC	Nonvaccinate	12	100.0	100.0	91.7	91.7	91.7	91.7	91.7	91.7	91.7
	Vaccinate	50	94.0	100.0	76.0	84.0	90.0	100.0	100.0	84.0	84.0

[a]Positive reactions were defined as follows: FIAX > 50 ng Ig, ELISA > 20 ng Ig, CF $\geq 3^+$ at a 1:10 serum dilution, RIV $\geq$ 1:25, CARD any visible agglutination, IgG1 > 33.6 ng Ig, IgG2 > 25.9 ng Ig, IgM > 40.8 ng Ig, IgA > 28.5 ng Ig.

[b]Nonvaccinates received FCA and saline on Day 0.

[c]Vaccinates received *B. abortus* cell surface protein on Day 0 and 6 weeks postvaccination.

TABLE 8. A comparison of vaccinates and nonvaccinates percent positive[a] serological reactors with regard to final culture and abortion status

Date	Treatment	Culture	Abortion	No.	FIAX % Pos	ELISA % Pos	CF % Pos	RIV % Pos	CARD % Pos	IgG1 % Pos	IgG2 % Pos	IgA % Pos	IgM % Pos
Day 0	Nonvaccinate[b]	+	+	10	0.0	30.0	10.0	0.0	0.0	0.0	0.0	0.0	10.0
		-	-	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Vaccinate[c]	+	+	33	0.0	0.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0
		+	-	5	0.0	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		-	+	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		-	-	10	0.0	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2WKPV	Nonvaccinate	+	+	10	90.0	70.0	60.0	70.0	80.0	90.0	90.0	20.0	60.0
		-	-	2	50.0	0.0	0.0	0.0	50.0	50.0	100.0	0.0	0.0
	Vaccinate	+	+	33	100.0	93.9	93.9	100.0	100.0	100.0	100.0	69.7	97.0
		+	-	5	100.0	100.0	100.0	100.0	100.0	100.0	80.0	100.0	80.0
		-	+	2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		-	-	10	100.0	90.0	100.0	100.0	100.0	100.0	100.0	50.0	100.0
4WKPV	Nonvaccinate	+	+	10	60.0	90.0	30.0	50.0	60.0	90.0	90.0	20.0	50.0
		-	-	2	0.0	50.0	0.0	0.0	0.0	50.0	100.0	0.0	0.0
	Vaccinate	+	+	33	90.9	100.0	39.4	75.8	84.8	97.0	100.0	51.5	78.8
		+	-	5	100.0	100.0	100.0	100.0	100.0	80.0	100.0	100.0	100.0
		-	+	2	100.0	100.0	50.0	100.0	100.0	100.0	100.0	50.0	50.0
		-	-	10	90.0	100.0	50.0	80.0	90.0	100.0	100.0	50.0	80.0
10WKPV	Nonvaccinate	+	+	10	0.0	60.0	0.0	0.0	0.0	40.0	80.0	0.0	10.0
		-	-	2	0.0	50.0	0.0	0.0	0.0	0.0	50.0	0.0	0.0
	Vaccinate	+	+	33	100.0	97.0	69.7	90.9	90.9	100.0	100.0	72.7	72.7
		+	-	5	100.0	100.0	80.0	100.0	100.0	100.0	100.0	100.0	80.0
		-	+	2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	50.0
		-	-	10	100.0	100.0	70.0	80.0	90.0	100.0	100.0	90.0	90.0
31WKPV	Nonvaccinate	+	+	10	0.0	10.0	10.0	0.0	0.0	20.0	0.0	0.0	10.0
		-	-	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Vaccinate	+	+	33	60.6	97.0	18.2	27.3	51.5	93.9	97.0	15.2	30.3
		+	-	5	80.0	100.0	60.0	0.0	100.0	100.0	100.0	20.0	20.0
		-	+	2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	50.0
		-	-	10	100.0	100.0	40.0	30.0	80.0	100.0	100.0	20.0	10.0
4WKPC	Nonvaccinate	+	+	10	100.0	100.0	70.0	80.0	90.0	100.0	100.0	70.0	90.0
		-	-	2	50.0	100.0	0.0	0.0	0.0	50.0	0.0	0.0	50.0
	Vaccinate	+	+	33	87.9	100.0	87.9	78.8	84.8	100.0	100.0	66.7	78.8
		+	-	5	40.0	100.0	40.0	0.0	60.0	100.0	100.0	0.0	0.0
		-	+	2	100.0	100.0	100.0	50.0	50.0	100.0	100.0	50.0	100.0
		-	-	10	50.0	100.0	20.0	20.0	20.0	100.0	90.0	20.0	40.0
ATA	Nonvaccinate	+	+	10	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		-	-	2	50.0	100.0	50.0	50.0	50.0	50.0	100.0	50.0	100.0
	Vaccinate	+	+	33	100.0	97.0	93.9	97.0	100.0	100.0	100.0	100.0	100.0
		+	-	5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		-	+	1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		-	-	8	100.0	100.0	62.5	25.0	62.5	100.0	100.0	25.0	62.5
13WKPC	Nonvaccinate	+	+	10	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		-	-	2	100.0	100.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
	Vaccinate	+	+	33	100.0	100.0	84.8	97.0	100.0	100.0	100.0	97.0	93.9
		+	-	5	100.0	100.0	60.0	80.0	100.0	100.0	100.0	80.0	100.0
		-	+	2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		-	-	10	70.0	100.0	50.0	40.0	50.0	100.0	100.0	40.0	40.0

[a]Positive reactions were defined as follows: FIAX > 50 ng Ig, ELISA > 20 ng Ig, CF $\geq 3^{+}$ at a 1:10 serum dilution, RIV $\geq$ 1:25, CARD any visible agglutination, IgG1 > 33.6 ng Ig, IgG2 > 25.9 ng Ig, IgM > 40.8 ng Ig, IgA > 28.5 ng Ig.

[b]Nonvaccinates received FCA and saline on Day 0.

[c]Vaccinates received *B. abortus* cell surface protein on Day 0 and 6 weeks postvaccination.

Table 9. A comparison of vaccinates and nonvaccinates percent positive[a] vaginal and corrected vaginal samples

Date	Treatment	No.	Vaginal IgG1 % Pos	Corrected Vaginal IgG1 % Pos	Vaginal IgG2 % Pos	Corrected Vaginal IgG2 % Pos	Vaginal IgA % Pos	Corrected Vaginal IgA % Pos
4WKPV	Nonvaccinate[b]	8	75.0	50.0	87.5	75.0	25.0	50.0
	Vaccinate[c]	41	82.9	68.3	80.5	65.9	39.0	29.3
31WKPV	Nonvaccinate	4	0.0	0.0	0.0	0.0	0.0	0.0
	Vaccinate	33	81.8	42.4	24.2	18.2	3.0	9.1
4WKPC	Nonvaccinate	12	83.3	33.3	50.0	33.3	66.7	33.3
	Vaccinate	51	82.4	58.8	47.1	31.4	49.0	27.5
ATA	Nonvaccinate	12	100.0	75.0	83.3	66.7	91.7	50.0
	Vaccinate	46	93.5	89.1	89.1	82.6	89.1	71.7
13WKPC	Nonvaccinate	4	75.0	0.0	50.0	0.0	75.0	0.0
	Vaccinate	25	84.0	40.0	72.0	24.0	84.0	28.0

[a]vaginal IgG1 > 13.8 ng Ig, corrected vaginal IgG1 > 30.1 ng Ig,
vaginal IgG2 > 34.0 ng Ig, corrected vaginal IgG2 > 57.6 ng Ig,
vaginal IgA > 12.6 ng Ig, corrected vaginal IgA > 19.1 ng Ig.
[b]Nonvaccinates received FCA and saline on Day 0.
[c]Vaccinates received B. abortus cell surface protein on Day 0 and 6 weeks postvaccination.

Table 10. A comparison of vaccinates and nonvaccinates percent positive[a] vaginal and corrected vaginal samples with regard to final culture and abortion status

Date	Treatment	Culture	Abortion	No.	Vaginal IgG1 % Pos	Corrected Vaginal IgG1 % Pos	Vaginal IgG2 % Pos	Corrected Vaginal IgG2 % Pos	Vaginal IgA % Pos	Corrected Vaginal IgA % Pos
4WKPV	Nonvaccinate[b]	+	+	7	85.7	57.1	100.0	85.7	28.6	57.1
		-	-	1	0.0	0.0	0.0	0.0	0.0	0.0
	Vaccinate[c]	+	+	25	72.0	64.0	80.0	60.0	36.0	28.0
		+	-	4	100.0	100.0	100.0	100.0	50.0	50.0
		-	+	2	100.0	100.0	100.0	100.0	100.0	100.0
		-	-	9	100.0	66.7	77.8	66.7	33.3	11.1
31WKPV	Nonvaccinate	+	+	4	0.0	0.0	0.0	0.0	0.0	0.0
	Vaccinate	+	+	17	88.2	41.2	35.3	17.6	5.9	5.9
		+	-	4	100.0	0.0	0.0	0.0	0.0	0.0
		-	+	1	100.0	100.0	100.0	100.0	0.0	0.0
		-	-	10	70.0	60.0	10.0	20.0	0.0	20.0
4WKPC	Nonvaccinate	+	+	10	90.0	40.0	60.0	40.0	80.0	40.0
		-	-	2	50.0	0.0	0.0	0.0	0.0	0.0
	Vaccinate	+	+	33	97.0	72.7	66.7	42.4	60.6	30.3
		+	-	5	60.0	20.0	0.0	0.0	20.0	0.0
		-	+	2	50.0	50.0	50.0	50.0	0.0	50.0
		-	-	10	50.0	30.0	0.0	0.0	30.0	20.0
ATA	Nonvaccinate	+	+	10	100.0	90.0	100.0	80.0	100.0	60.0
		-	-	2	100.0	0.0	0.0	0.0	50.0	0.0
	Vaccinate	+	+	33	100.0	100.0	100.0	93.9	100.0	90.9
		+	-	5	80.0	80.0	80.0	60.0	80.0	20.0
		-	+	1	100.0	100.0	100.0	100.0	100.0	100.0
		-	-	7	71.4	42.9	42.9	42.9	42.9	14.3

