FLUOROMETRIC MEASUREMENT OF IgG ANTIBODY RESPONSES IN SERUM AND CERVICO-VAGINAL MUCUS IN CATTLE VACCINATED WITH *TRITRICHOMONAS FOETUS*

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To my husband, Shan Collett, DVM, I would like to express my love and gratitude for his encouragement and help throughout my master's program.

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Introduction

Chapter I

Bovine genital trichomoniasis is caused by *Tritrichomonas foetus* and is characterized by infertility, early embryonic death, abortion, or reduced calving rates.[2,20] Treatment of the infection is difficult in bulls, although oral dimetridazole[23] and ipronidaxole[27] have shown some success. Treatment is not as crucial in cows because the infection is self-limiting, persisting for up to 95 days.[24]

Past attempts to produce a vaccine against *T. foetus* using membrane proteins or membrane glycoproteins administered in a mineral oil adjuvant met limited success.[5,6] A recently developed *Trichomonas foetus* vaccine (Fort Dodge Laboratories, Ft. Dodge, IA) consisting of killed whole cell *Tritrichomonas foetus* in a dual adjuvant system formulated to enhance the immune response and the product brochure indicates that significantly higher titers were produced than with other vaccines.

Herd disease prevention programs seem to be the best methods to control the parasites. Prevention involves identifying infected animals and either removing them from the herd or treating them. The most common detection method is by cultivation/microscopic examinations of prepucial or vaginal washings.[24] However, these tests require 3-4 weeks, and not all laboratories perform the tests.

Several serologic methods have been investigated for detection of trichomoniasis. An ELISA test was developed but exhibited problems with non-specific antigen cross-reactivity resulting in frequent false-positive results.[30] However it is possible to detect specific antibodies, such as IgG and IgA, in serum and seminal plasma using either ELISA[5] or slide IFA methods.[18,27] A DNA hybridization assay was developed and showed some promise, but the tests require days to run and are too expensive for practical field

application.[1,30]

Tritrichomonas foetus does not invade the epithelium of the cow's reproductive tract, but remains within the lumen or on the mucosal surface.[25,28] Because the bovine reproductive tract is a component of the secretory immune system, local responses to infection would be expected instead of a systemic response. A study done by Skirrow and BonDurant attempted to define the isotypes and temporal pattern of specific anti-*T. foetus* antibodies produced in both the female reproductive tract and circulation during an experimental trichomonad infection. They showed evidence of humoral immune response in all regions of the reproductive tract, and to a lesser extent, in the systemic circulation.[28]

Tritrichomonas foetus colonizes all parts of the reproductive tract of a cow within two weeks of the initial infection. The numbers of parasites in the cervico-vaginal mucus fluctuates during the estrous cycle, with largest numbers a few days before estrous. Cows may maintain infection throughout pregnancy and up to six to nine weeks postpartum, thus serving as a source of possible infection for bulls.[32]

The immunoglobulin subclasses in the bovine reproductive tract tend to vary within different regions of the tract.[28] Corbeil *et al*[7,11] demonstrated with *Campylobacter fetus* var *venerialis* infections that IgA predominated in the vagina and IgG in the uterus; this was similarly revealed using *Mycoplasma* sp.[8] Antibodies of the IgG class were capable of immobilizing and opsonizing *C. fetus* sub sp *fetus* and appearance correlated with early clearance of organisms from the uterus. In the vagina, immunoglobulins of the classes IgM, IgG and IgA simultaneously appear when organisms undergo antigenic variation. IgA was thought to block IgG from opsonizing organisms, keeping them from being entirely cleared from the vagina.[11] Immobilizing but non-opsonic IgA in the vagina

coupled with antigenic variation permits persistence of organisms in the vagina but prevents uterine colonization because of uterine IgG opsonins. The resulting continuous antigenic stimulus keeps protective antibody levels high.[10,11]

The antibody response to *T. foetus* appears to differ from that of *Campylobacter fetus*. Skirrow and BonDurant[28] showed that natural antibodies to *T. foetus* present in bovine serum are responsible for preinfection reactivity against *T. foetus*. It was shown that *T. foetus* cross-reacted with *C. fetus* and trauma, contamination and inflammation from collection procedures may stimulate both local production of cross-reactive antibodies and the infiltration of systemic antibodies into the reproductive tract. IgG and IgA were the predominant immunoglobulins in the reproductive tract. They also showed marked fluctuations in immunoglobulins following the initial response to *T. foetus*. This phenomenon has also been shown with campylobacteriosis and brucellosis, and may be attributed to the fact that reproductive secretions are intrinsically cyclical in nature.[28]

An early study done by Pierce[25] reported that an agglutinin to *T. foetus* develops in vaginal discharges of infected animals. The appearance of the agglutinin coincides with the disappearance of the active organism. There is a reduction in concentration of the agglutinin in the vaginal mucus when estrus returns or a uterine discharge is present. This is associated with the reappearance of active organisms.

Mucus is produced primarily by cervical goblet cells and the amount produced varies throughout the estrous cycle.[28] Postestrual hemorrhage can expose the tract to serum components through leakage from congested endometrial capillaries. The predominant immunoglobulin in the mucus secretions was IgG_1, and radioimmunoassay showed that it appears to predominate in the secretions of the respiratory, alimentary

and genital tracts of cattle.[13] It was suggested that only a small proportion of the isotype IgG_1 were derived from the plasma, and remainder synthesized locally by mucosal lymphoid cells found in the lamina propria and at the base of the villi.[13] A study done by Soto *et al,* demonstrated IgG_2 as the predominant serum antibody, while those isolated from vaginal mucus were characterized as IgG_1.[30]

Other important immunoglobulins in the reproductive tract are IgM and IgA. IgA is a minor serum component but a predominant immunoglobulin in nasal secretions, tears and saliva; its relative concentration in vaginal mucus is lower than IgG but higher proportionally than in serum.[4,14]

BonDurant *et al*[3] immunized virgin cows(heifers) with immunoaffinity-purified *Tritrichomonas foetus* antigen in incomplete Freund's adjuvant. They showed clearance of infection faster than with adjuvant controls. Isotype-specific enzyme-linked immunosorbent assay showed serum IgG_1 and IgG_2 antibody responses along with cervicovaginal mucus IgG_1 and IgA antibodies peaked about the time of clearance of infection in vaccinated animals. Control cattle developed cervicovaginal mucus IgA antibody responses as expected with a primary local immune response. This study showed the importance of early clearance of the *T. foetus* organism as uterine infection persisting beyond 7 weeks result in endometritis, placentitis, or fetal loss.[3,22] Another study done by Gault *et al*[17], provided similar results by showing vaccine efficacy to *T. foetus* infection is attributable to systemic immunization, leading to enhanced localized immune response.

Parsonsen *et al*[24] saw no placental or genital lesions prior to 7 weeks and fetal loss did not occur until 9 weeks into experimental infections with *T. foetus*. Thus, pregnancies would be less likely to be adversely affected if heifers or cows clear

infections within 7 weeks. Infertility can be prevented by protection from cervicitis, endometritis, placentitis, and/or embryonic death, so that reproductive failure should be prevented by herd immunity if all cows are vaccinated.[3] Immunization has also proven to be effective in curing the cervicovaginal carrier state in venereal vibriosis.[26]

CHAPTER II

Seroconversions in Cattle Vaccinated with *Tritrichomonas foetus*

The purpose of Experiment I was to develop a simplified serologic test to measure seroconversion in cattle vaccinated with a *T. foetus* vaccine. The intent was to monitor seroconversion in vaccinated cattle, but the feasibility of detecting anti-*T. foetus* antibodies in naturally infected animals was also examined. An objectively quantitative fluorescent immunoassay system (FIAX 100™ fluorometer, Bio-Whittaker Inc., Walkersville, Maryland) was chosen for this purpose. Tests using FIAX technology have been reported to be rapid and economical, to be easy to adapt to many types of parasites, to provide reasonable precise estimates of antibody levels and to have the flexibility to detect IgG, IgM or other antibody classes.[16]

MATERIALS AND METHODS

Animals: Four one-year-old virgin Hereford-Angus heifers were purchased from a single herd in Oklahoma. They were grouped together in an isolation paddock and fed hay, grain and water *ad libitum*. Three of the heifers were vaccinated with the Fort Dodge vaccine and one served as a non-vaccinated control. Vaccine was given in two doses two weeks apart consistent with manufacturer recommendations. Blood samples were drawn from each heifer on a weekly basis for approximately 11 weeks.

Reference Sera and Vaccine: Reference sera collected during a previous cattle vaccination study and slide IFA titers were obtained from researchers at the University Nevada-Reno, Reno, NV (Drs. M. Hall and W.G. Kvasnicka, School of Veterinary Medicine). The Trichomonas Foetus Vaccine™ was provided by Fort Dodge Laboratories, Fort Dodge, Kansas, and consisted of formalin-fixed whole cells in oil adjuvant.

Organisms: A *T. foetus*[21] isolate was cultured in modified Diamond's medium. Modified

media consisted of 10.0 ml of Diamond's medium without agar to which was added 1.5 ml bovine serum and 0.2 ml of an antibiotic solution containing 1,000 Units of Penicillin and 50 µg of Streptomycin to inhibit bacterial growth. *Tritrichomonas foetus* were aseptically transferred every 4 days in 0.5 ml volumes to fresh media. The cultures were incubated at 37 C and harvested at peak growth phase.

Antigen Preparation: At peak growth, the *T. foetus* cultures were placed in a refrigerator at 4 C overnight to allow the organisms to settle to the bottom of the tube. The settled cultures were centrifuged at 2000 x g for 10 minutes, and the supernatant extracted from the tube using a vacuum pump. The *T. foetus* pellets were transferred to graduated conical centrifuge tubes and resuspended with physiological saline to 15.0 ml. The resuspended material was centrifuged for 10 minutes at 2000 x g and the supernatant again removed. The latter process was repeated twice to remove excess media. After the final wash, the pellet of whole organisms was resuspended in 1.0 ml of physiological saline and frozen at -20 C.

Fluorometric Assay: The antigen consisting of freeze-thawed whole cells was titrated against positive control sera to determine the optimum working concentration. Whole-cell *T. foetus* antigen was diluted by serial 2-fold dilutions, spotted in 25 µl amounts onto cellulosenitrate disks attached to StiQ™ samplers (Bio-Whittaker Inc., Walkersville, Maryland) and allowed to dry overnight. The StiQs™ with adsorbed antigen were transferred through diluted serum (25 min), a wash buffer (5 min), fluorescein isothiocyanate (FITC)-conjugated rabbit antibovine IgG (15 min), and a final wash (5 min). Sera were tested in 1.0 ml aliquots at a dilution of 1:100 in phosphate-buffered saline at pH 7.3 and containing 0.15% polyoxyethylene sorbitan monolaurate (Tween 20). The washes were done in 0.6 ml volumes of buffer and the conjugate was diluted 1:200

and used in 0.5 ml aliquots. Tween 20 buffer was used as diluent in all steps and all samples were tested in duplicate at room temperature.

Antibody binding was determined by measuring the amount of fluorescence produced by the bound FITC-conjugated antibovine IgG. A FIAX 100™ fluorometer was used to measure the fluorescence on the StiQ samplers. The fluorometer measured fluorescence at a wavelength of 540 nm when excited by light of 475 nm.[15] Antibody levels were compared to positive and negative sera selected from a series of test sera collected from one of the OSU vaccinated heifers. The positive control produced the highest fluorescent readings (comparable to ELISA optical density readings) and the negative control the lowest for that animal. Antibody levels for the samples were expressed as FIAX values; FIAX values are rankings and not absolute expressions of titer. FIAX values were extrapolated from regression curves based on log-log data conversions of the StiQ fluorescence measured for duplicate controls in each test and the assigned standard FIAX values for the negative and positive controls. Standard FIAX values were arbitrarily assigned to the control sera to provide a scale for ranking samples according to antibody levels of all other tested sera. The extrapolated FIAX values for all samples were paired means.

Statistical Analysis: FIAX values representative of negative, suspect and positive antibody levels were estimated from the extrapolated FIAX values. The mean and standard deviation for all prevaccination samples and the control animal samples were used to calculate the upper value for negative sera using a 95% confidence limit (mean + 1.96 x SD); values greater than the calculated value were considered positive. A Systat statistical program (Systat, Evanston, Illinois) was used to construct probability plots, quantile plots and stem and leaf plots and to compute statistics on the data. The suspect

or transitional range was derived from a SYSTAT stem and leaf plot of the entire set of data; values between the upper hinge (75th percentile) and the calculated upper limit for negative sera were designated suspect. Quantile and probability plots were used to visually verify the point at which FIAX values deviated from the normal distribution of the negative range.

RESULTS

Control sera were chosen from one of the vaccinated heifers to provide a scale to rank serum antibody levels. The negative control was assigned a FIAX value of 25 and the positive control 200; all other sera were ranked with regard to the controls.

Figure 1 shows a frequency distribution of FIAX values of all OSU sera. Visual inspection of this graph and the quantile and probability plots (not shown) indicated that seroconversion occurred at FIAX values of approximately 60. A Systat stem and leaf plot of the data indicated that FIAX values greater than 74 were designated "outside values" or what could be considered seropositive. FIAX values higher than 72 were excluded and statistics were calculated for the remaining data. The mean (46.67) was added to 1.96 times the standard deviation (=25.40) to estimate the highest nonpositive value (72.07). Therefore, FIAX values between 56 (the upper hinge of a stem and leaf plot) and 72 (the highest calculated negative value) were designated to represent the suspect range.

Figure 2 shows the IgG response curve for the OSU cattle vaccinated with the *T. foetus* vaccine. The highest FIAX value (IgG level) in a vaccinated cow was 237. FIAX values for the negative control animal ranged between 28 and 55. Figure 3 shows that the FIAX values for 20 selected bovine reference sera obtained from a Reno vaccine trial were similar to those in the OSU study. By the second week postvaccination, all animals

exhibited a dramatic increase in IgG antibody levels. The highest FIAX value observed in the Reno sera was 193 and IgG antibody apparently remained near that level for several weeks. To convert FIAX values to titers, a regression analysis was performed using the FIAX values generated in the OSU study and the slide IFA titers of the Reno reference sera. Table 1 shows the regression results indicating that the highest FIAX value of 193 was approximately equivalent to an estimated IFA titer of 7732.