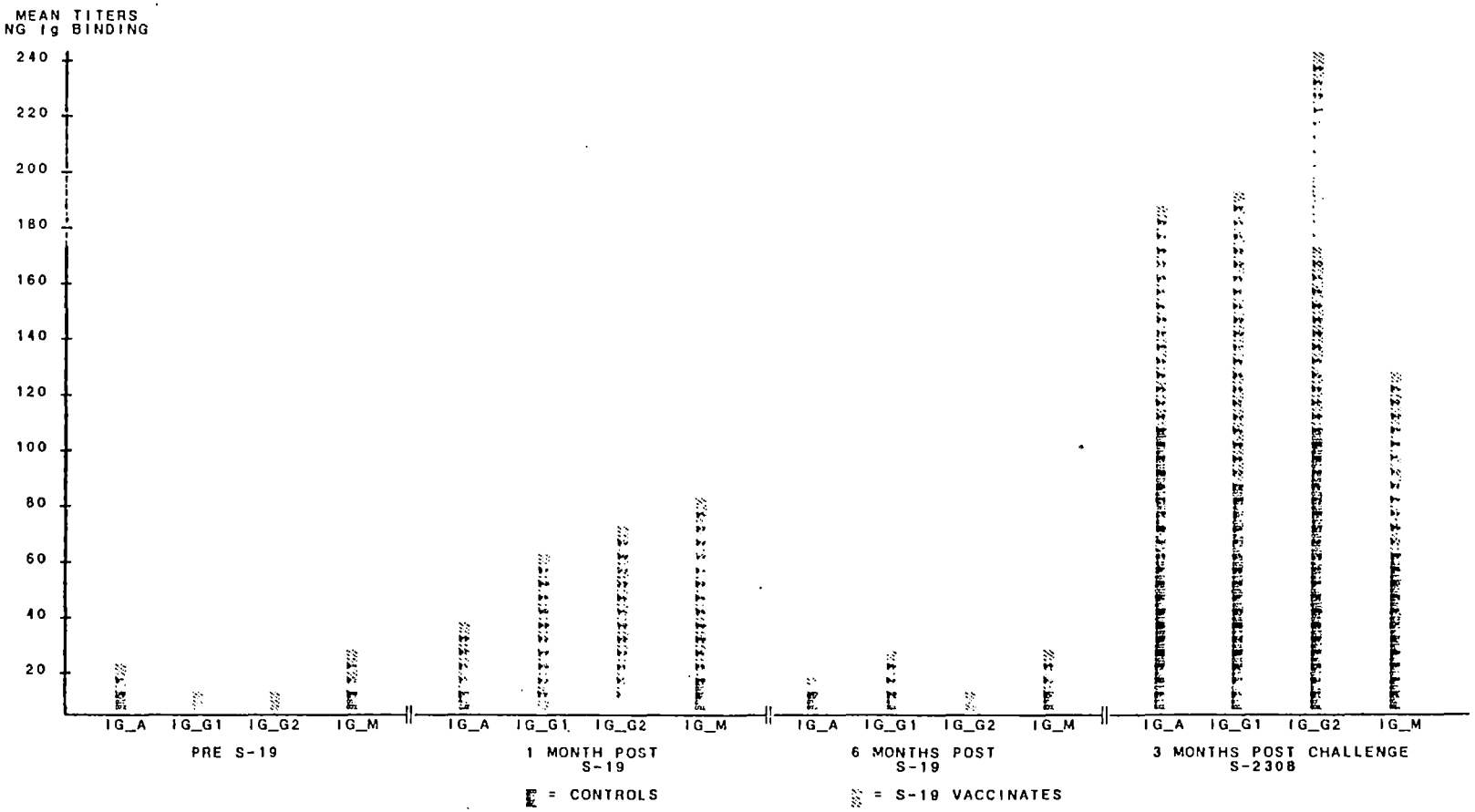
Table 10 (Continued)

Date	Treatment	Culture	Abortion	No.	Vaginal IgG1 % Pos	Corrected Vaginal IgG1 % Pos	Vaginal IgG2 % Pos	Corrected Vaginal IgG2 % Pos	Vaginal IgA % Pos	Corrected Vaginal IgA % Pos
13WKPC	Nonvaccinate	+	+	4	75.0	0.0	50.0	0.0	75.0	0.0
	Vaccinate	+	+	19	89.5	36.8	73.7	31.6	89.5	31.6
		+	-	2	100.0	50.0	100.0	0.0	100.0	0.0
		-	+	1	100.0	100.0	100.0	0.0	100.0	100.0
		-	-	3	33.3	33.3	33.3	0.0	33.3	0.0

[a]vaginal IgG1 > 13.8 ng Ig, corrected vaginal IgG1 > 30.1 ng Ig,
vaginal IgG2 > 34.0 ng Ig, corrected vaginal IgG2 > 57.6 ng Ig,
vaginal IgA > 12.6 ng Ig, corrected vaginal IgA > 19.1 ng Ig.
[b]Nonvaccinates received FCA and saline on Day 0.
[c]Vaccinates received B. abortus cell surface protein on Day 0 and 6 weeks postvaccination.

APPENDIX

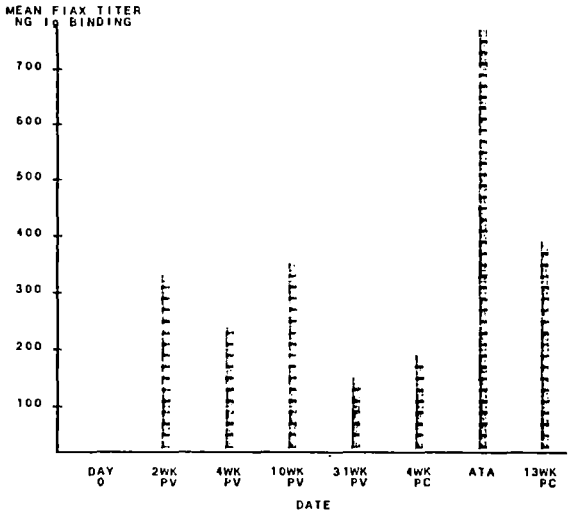
SERUM Ig CLASS & SUBCLASS RESPONSE TO S-19