Figure 1. Frequency distribution of FIAX values for control and vaccinated cattle included in the OSU vaccination trial. The minimum positive value was calculated from the mean and standard deviation using a 95% confidence limit

Distribution of Serum IgG FIAX Values to *Tritrichomonas foetus*

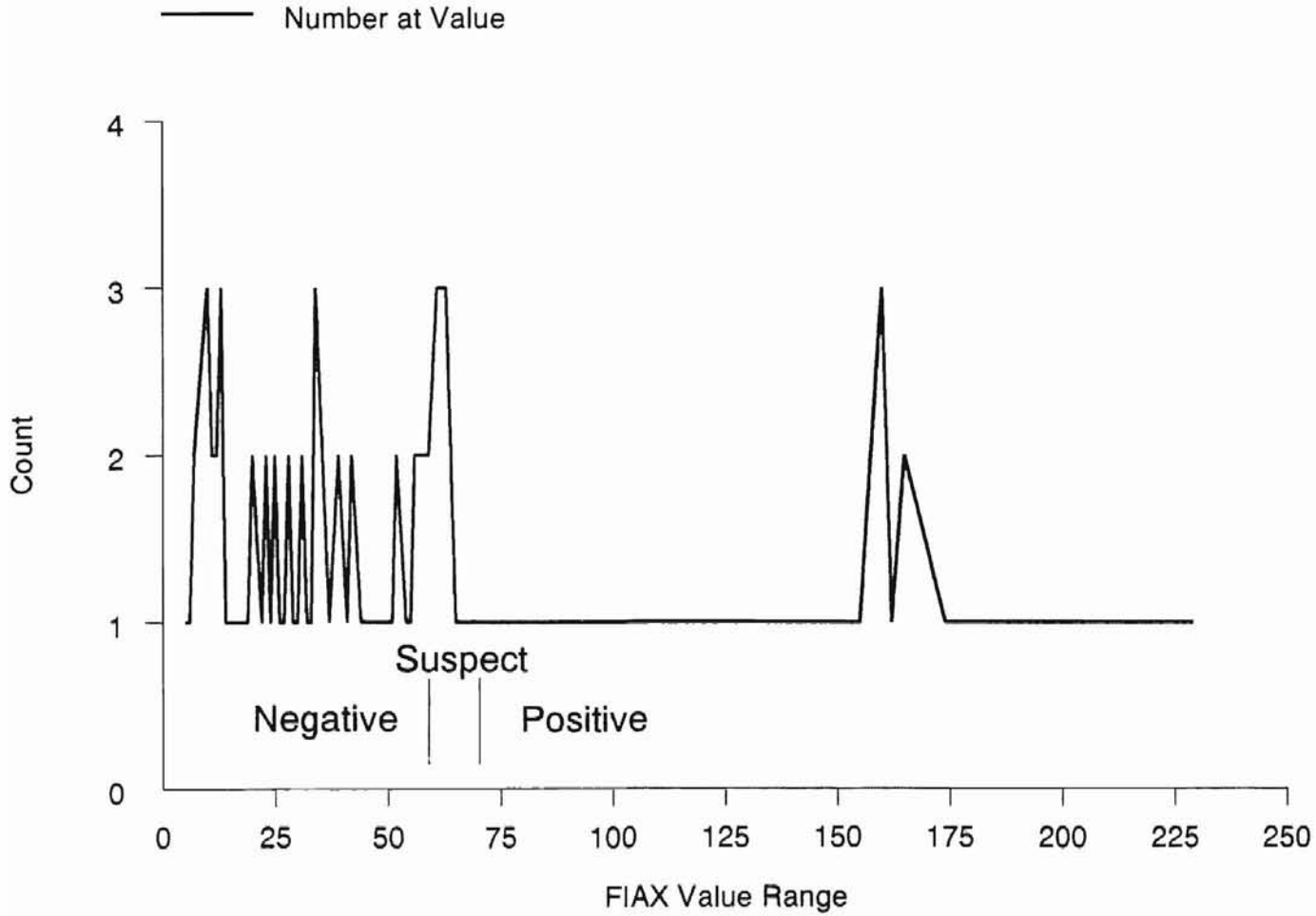

Figure 2. Antibody response curves for cattle vaccinated once with Fort Dodge Trichomonas Foetus Vaccine™. Dotted line represents minimum positive FIAX value.

IgG Responses Following Vaccination with *Tritrichomonas foetus*

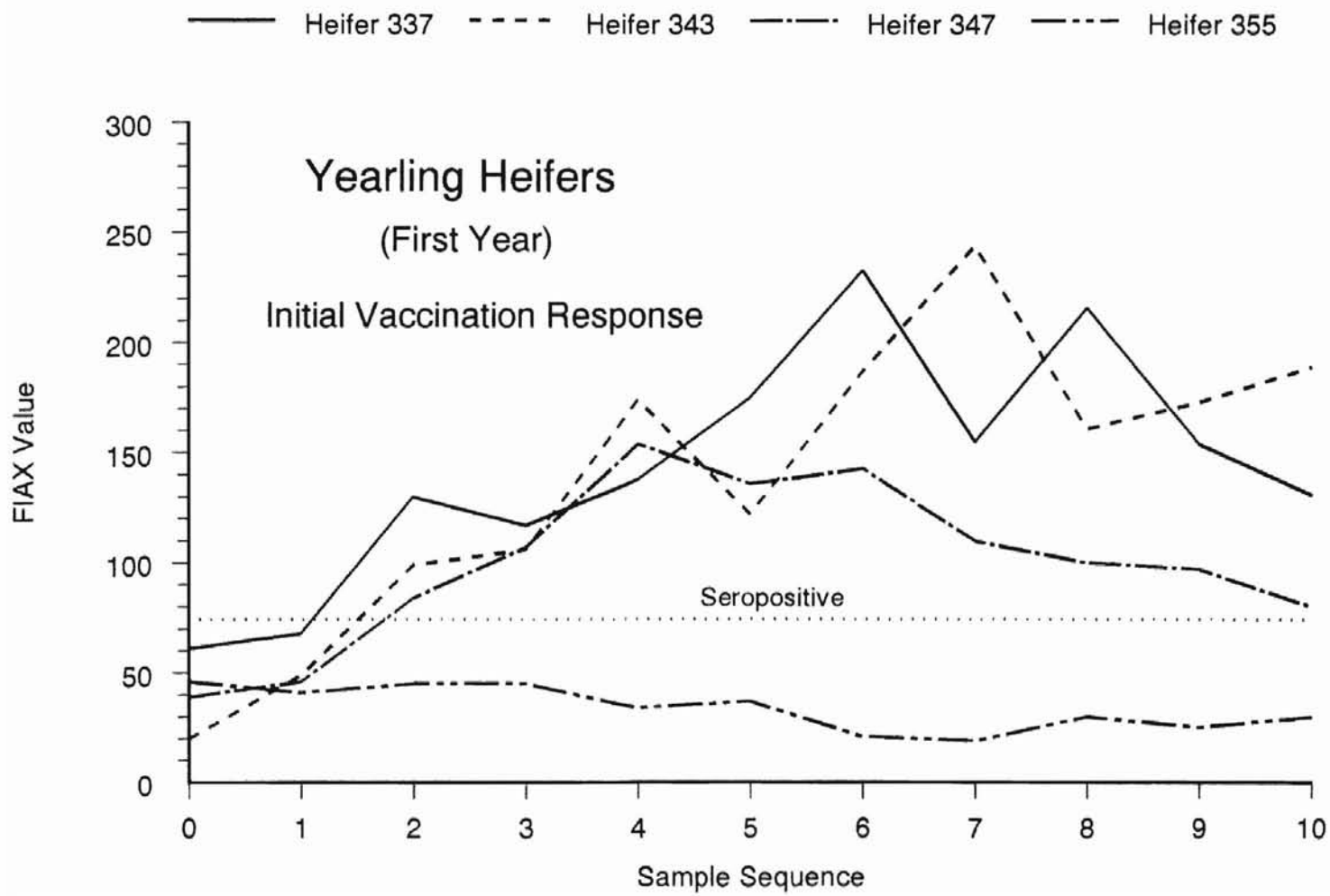

Figure 3. Antibody response curves for reference sera from a Reno *Tritrichomonas foetus* vaccination trial in cattle. Dotted line represents minimum positive FIAX value.

RENO *Tritrichomonas foetus* VACCINATION TRIAL

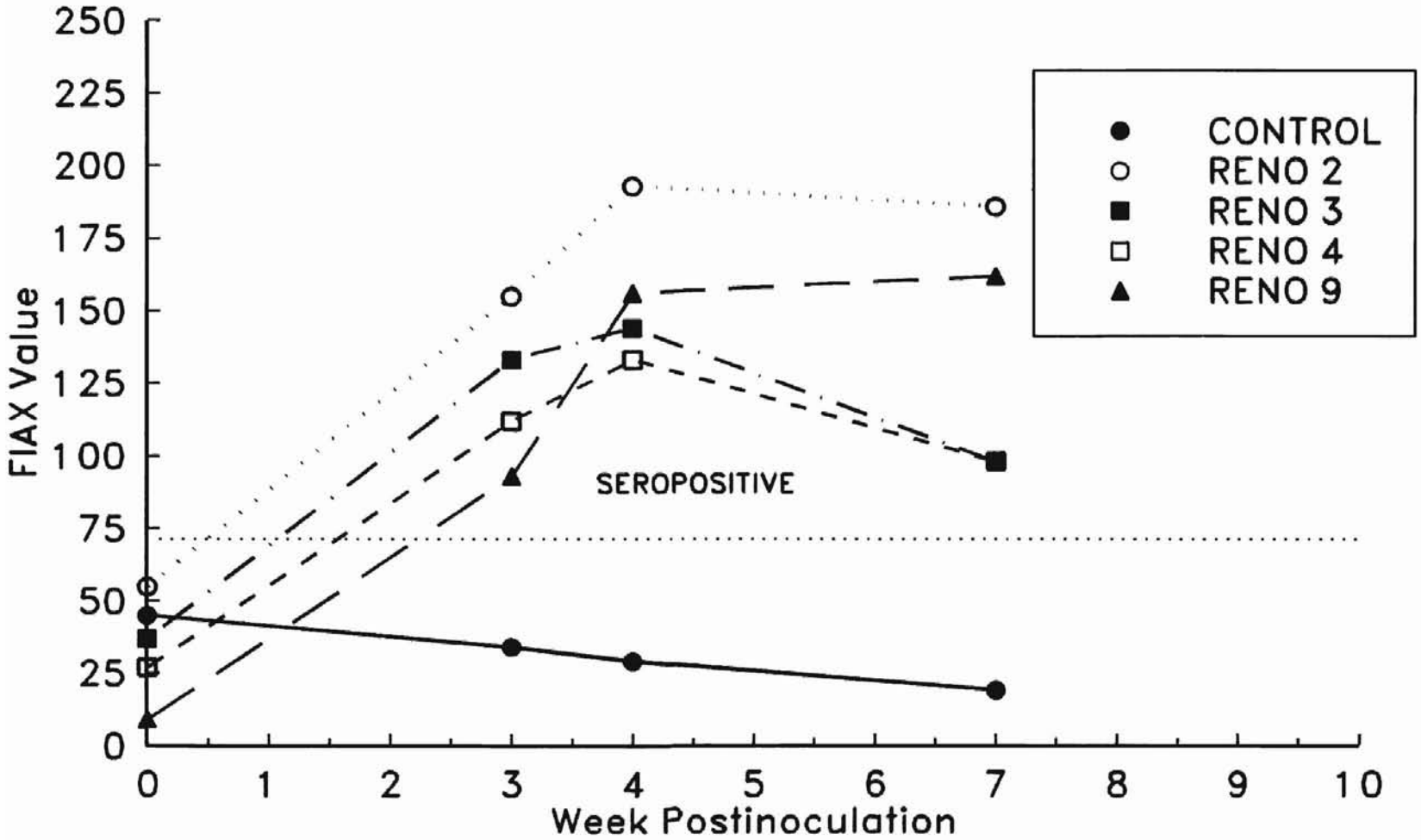

Table 1. Regression estimated IFA titers for 20 reference serum samples from a Reno vaccine trial estimated from FIAX values and the corresponding slide IFA titers for individual samples.

FIAX value	Slide IFA titer	Estimated IFA titer[1]
9	200	0
19	200	0
27	200	0
29	200	0
34	200	0
37	200	0
45	200	336
55	200	835
93	2000	2735
98	2000	2984
98	2000	2984
112	2000	3684
133	2000	4734
133	2000	4734
144	2000	5283
155	2000	5833
156	10000	5883
162	10000	6183
186	10000	7382
193	10000	7732

[1]Negative estimated values listed as 0

CHAPTER III

Challenge infections in cattle with single or double vaccinations with *Tritrichomonas foetus* vaccine

Animals from Experiment I were included in a second vaccination experiment a year later to determine if the twice-vaccinated heifers would clear challenge infections with *Tritrichomonas foetus* differently than single-vaccination animals. Cervico-vaginal mucus samples were also obtained to measure IgG antibody.

MATERIALS AND METHODS

Experiment II

Animals: The four initial heifers from Experiment I (now two years old) were retained for one year for a second vaccination study. Four additional one-year-old virgin Hereford Angus heifers were purchased from a single herd in Oklahoma. One two-year-old and one one-year-old heifer served as negative controls. All animals were housed together in an isolation paddock and fed hay, grain and water *ad libitum*. There were three groups of cattle. Group I consisted of three previously vaccinated heifers (from Experiment I); Group II included three unvaccinated animals; and Group III consisted of two unvaccinated controls. Group II heifers were challenged in the same fashion as Group I. Both Group I and II heifers were vaccinated with the same whole cell Fort Dodge *T. foetus* vaccine used in Experiment I. Vaccine was administered consistent with manufacturer recommendations (see Chapter II).

Antigen for serologic tests: Antigen was prepared as described in Chapter II.

Blood sample collection: Approximately 10.0 cc of blood was drawn from the jugular vein into serum vacutainer tubes of each heifer on a weekly basis for approximately 17

weeks for Group I heifers and 13 weeks for Group II heifers. Negative controls were sampled with their respective age groups.

Cervico-vaginal mucus collection: Cervical fluid samples were taken from each heifer on a weekly basis for approximately 13 weeks. Cervico-vaginal mucus samples from Group I and Group II animals were collected post-exposure to *T. foetus*. To obtain cervical samples, the external vaginal area was cleaned with diluted Nolvasan solution prior to collection. A sterile artificial insemination pipette was used to aspirate the sample. The sterile pipette within its protective plastic sleeve was guided through the vulva to the fornix of the cervix with the left hand, while the fornix was stabilized with the right hand per rectum. When in place, the pipette tip was then pushed through the plastic sleeve. A 35 cc syringe was placed on the end of the pipette and negative pressure applied to aspirate the cervical fluid. The pipette was then pulled back into the sleeve and both withdrawn from the vagina. The cervico-vaginal mucus sample was then placed in 1 ml of sterile 0.9% saline.

Fluorometric assay: Serum antibody assay was described in Chapter II. The fluorometric assay for the cervical wash samples was conducted in a similar manner as for the serum samples. Cervical wash samples were diluted 1:1 with sterile 0.9% saline. The samples were tested in 1.0 ml aliquots at a dilution of 1:18 in phosphate-buffered saline at pH 7.3 and containing 0.15% polyoxyethylene sorbitan monolaurate (Tween 20). The washes were done in 0.6 ml volumes of buffer and the conjugate was diluted 1:300 and used in 0.5 ml aliquots. Tween 20 buffer was used as diluent in all steps and all samples were tested in duplicate at room temperature.

The antibody concentrations were determined in the same manner as previously described in the **Fluorometric Assay** section of Chapter II using the positive serum

samples as controls, and antibody concentration was expressed as FIAX values.

Inoculation of *T. foetus* organisms: A *T. foetus*[21] isolate was cultured as described in Chapter II. At peak growth phase a sterile artificial insemination(AI) pipette was used to inoculate approximately 7.0 x 10^6 organisms/ml near the cervix of all eight virgin heifers (vaccinates and controls). This procedure was done twice in Group II animals, with a one week interval between inoculations. Once the heifers were inoculated, weekly samples of cervico-vaginal mucus were taken. A direct smear was examined by placing a drop of the mucus sample with a drop of normal saline on a slide. A sample was considered positive if *T. foetus* organisms were seen. When a direct smear sample was found to be negative, the cervical wash material was then cultured in Diamond's media as described in Chapter II. The samples were observed microscopically at 24, 48, 72, or 96 hours for the presence of organisms. A heifer was considered cleared of the *T. foetus* organism when three consecutive cervical fluid cultures were negative.