SERUM Ig CLASS & SUBCLASS RESPONSE TO S-19

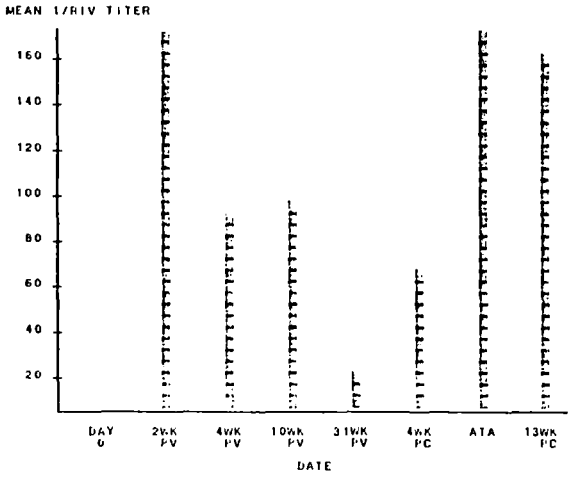
STANDARD SEROLOGICAL TESTS

FIAX



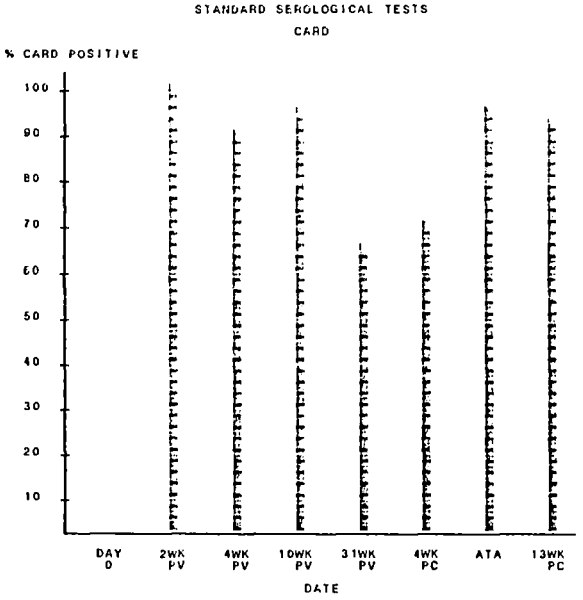
STANDARD SEROLOGICAL TESTS

RIV



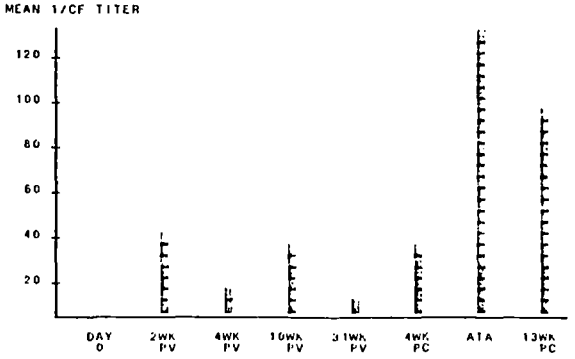
STANDARD SEROLOGICAL TESTS

CARD



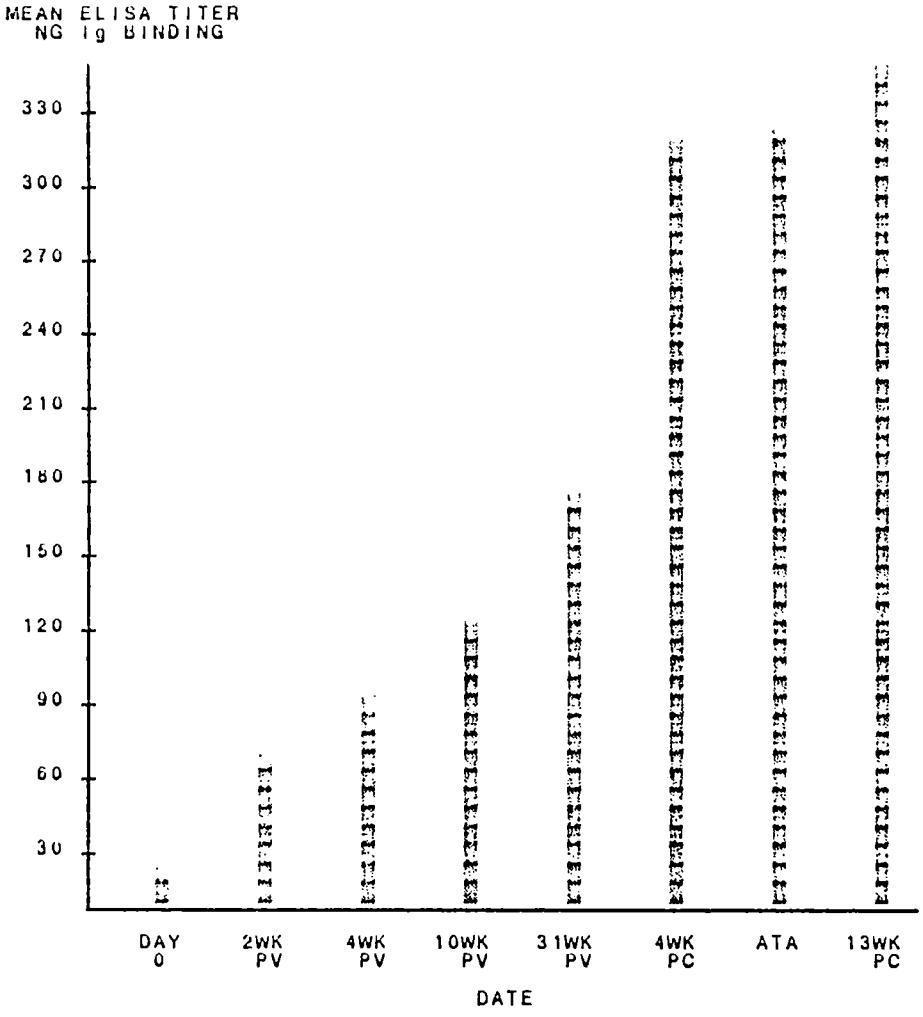
STANDARD SEROLOGICAL TESTS

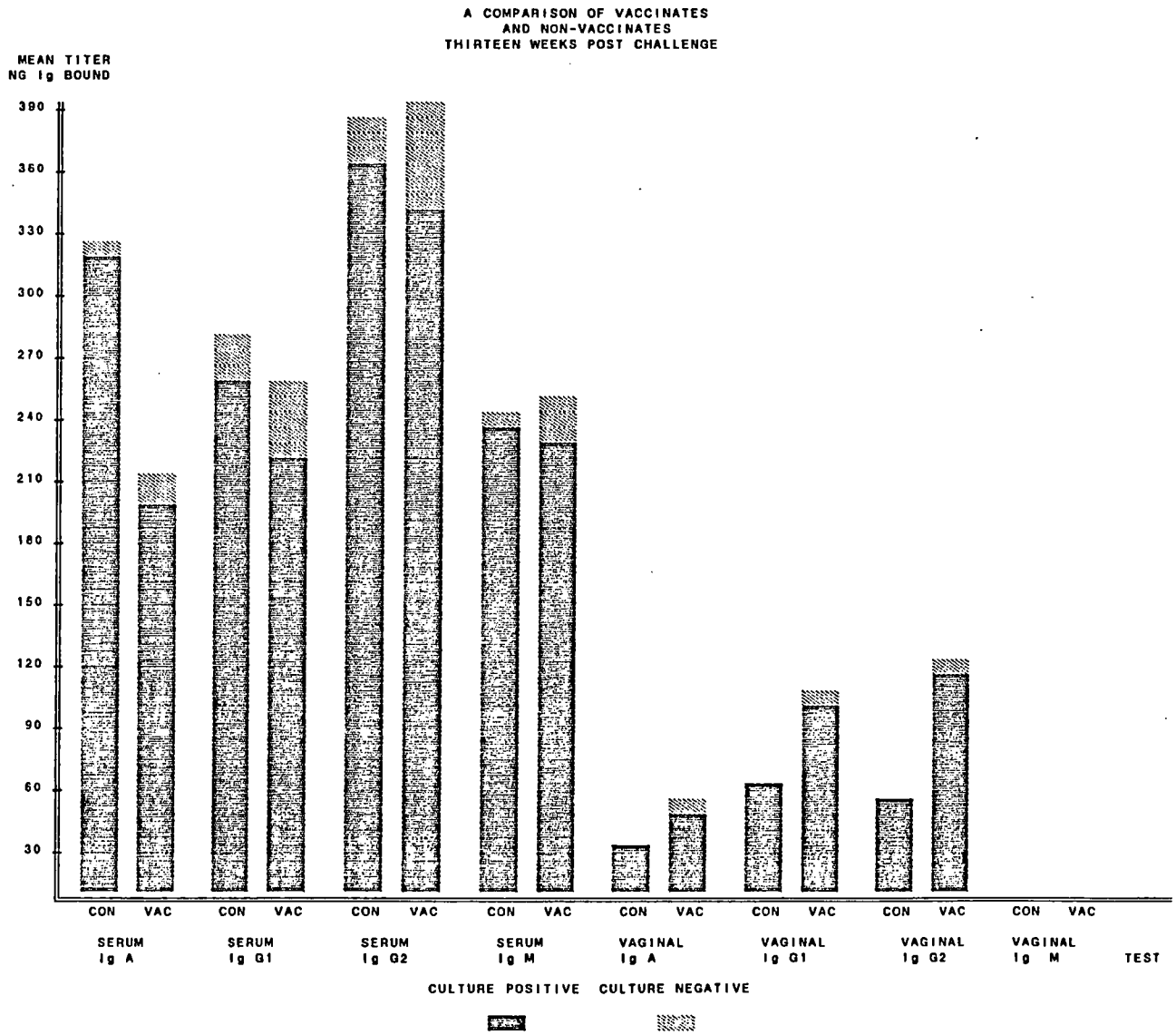
CF

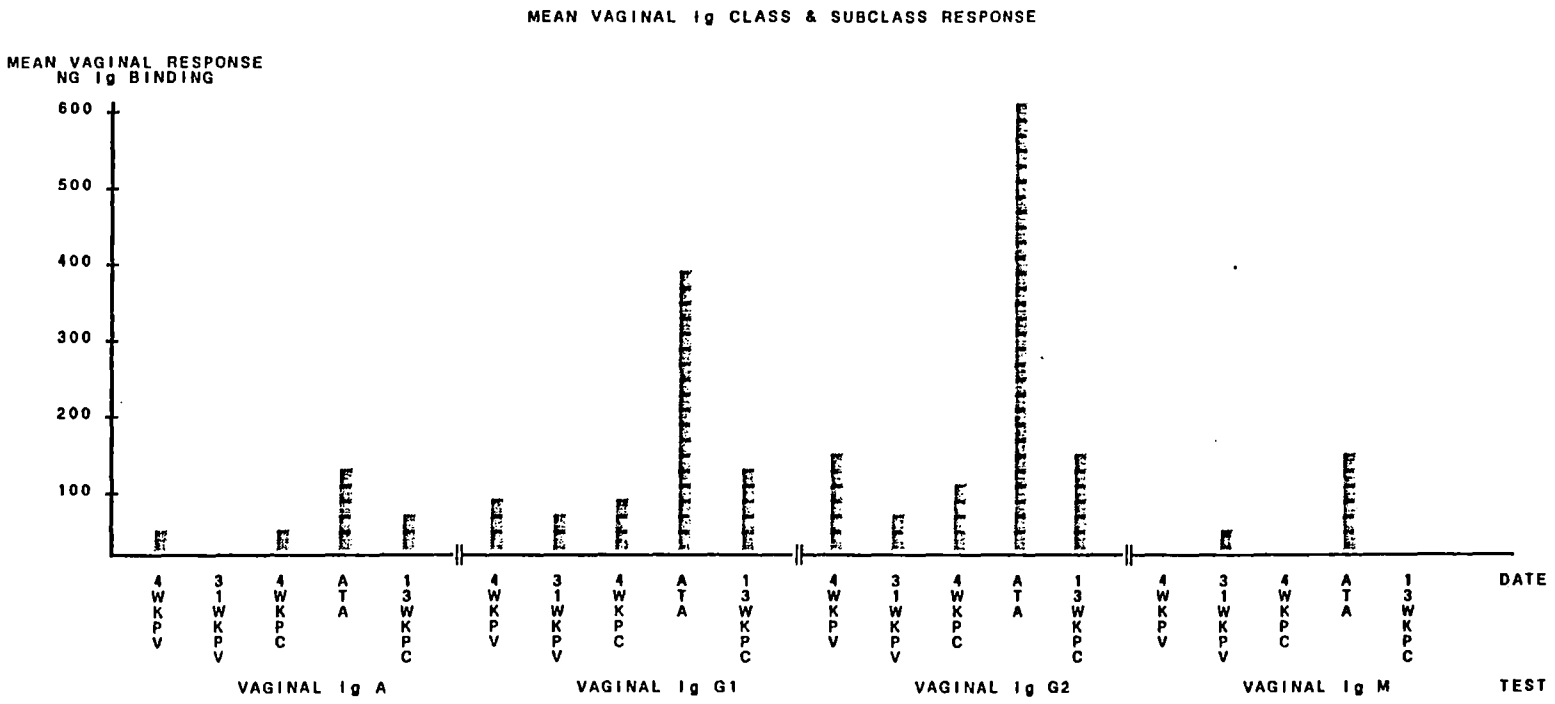