RESULTS

Control sera from Experiment I were used to provide a scale to rank serum and cervical fluid antibody levels. The negative serum control had a FIAX value of 25 and the positive control 200; all sera and cervical fluids were ranked with regard to the serum controls.

Figures 4-7 show specific IgG levels in serum and cervico-vaginal fluid in the second year vaccinate cattle(Group I). This study ran for 17 weeks. There were no serum FIAX values in Fig. 4 after week 10 because this heifer (#337) cleared the *T. foetus* infection and no other cervico-vaginal samples were taken after that time. There were no FIAX values in Fig. 5 and 7 after week 14 due to sample loss.

The negative control for the single-vaccinated group was negative on direct smear by two weeks post-challenge with *T. foetus* (Table III). This is in contrast to the negative control animal for the double-vaccinated group, which did not become negative on direct smear until four weeks post-challenge (Table II). The single-vaccinate animal (# KG4) had elevated IgG levels in the serum prior to challenge. Tables 2-5 show the direct smear and culture results from Group I and Group II heifers. The cattle were inoculated with *Tritrichomonas foetus* at week zero and week one. All cattle became infected with the organism. By week 6 all vaccinated and control cattle became negative on direct smear, and samples were routinely cultured on Diamond's media. Only twice-vaccinated heifer #337 from Group I was culture negative by week 8, whereas the remaining heifers were culture positive to the end of the observation period (13-17 weeks).

Figures 8-11 show specific IgG levels in serum and cervico-vaginal fluid in the first year vaccinate cattle(Group II). This study ran for 13 weeks. The highest recorded serum FIAX value(IgG level) in Group I was 225, and the highest serum value in Group II was 275. The FIAX serum values for the negative control animal from Group I ranged between 15 and 75, and the serum values from Group II between 50 and 120. By the second week post-vaccination, all animals exhibited increased serum IgG levels.

The highest cervical fluid FIAX value(IgG level) in Group I was 75, and the highest cervical fluid value in Group II was 78. The FIAX cervical fluid values for the negative control animal from Group I ranged between 1 and 25, and the cervical fluid values from Group II between 0 and 15. Because the cervico-vaginal fluid was tested 5 times more concentrated, the specific IgG in the vagina was much lower than in serum.

FIAX tests were run on each serum and cervico-vaginal fluid sample at least four times with the respective serum sample. Specific IgG antibody was found in the cervical fluid samples. FIAX values of IgG antibodies for cervical fluid samples were significantly lower than for serum samples.

Figure 4. Specific IgG levels in serum and cervico-vaginal fluid in second year vaccinate heifer (337) vaccinated with *Tritrichomonas foetus* vaccine. Arrow denotes challenge with *T. foetus* organism. Dottedline represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

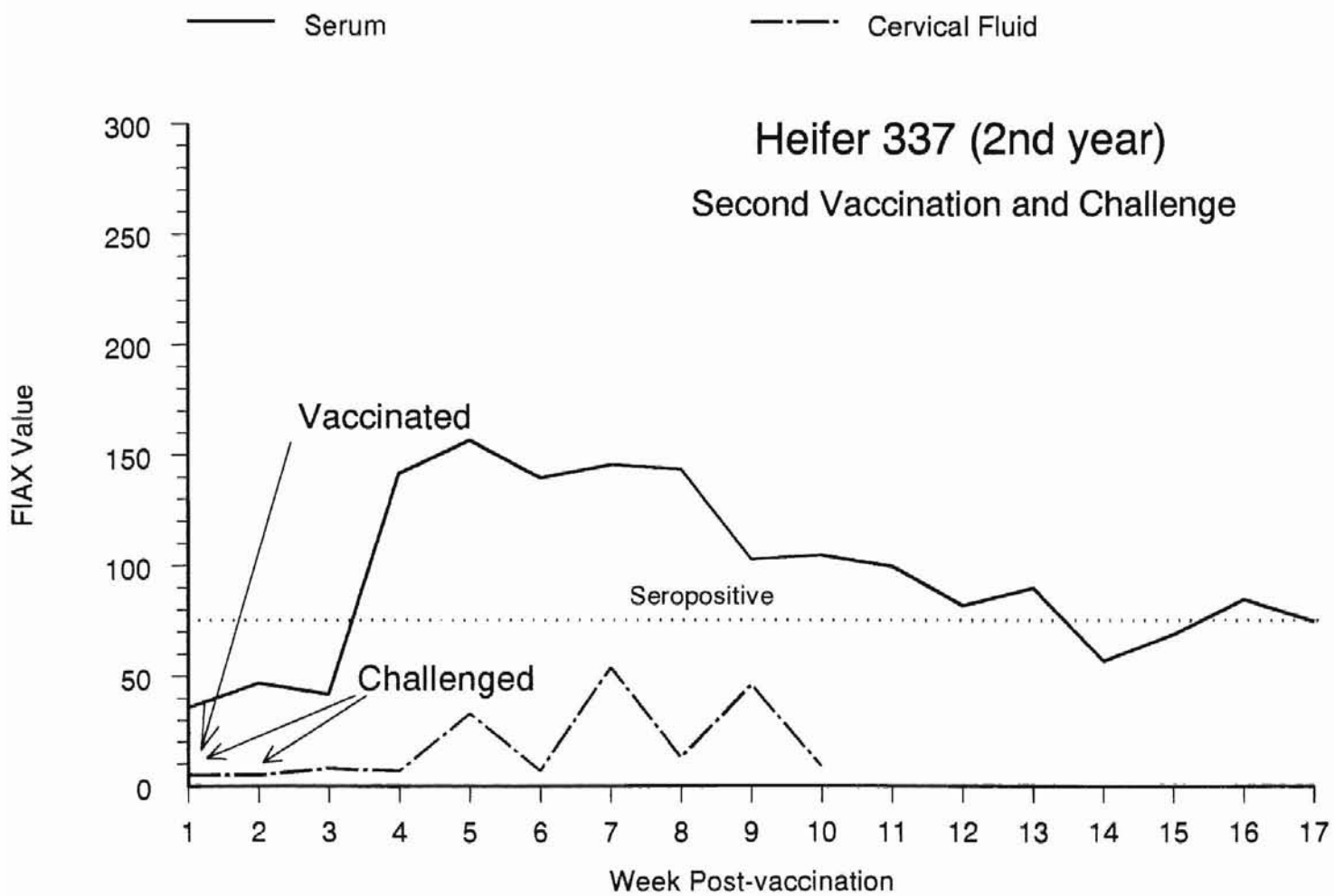

Figure 5. Specific IgG levels in serum and cervico-vaginal fluid in second year vaccinate heifer (343) vaccinated with *Tritrichomonas foetus* vaccine. Arrow denotes challenge with *T. foetus* organism. Dotted line represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