STANDARD SEROLOGICAL TESTS

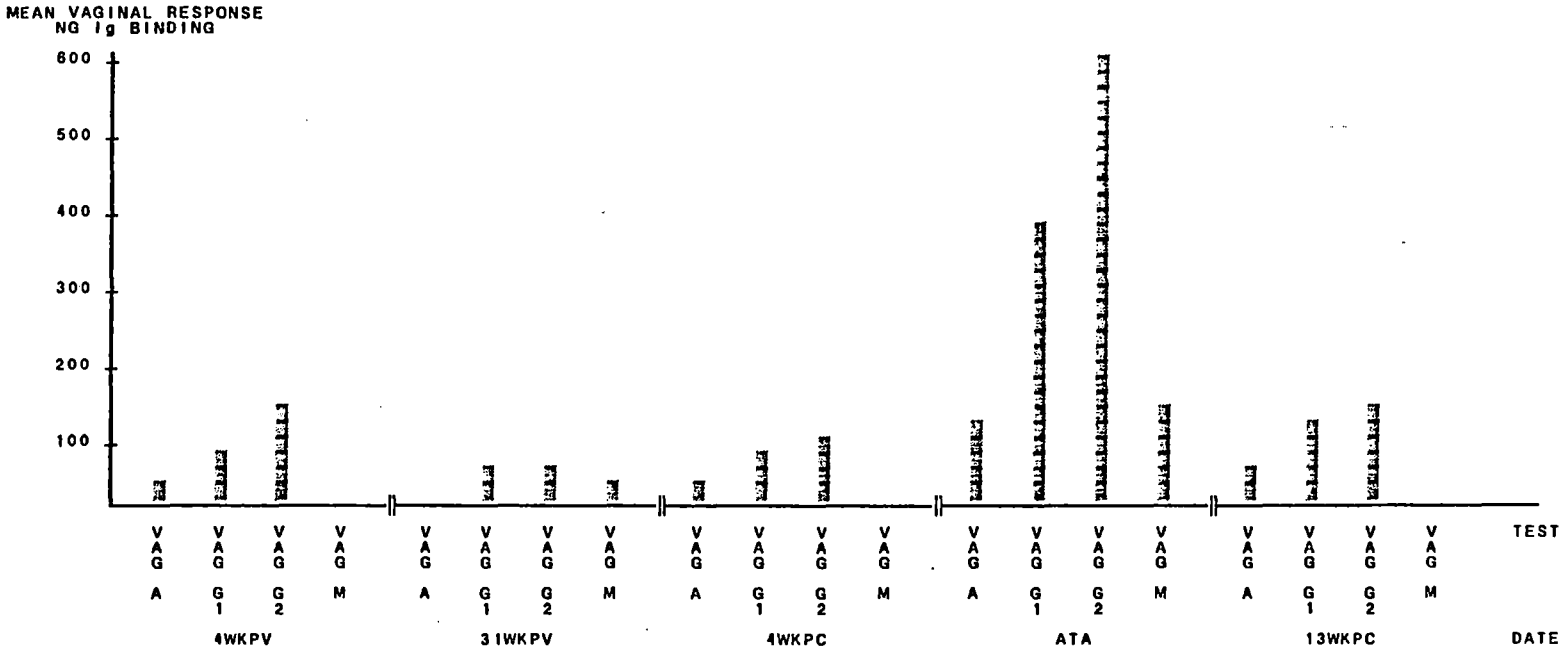
ELISA



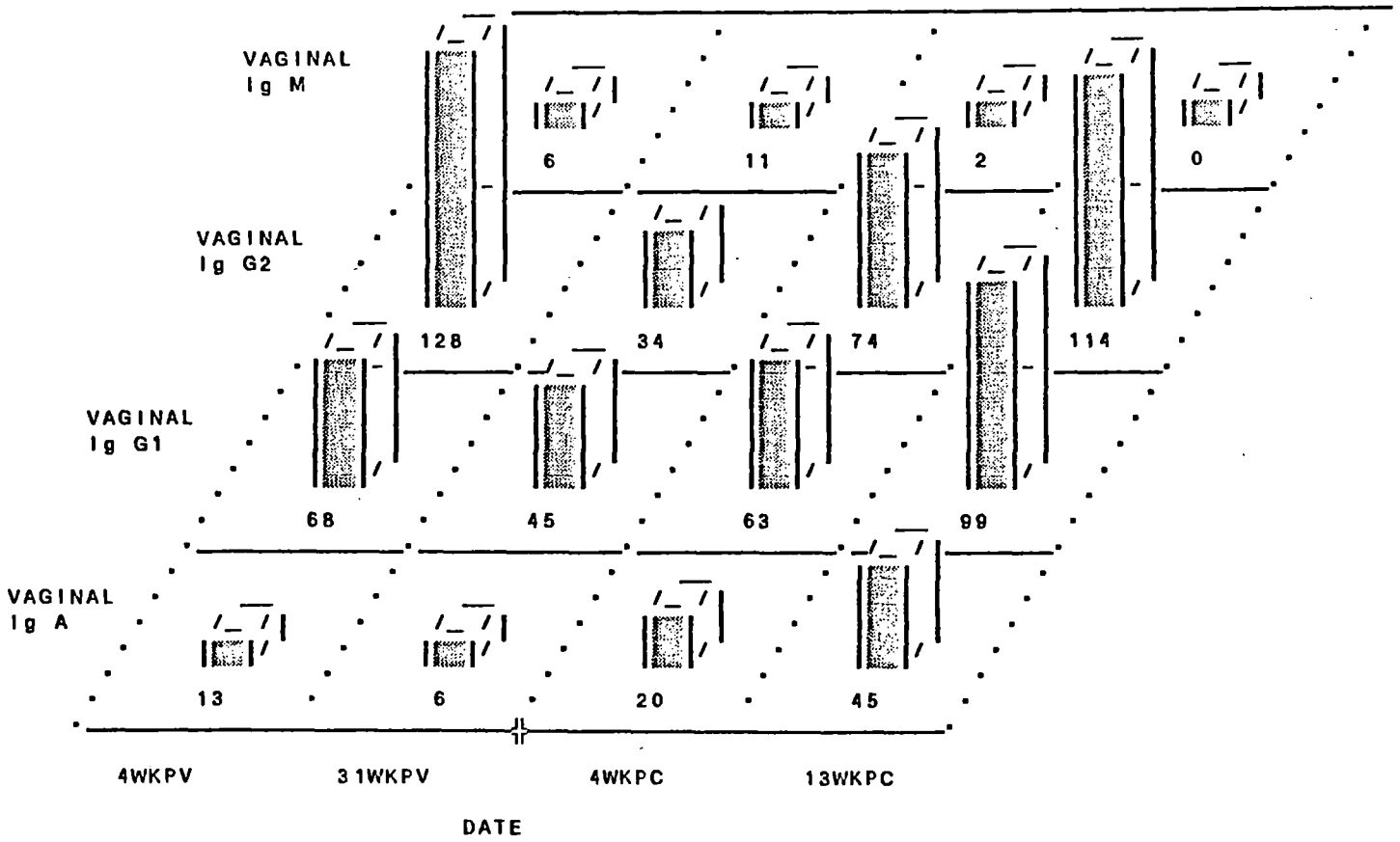

A COMPARISON OF VACCINATES
AND NON-VACCINATES
THIRTEEN WEEKS POST CHALLENGE
MEAN TITER
NG Ig BOUND
390
360
330
300
270
240
210
180
150
120
90
60
30
CON VAC
SERUM
Ig A
SERUM
Ig G1
SERUM
Ig G2
SERUM
Ig M
VAGINAL
Ig A
VAGINAL
Ig G1
VAGINAL
Ig G2
VAGINAL
Ig M
TEST
CULTURE POSITIVE
CULTURE NEGATIVE

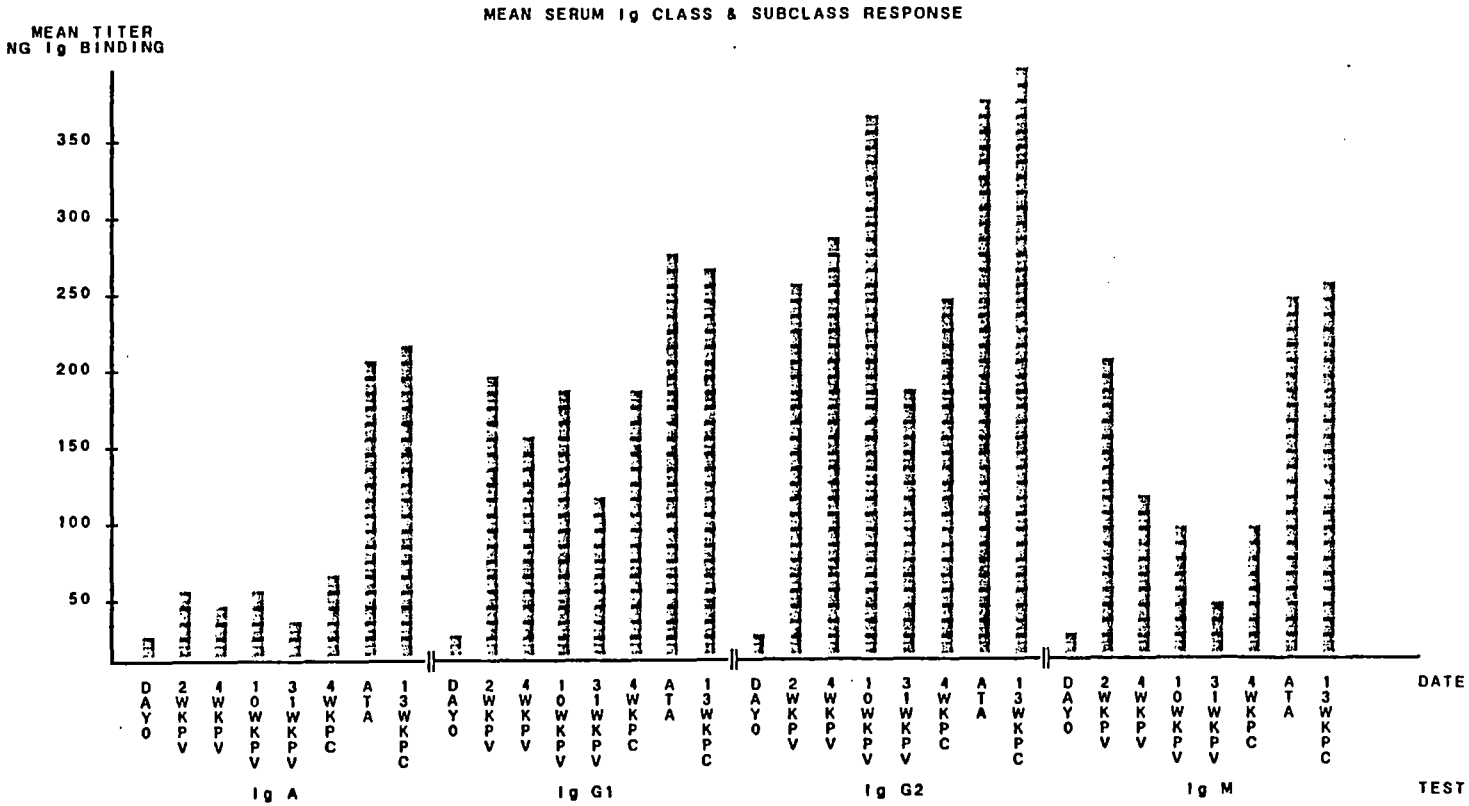

MEAN VAGINAL Ig CLASS & SUBCLASS RESPONSE
MEAN VAGINAL RESPONSE
NG Ig BINDING
600
500
400
300
200
100
4 WKPV
31 WKPV
4 WKPC
ATA
13 WKPC
DATE
VAGINAL Ig A
VAGINAL Ig G1
VAGINAL Ig G2
VAGINAL Ig M
TEST