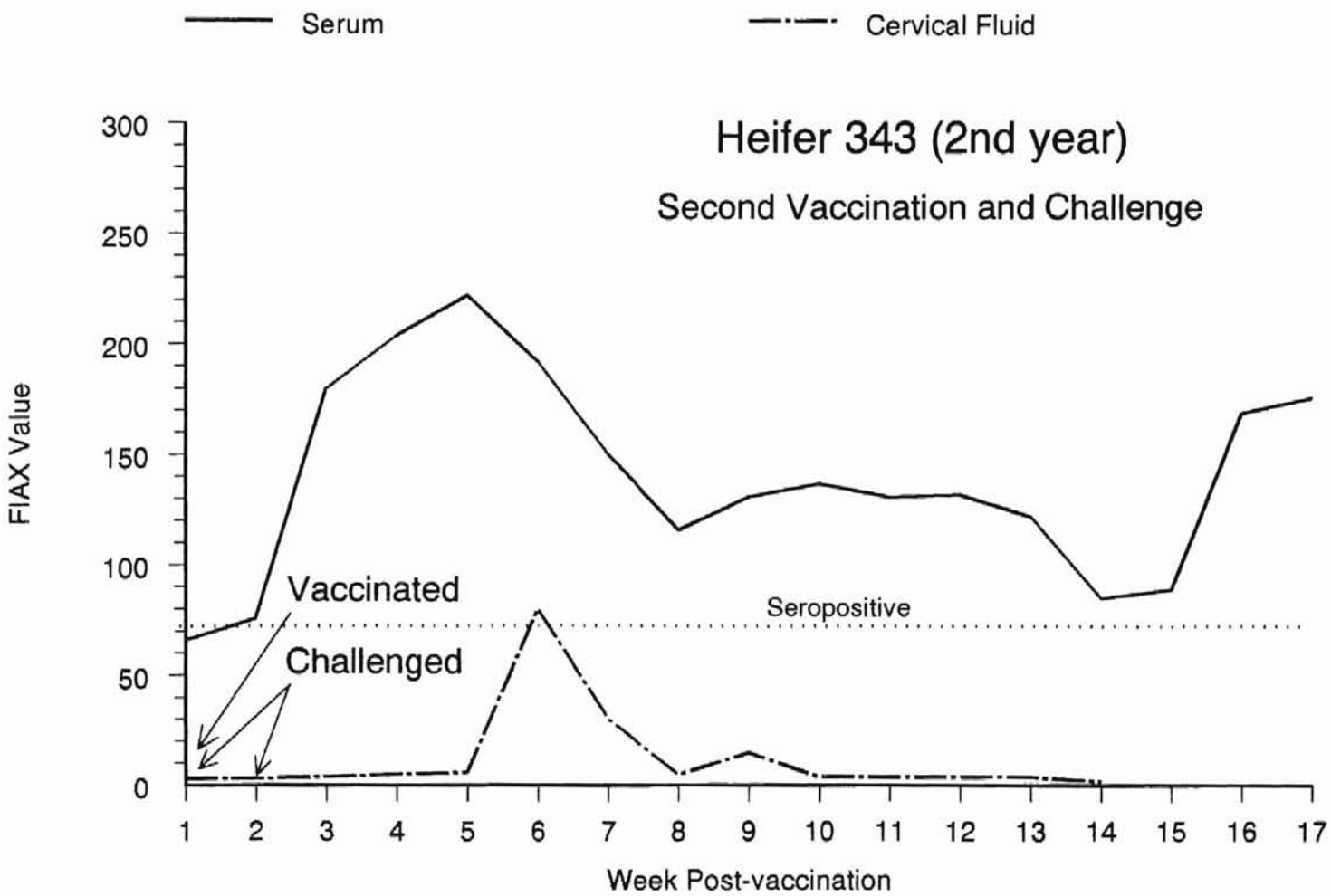

Figure 6. Specific IgG levels in serum and cervico-vaginal fluid in second year vaccinate heifer (347) vaccinated with *Tritrichomonas foetus* vaccine. Arrow denotes challenge with *T. foetus* organism. Dotted line represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

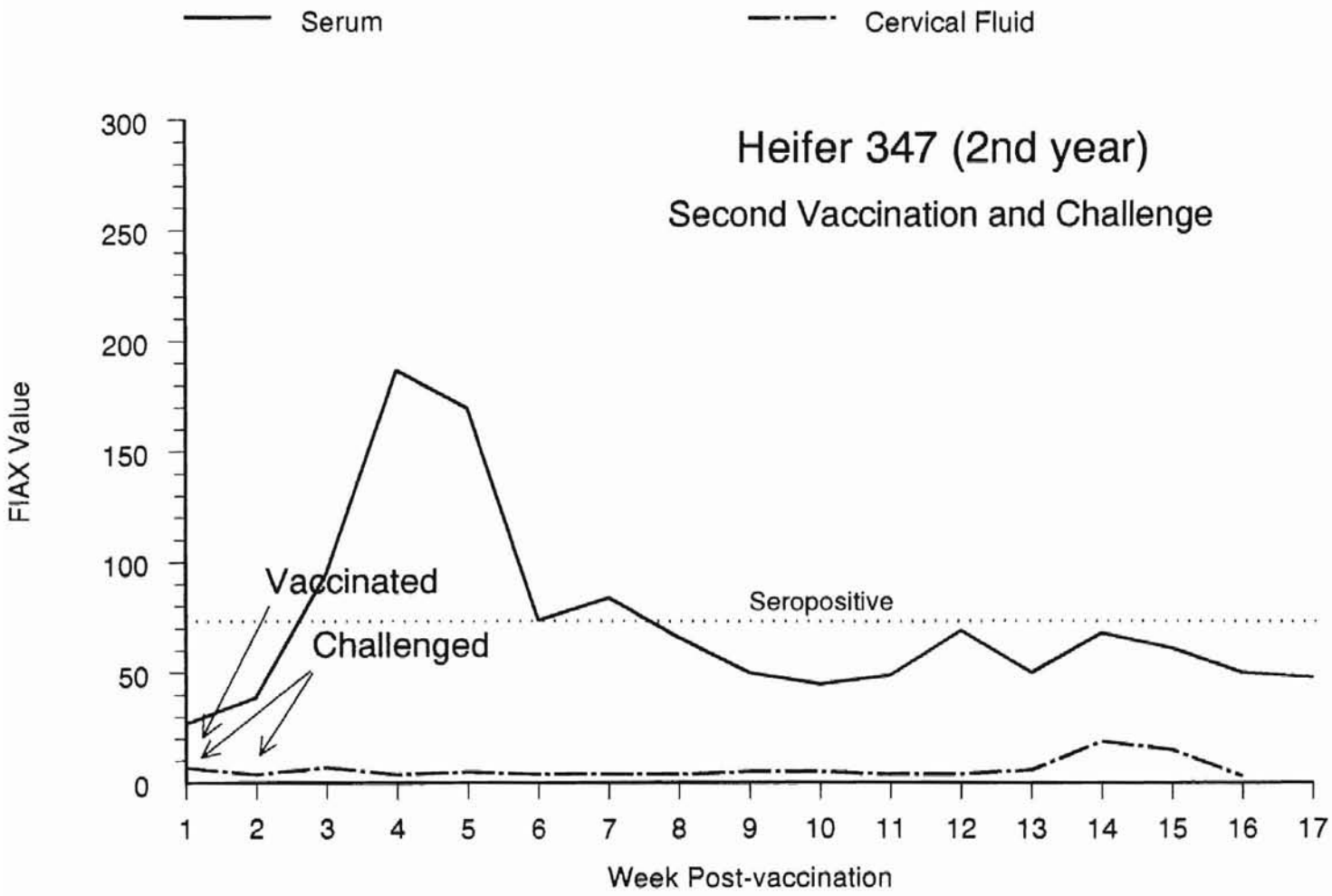

Figure 7. Specific IgG levels in serum and cervico-vaginal fluid in non-vaccinated control heifer (355). Arrow denotes challenge with *T. foetus* organism. Dotted line represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