MEAN VAGINAL Ig CLASS & SUBCLASS RESPONSE

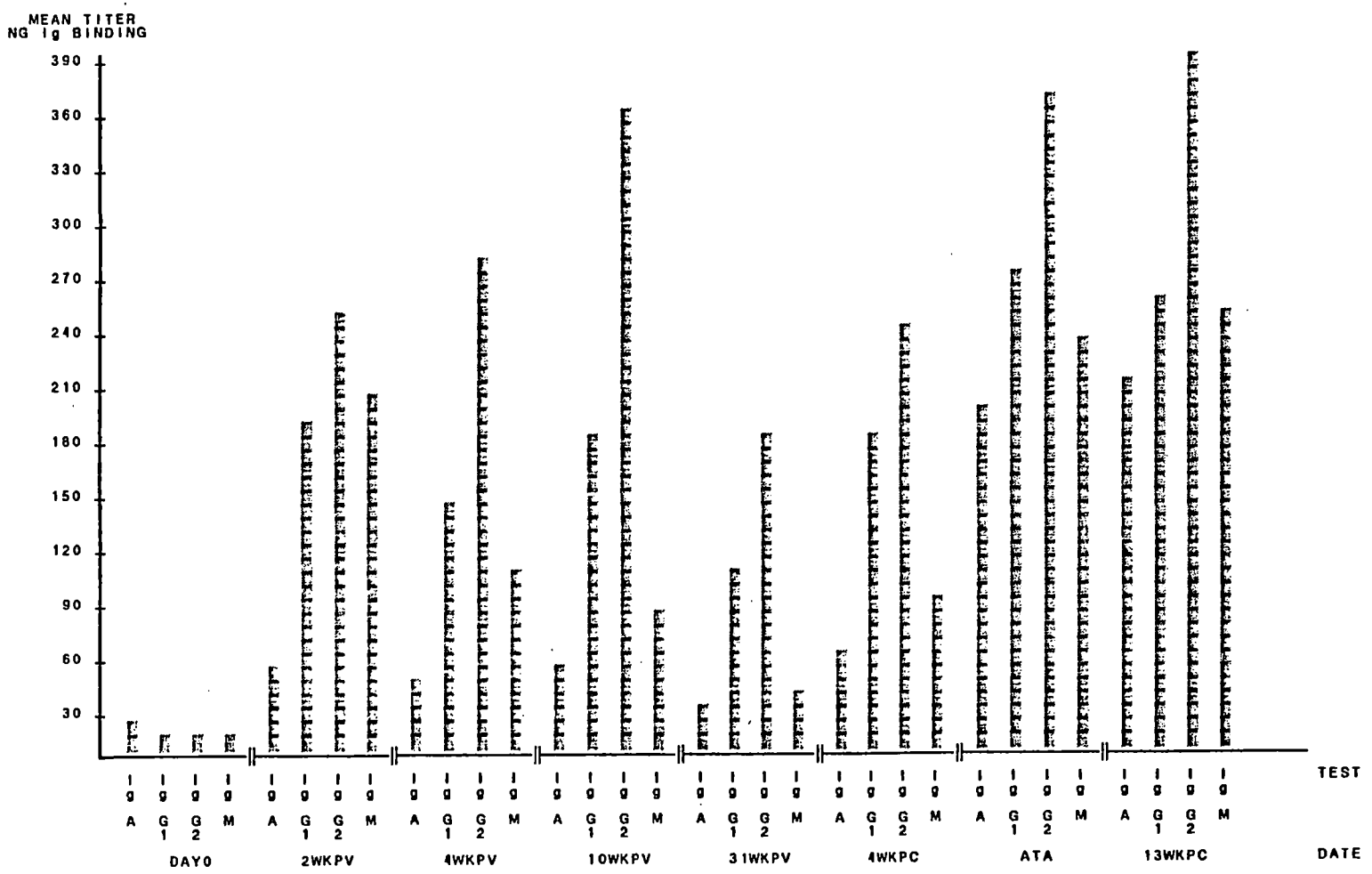


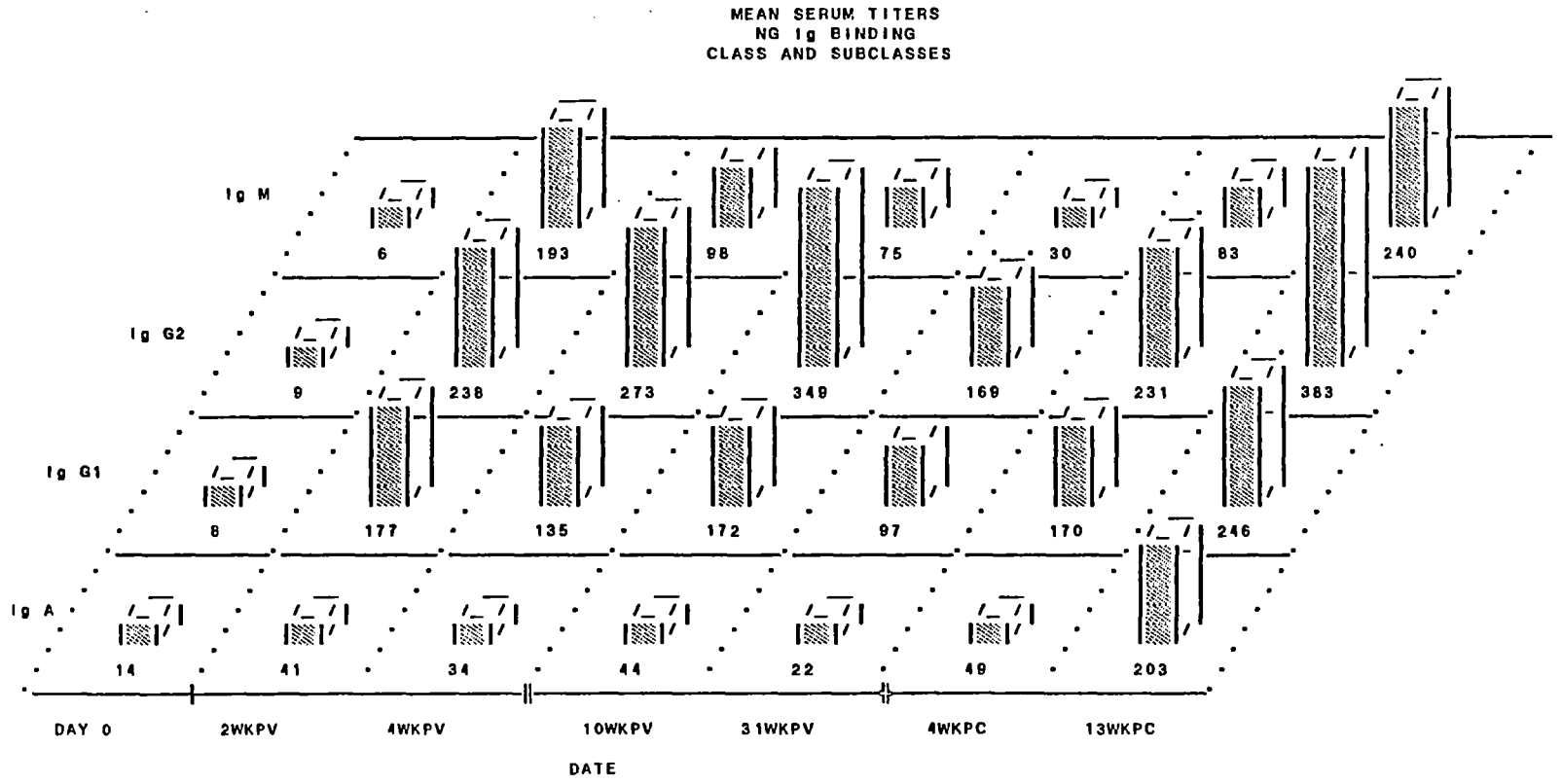
MEAN VAGINAL TITERS
NG Ig BINDING
CLASS AND SUBCLASSES