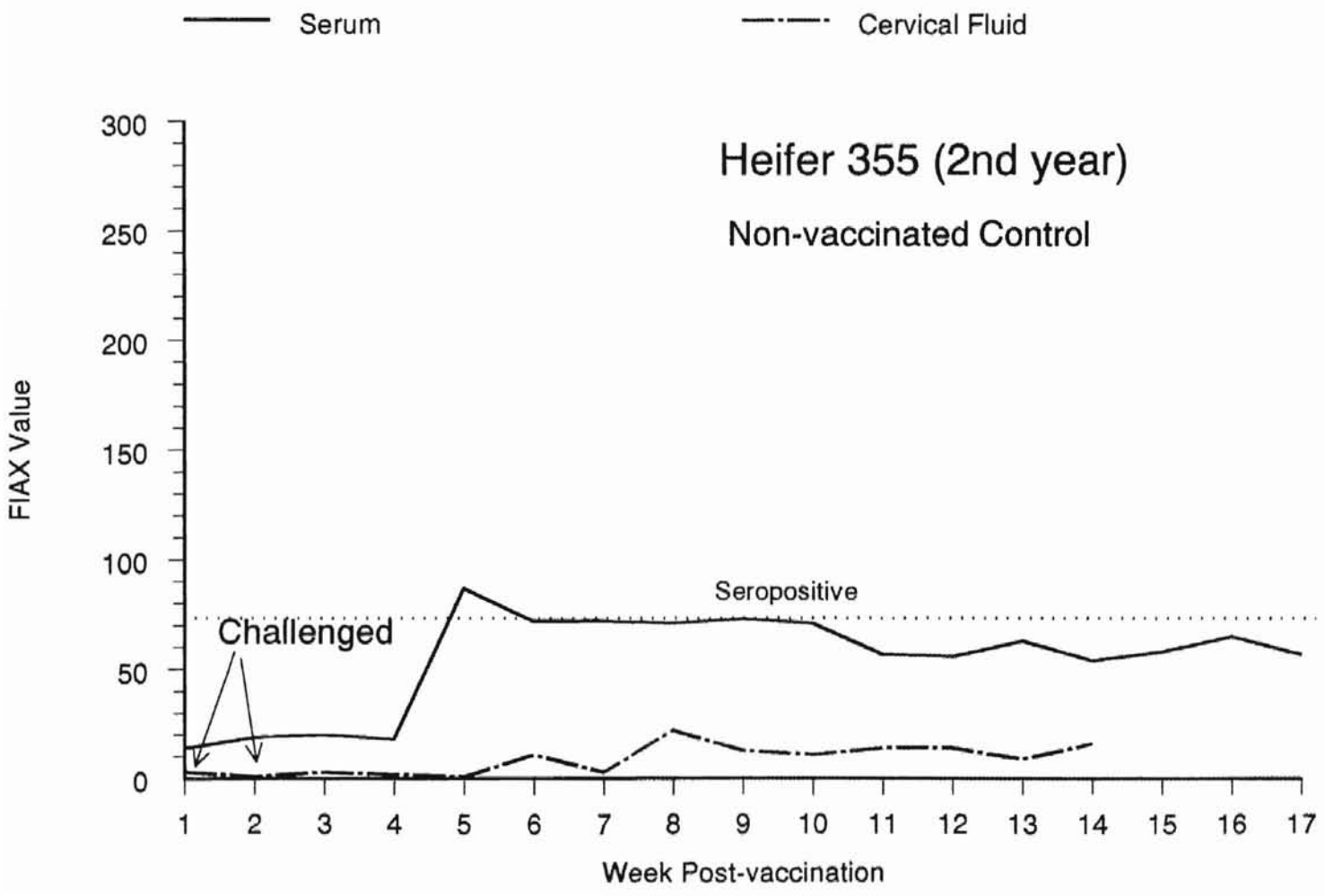

Figure 8. Specific IgG levels in serum and cervico-vaginal fluid in first year vaccinate heifer (KG1) vaccinated with *Tritrichomonas foetus* vaccine. Arrow denotes challenge with *T. foetus* organism. Dotted line represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

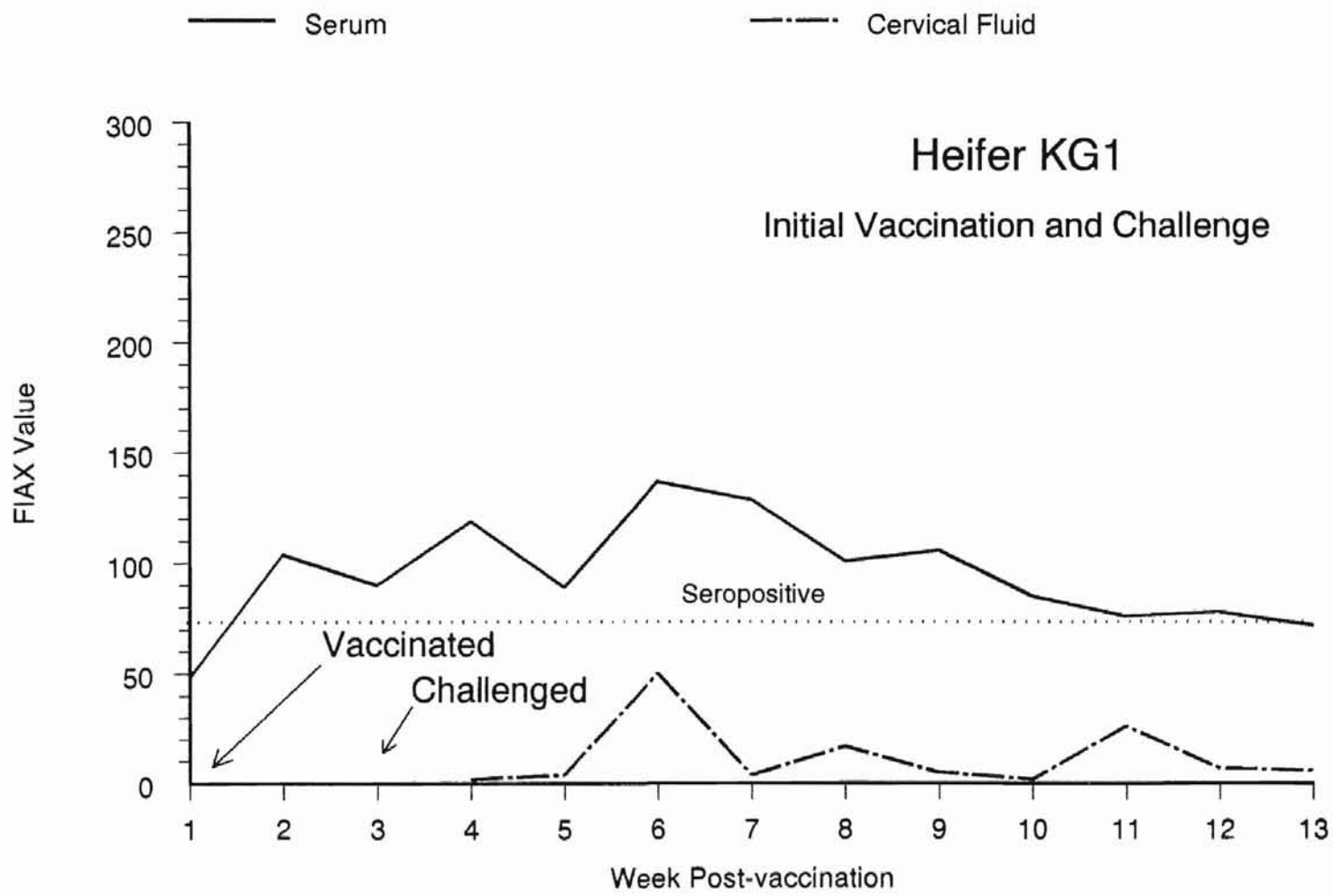

Figure 9. Specific IgG levels in serum and cervico-vaginal fluid in first year vaccinate heifer (KG2) vaccinated with *Tritrichomonas foetus* vaccine. Arrow denotes challenge with *T. foetus* organism. Dotted line represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