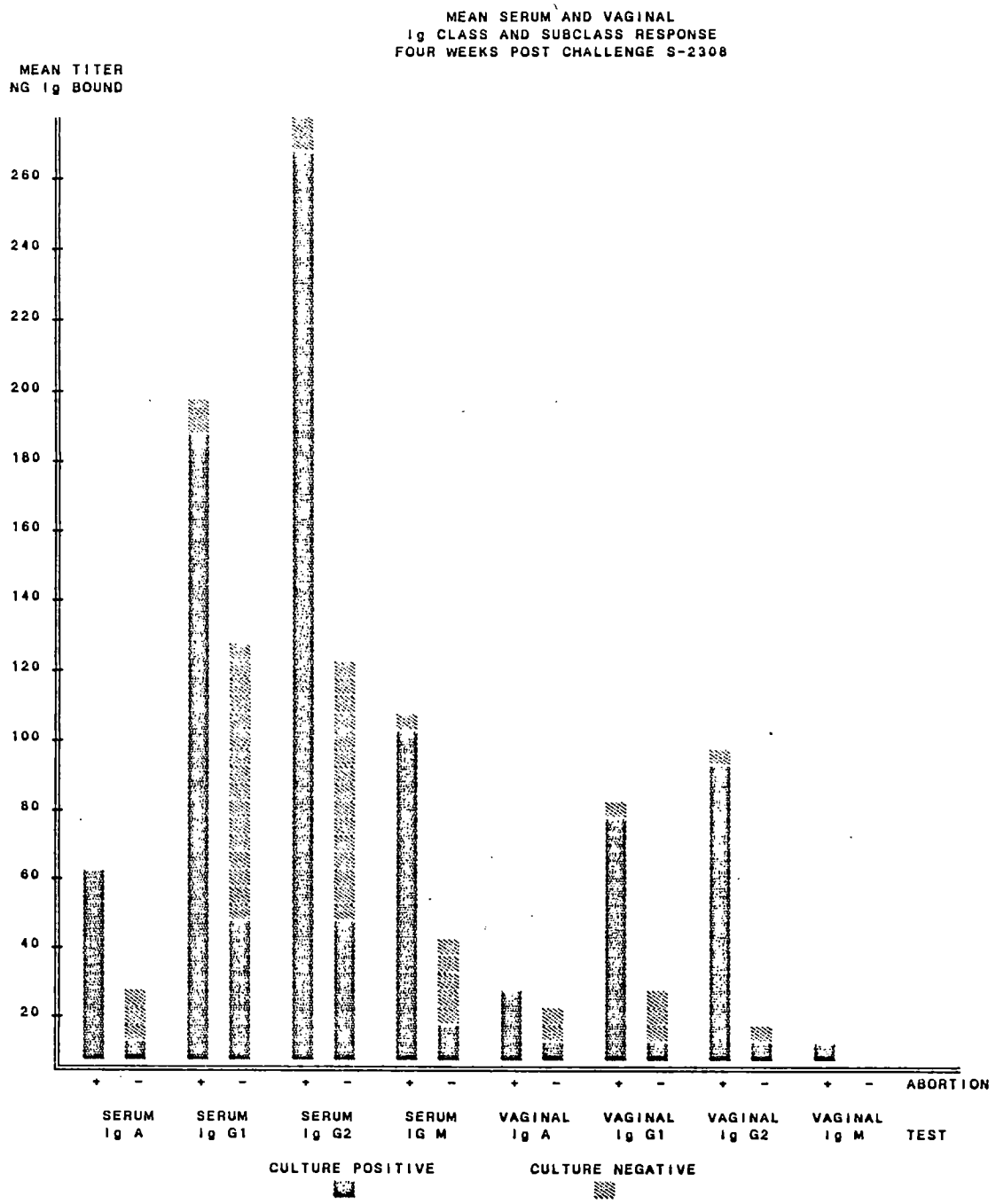


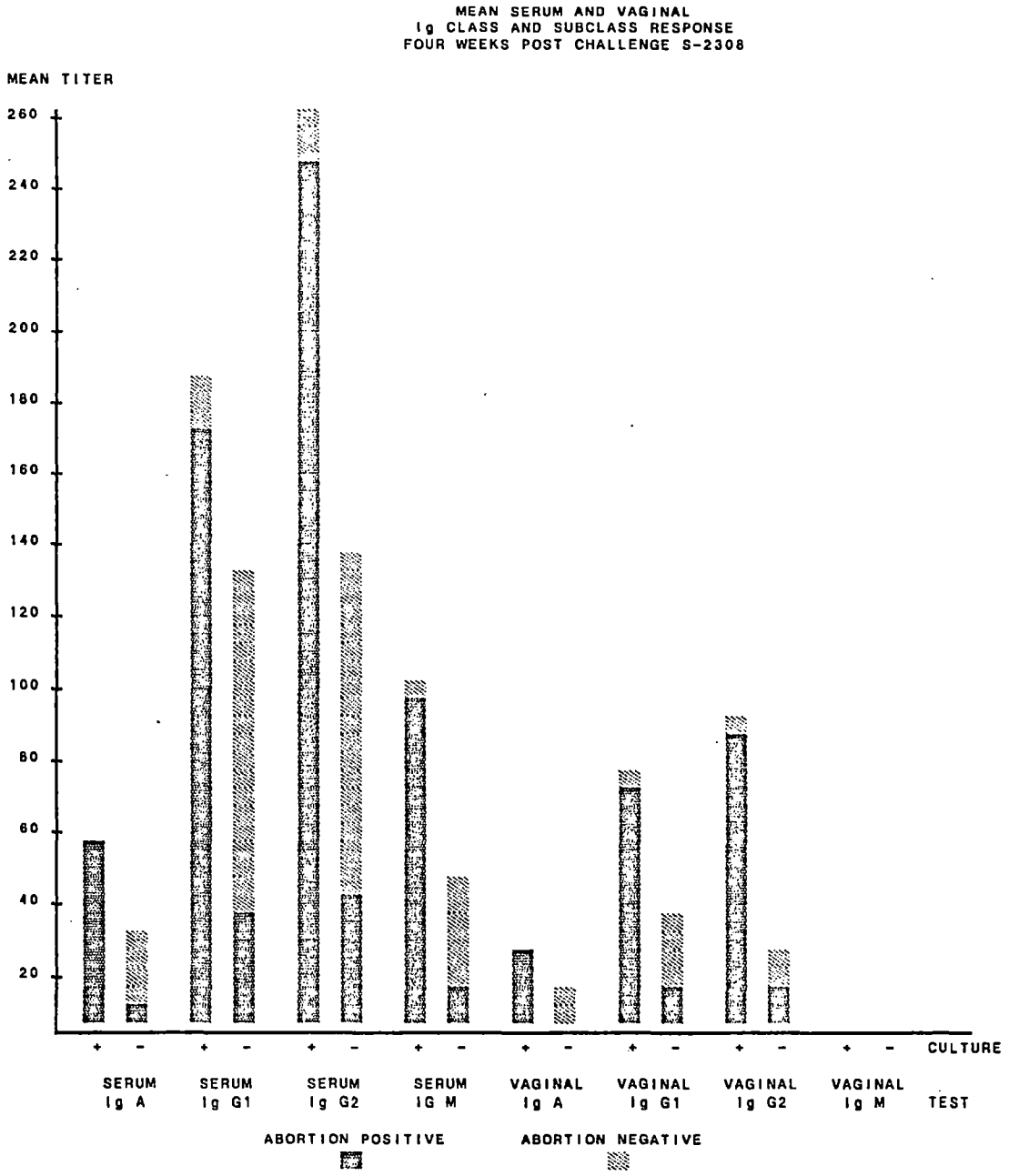

MEAN SERUM Ig CLASS & SUBCLASS RESPONSE
MEAN TITER
NG Ig BINDING
350
300
250
200
150
100
50
DAY 0
2WKPV
4WKPV
10WKPV
31WKPV
4WKPC
ATA
13WKPC
Ig A
Ig G1
Ig G2
Ig M
DATE
TEST

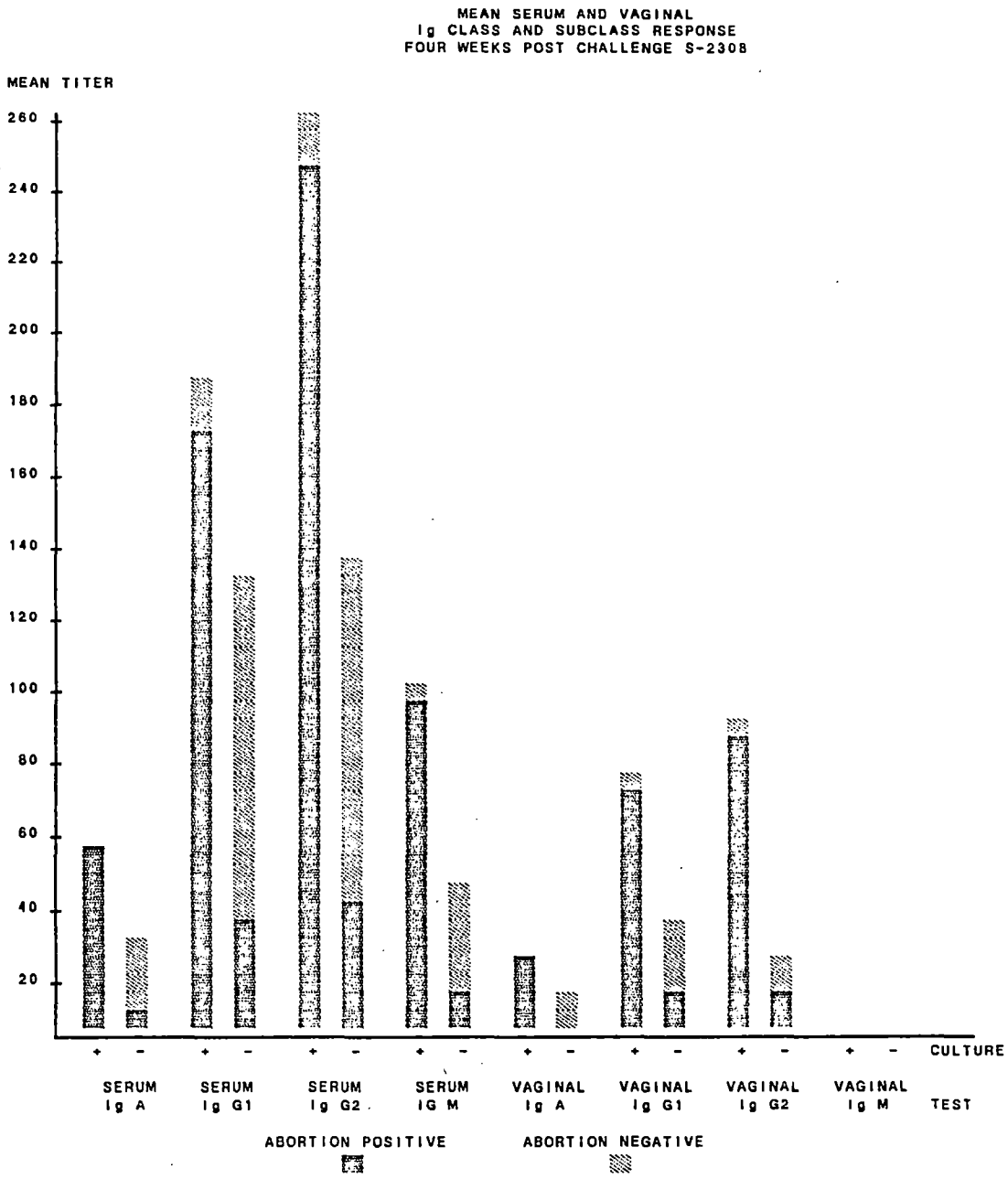
MEAN SERUM Ig CLASS & SUBCLASS RESPONSE




MEAN SERUM TITERS
NG Ig BINDING
CLASS AND SUBCLASSES
Ig M
Ig G2
Ig G1
Ig A
6
193
98
75
30
83
240
9
238
273
349
169
231
383
8
177
135
172
97
170
246
14
41
34
44
22
49
203
DAY 0
2WKPV
4WKPV
10WKPV
31WKPV
4WKPC
13WKPC
DATE


MEAN SERUM AND VAGINAL
Ig CLASS AND SUBCLASS RESPONSE
FOUR WEEKS POST CHALLENGE S-2308
MEAN TITER
NG Ig BOUND
260
240
220
200
180
160
140
120
100
80
60
40
20
+ - + - + - + - + - + - + - + - ABORTION
SERUM Ig A
SERUM Ig G1
SERUM Ig G2
SERUM IG M
VAGINAL Ig A
VAGINAL Ig G1
VAGINAL Ig G2
VAGINAL Ig M
TEST
CULTURE POSITIVE
CULTURE NEGATIVE


MEAN SERUM AND VAGINAL
Ig CLASS AND SUBCLASS RESPONSE
FOUR WEEKS POST CHALLENGE S-2308
MEAN TITER
260
240
220
200
180
160
140
120
100
80
60
40
20
+ - + - + - + - + - + - + - + - CULTURE
SERUM Ig A
SERUM Ig G1
SERUM Ig G2
SERUM IG M
VAGINAL Ig A
VAGINAL Ig G1
VAGINAL Ig G2
VAGINAL Ig M
TEST
ABORTION POSITIVE
ABORTION NEGATIVE


MEAN SERUM AND VAGINAL
Ig CLASS AND SUBCLASS RESPONSE
FOUR WEEKS POST CHALLENGE S-2308
MEAN TITER
260
240
220
200
180
160
140
120
100
80
60
40
20
+ - + - + - + - + - + - + - + - CULTURE
SERUM Ig A
SERUM Ig G1
SERUM Ig G2
SERUM IG M
VAGINAL Ig A
VAGINAL Ig G1
VAGINAL Ig G2
VAGINAL Ig M
TEST
ABORTION POSITIVE
ABORTION NEGATIVE

BASIC Program for Two Class Discrimination

```
10    PRINT CHR$(12)                : REM CLEAR THE SCREEN
20    GOSUB 1340
30    PRINT "MEAN FOR ";A$;" = ";
40    INPUT M1
50    PRINT "MEAN FOR ";B$;" = ";
60    INPUT M2
70    PRINT "# OF ";A$;"'S = ";
80    INPUT A1
90    PRINT "# OF ";B$;"'S = ";
100   INPUT A2
110   PRINT "DO YOU WANT TO ENTER: "
120   PRINT "1. STANDARD DEV."
130   PRINT "2. VARIANCE      "
140   PRINT "3. STANDARD ERROR OF MEAN"
150   PRINT
160   INPUT C$
170   IF C$="1" GOTO 210
180   IF C$="2" GOTO 330
190   IF C$="3" GOTO 270
200   GOTO 110
210   PRINT A$;" STD = ";
220   INPUT V1
230   PRINT B$;" STD = ";
240   INPUT V2
250   V1=V1^2 : V2=V2^2
260   GOTO 370
270   PRINT A$;" STD ERROR = ";
280   INPUT V1
290   PRINT B$;" STD ERROR = ";
300   INPUT V2
310   V1=(V1^2)*A1 : V2=(V2^2)*A2
320   GOTO 370
330   PRINT A$;" VARIANCE = ";
340   INPUT V1
350   PRINT B$;" VARIANCE = ";
360   INPUT V2
370   Z1=SQR(V1) : Z2=SQR(V2)
380   GOSUB 1440
390   GOSUB 1590
400   GOSUB 1710
410   PRINT TAB(23);A$;TAB(48);B$
420   PRINT
430   PRINT"MEAN"; TAB(25)M1;TAB(50)M2
440   PRINT
450   PRINT"VAR.";TAB(25)V1;TAB(50)V2
460   PRINT
470   PRINT"STD.";TAB(25)Z1;TAB(50)Z2
480   PRINT
490   PRINT"The F-value of These Two Variances is = ";FF
500   PRINT"With Numerator Degrees of Freedom     = ";DN
510   PRINT"   And Denominator Degrees of Freedom = ";DD
```

```
520   PRINT
530   PRINT"The Probability of a Greater Value of F is = ";FP;"
      (one-tailed)"
540   IF FP < .05 GOTO 580
550   PRINT"The Probability of a Greater Value of  F  Was
      > .05  Thus"
560   PRINT"     Equal Variances Are Used In Calculating T-test
      Values"
570   GOTO 600
580   PRINT"The Probability of a Greater Value of F Was   < .05
      Thus "
590   PRINT"  Un-equal Variances Are Used in Calculating T-test
      values"
600   PRINT
610   PRINT"The T-test Value For The Two Sample Means = ";T
620   PRINT
630   PRINT"The Probability of a > Value of  T (two-tailed)
      = ";PF;" t-df= ";DF
640   IF PF > .05 THEN GOTO 1760
650   PRINT
660   INPUT "Enter 'RETURN' To Continue ";C$
670   PRINT CHR$(12)              : REM CLEAR THE SCREEN
680   PRINT"Sample Value of :";TAB(23)"% PROB. of "A$;TAB(48)"
      % PROB. of "B$
690   PRINT
700   GOTO 760
710   P2=INT(P2*100000!+.5)/1000 :
      REM ROUND-OFF f PRINT DISCRIM PROBABILITIES
720   P1=INT(P1*100000!+.5)/1000
730   PRINT X,TAB(23) P1;TAB(48)P2
740   PRINT
750   RETURN     : REM RETURN TO LINE 1150 OR 1250
760   YL=.05     : REM SET-UP THEN GOSUB PROBABILITY LEVELS
770   GOSUB 830 : REM     YL= DESIRED TESTING LEVELS
780   YL=.5
790   GOSUB 830
800   YL=.95
810   GOSUB 830
820   GOTO 1090
830                REM NEWTON'S METHOD SUCCESSIVE APPROXIMATION
840   LI=.0001   : REM LIMITS CLOSENESS OF PROB. CALCULATION
850   XD=1       : REM SETS DIRECTION TO MOVE X (+ OR -)
860   IF V1 < V2 THEN X = M1:
      REM SET X AS APPROXIMATION START POINT
870   IF V2 < V1 THEN X = M2
880   IF V1=V2 THEN X=(M1+M2)/2
890   XA=X/2         : REM XA=VALUE FOR LARGE INCREMENT MOVE OF X
900   GOSUB 1230    : REM SUB CALCULATE DISCRIM FUNCTIONS
910   DS=ABS(P1-YL): REM HOW CLOSE IS ESTIMATE
920   IF DS=0 GOTO 1060 : REM EXACTLY CORRECT
930   X=X+X*.1   : REM VALUE FOR SMALL INCREMENT MOVE OF X
940   GOTO 960
950   X=X+(XA*XD)    : REM LARGE INCREMENT CHANGE MADE TO X
960   OD=DS          : REM STORES APPROXIMATION FROM LINE 1000
```

```
970   GOSUB 1230     : REM RE-CALCULATE NEW DISCRIM FUNCTIONS
980   DS=ABS(P1-YL) : REM HOW CLOSE IS NEW ESTIMATE
990   IF DS < LI GOTO 1060 :
      REM IF TRUE,CLOSE ENOUGH SO GO PRINT
1000  IF DS < OD GOTO 950 : REM IF TRUE,NOT CLOSE ENOUGH,
      RIGHT DIRECTION
1010  IF XA < .000001 GOTO 1060 : REM IF TRUE,CAN NOT REACH,
      GIVE-UP, GO PRINT
1020  IF DS=OD GOTO 950      : REM DID NOT CHANGE, CONTINUE
1030  XD=-XD                 : REM MOVING WRONG WAY,
      CHANGE DIRECTION
1040  XA=XA/2
1050  GOTO 950
1060  GOSUB 710              : REM SUB TO PRINT DISCRIM PROB.
1070  PRINT
1080  RETURN
1090  INPUT "Sample Observation or '999' to END or '9999'
      for NEW VALUES ";X
1100  IF X=999 THEN END
1110  IF X=9999 GOTO 1130
1120  GOTO 1150
1130  PRINT "-------------------------------------------------"
1140  GOTO 10
1150  CK=1    : REM CK USED TO INHIBIT WARNING MESSAGE
1160  GOSUB 1230
1170  PRINT
1180  PRINT "Sample Value of:";TAB(23)"% Prob. of "A$;
      TAB(48)"% Prob. of "B$
1190  PRINT
1200  GOSUB 710 : REM SUB PRINT DISCRIM PROBABILITIES
1210  PRINT
1220  GOTO 1090
1230  D1=(X-M1)^2/V1+LOG(V1)     :
      REM GENERALIZED ^2 DISTANCE CLASS 1
1240  D2=(X-M2)^2/V2+LOG(V2)     :
      REM GENERALIZED ^2 DISTANCE CLASS 2
1250  P1=1
1260  P2=1
1270  TZ=EXP(-.5*D1)+EXP(-.5*D2):
      REM CHECK FOR DIVIDE BY ZERO CONDITION
1280  IF TZ=0 GOTO 1320
1290  P1 =EXP(-.5*D1)/(EXP(-.5*D1)+EXP(-.5*D2)) :
      REM CALCULATE PROB. CLASS 1
1300  P2=EXP(-.5*D2)/(EXP(-.5*D1)+EXP(-.5*D2))   :
      REM CALCULATE PROB. CLASS 2
1310  GOTO 1430
1320  IF CK = 0 GOTO 1430
1330  RETURN
1340  PRINT
1350  PRINT
1360  INPUT "Enter Name For Class 1";A$
1370  A$=LEFT$(A$,8)
1380  PRINT
1390  PRINT
```

```
1400  INPUT "Enter Name For Class 2";B$
1410  PRINT
1420  B$=LEFT$(B$,8)
1430  RETURN
1440  F=1                        : REM CALCULATES F-VALUES
1450  IF V1 < V2 THEN F=(V2/V1)
1460  IF V1 > V2 THEN F= (V1/V2)
1470  D1=(A1-1)                  :
      REM CALCULATES F-VALUE DEGREES OF FREEDOM
1480  D2=(A2-1)
1490  IF V1 < V2 THEN D1=(A2-1)
1500  IF V1 < V2 THEN D2=(A1-1)
1510  AD=2/9/D1                  :
      REM CALCULATES PROBABILITY OF > VALUE OF F
1520  BD=2/9/D2
1530  CP=ABS((1-BD)*F^(1/3)-1+AD)/SQR(BD*F^(2/3)+AD)
1540  IF D2 < 4 THEN CP=CP*(1+.08*CP^4/D2^3)
1550  PF=.5/(1+CP*(.196854+CP*(.115194+CP*
      (.000344+CP*.019527))))^4
1560  PF= INT(PF*10000+.5)/10000
1570  IF F < 1 THEN PF=(1-PF)    :
      REM CORRECT FOR SMALL T PROBABILITIES
1580  RETURN                        : REM RETURN TO LINE 430 OR 450
1590  IF PF < .05 GOTO 1650
1600  DF=(A1+A2)-2
1610  SQ=((A1-1)*V1+(A2-1)*V2)/DF
1620  SY=SQR(SQ*(1/A1+1/A2))
1630  T=ABS(M1-M2)/SY
1640  RETURN                   :
      REM RETURN TO LINE 440 (EQUAL VAR.)
1650  SA=(V1/A1)+(V2/A2)     :
      REM SATTERTHWAITE'S APPROX. DEG. OF FREEDOM
1660  SB=(V1/A1)^2/(A1-1)
1670  SC=(V2/A2)^2/(A2-1)
1680  DF=(SA)^2/(SB+SC)
1690  T=ABS(M1-M2)/SQR(SA)
1700  RETURN                        :
      REM RETURN TO LINE 440 (FOR UNEQUAL VAR.)
1710  FP=PF : FF=F : DN=D1 : DD=D2 :
      REM STORE VALUES FOR F BEFORE SUB FOR T
1720  F=T^2
1730  D1=1
1740  D2=DF
1750  GOTO 1510
1760  PRINT "WARNING: MEANS NOT STATISTICALLY DIFFERENT --
      DISCRIM FUNCTIONS CAN NOT BE CALCULATED"
1770  PRINT "CAN NOT CALCULATE DISCRIM FUNCTIONS --
      MEANS NOT STATISTICALLY DIFFERENT"
1780  GOTO 1090
```

VITA

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Candidate for the Degree of

Doctor of Philosophy

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