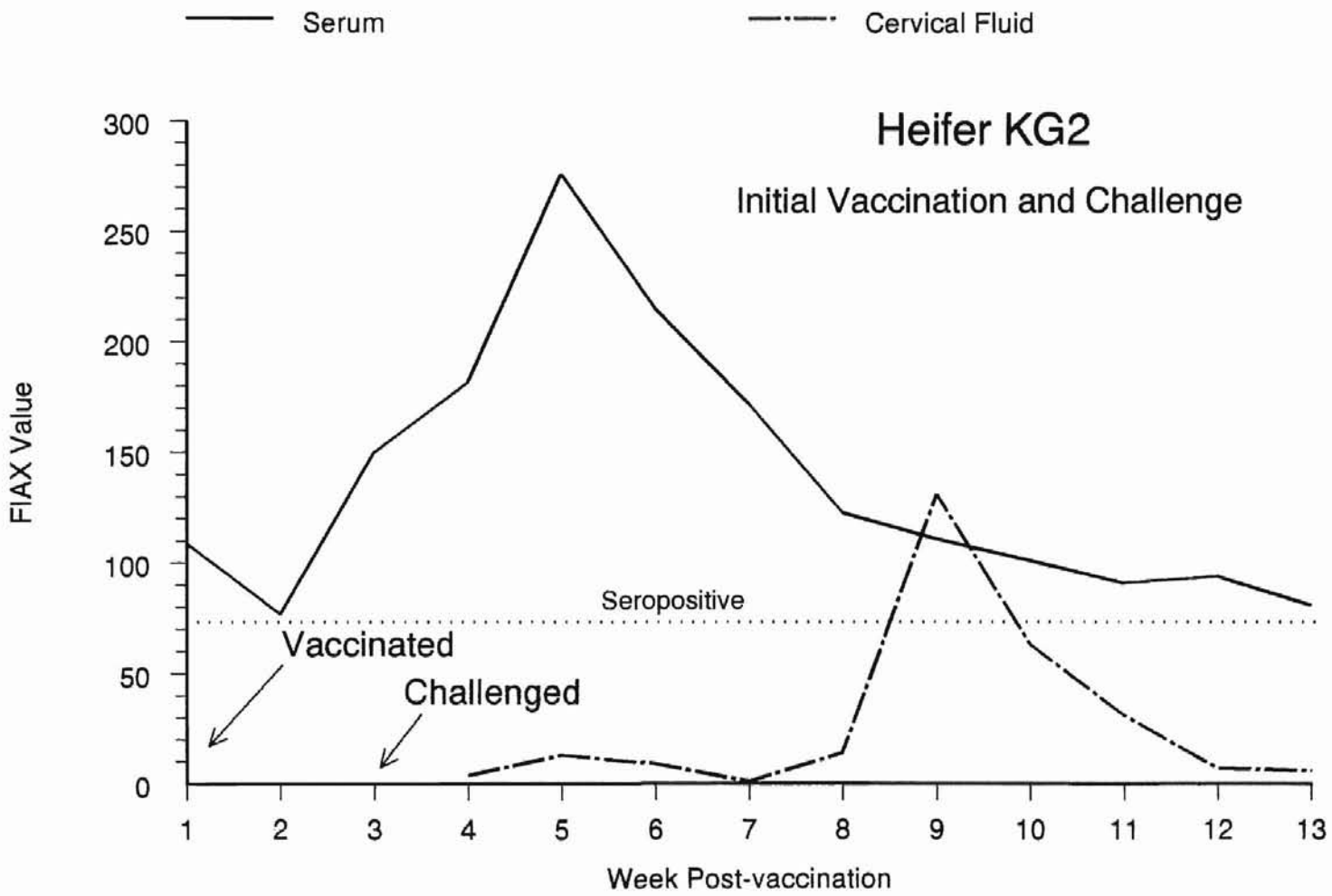

Figure 10. Specific IgG levels in serum and cervico-vaginal fluid in first year vaccinate heifer (KG3) vaccinated with *Tritrichomonas foetus* vaccine. Arrow denotes challenge with *T. foetus* organism. Dotted line represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

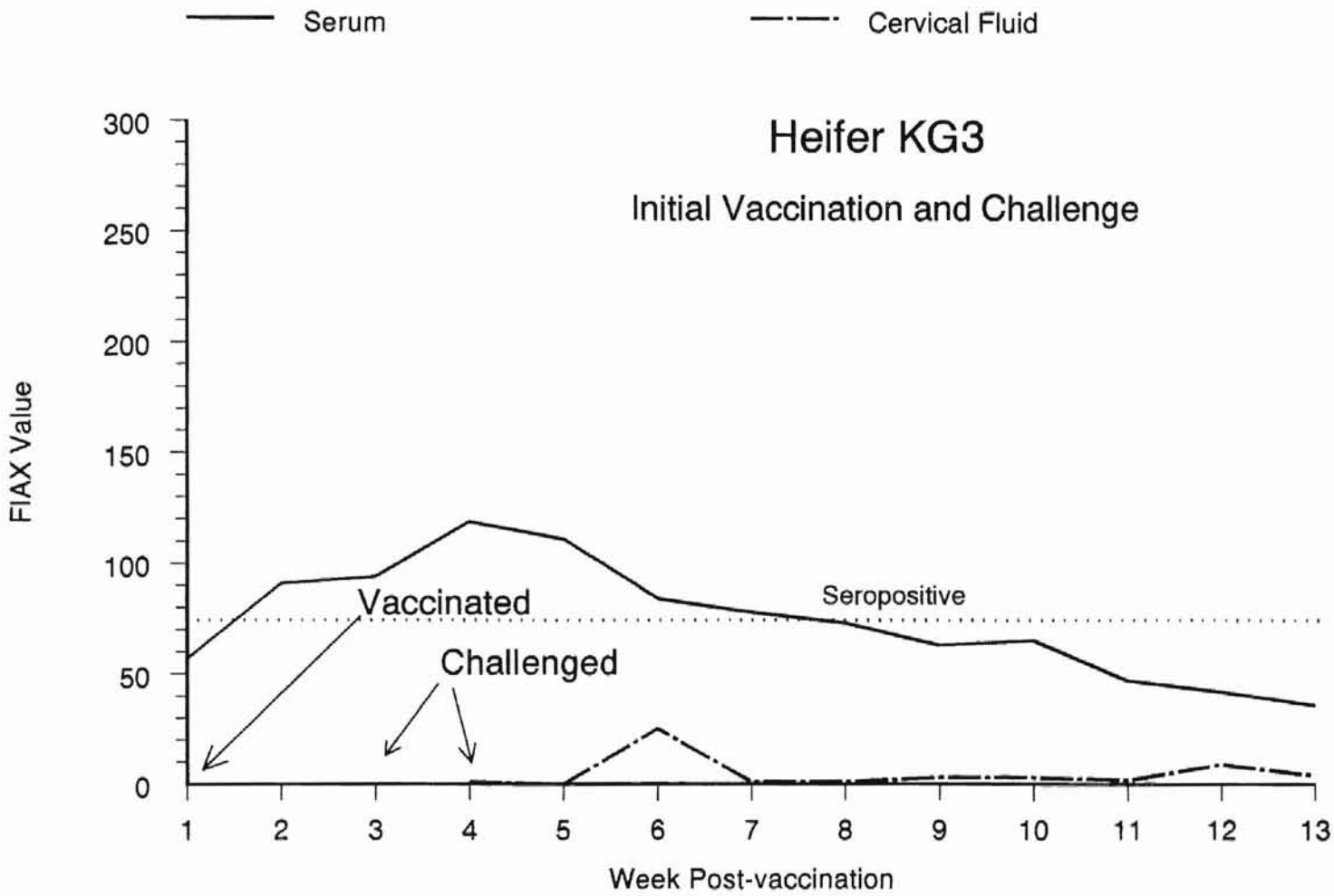

Figure 11. Specific IgG levels in serum and cervico-vaginal fluid in non-vaccinated control (KG4). Arrow denotes challenge with *T. foetus* organism. Dotted line represents minimum FIAX value.

Serum and Cervical Fluid IgG Levels to *Tritrichomonas foetus*

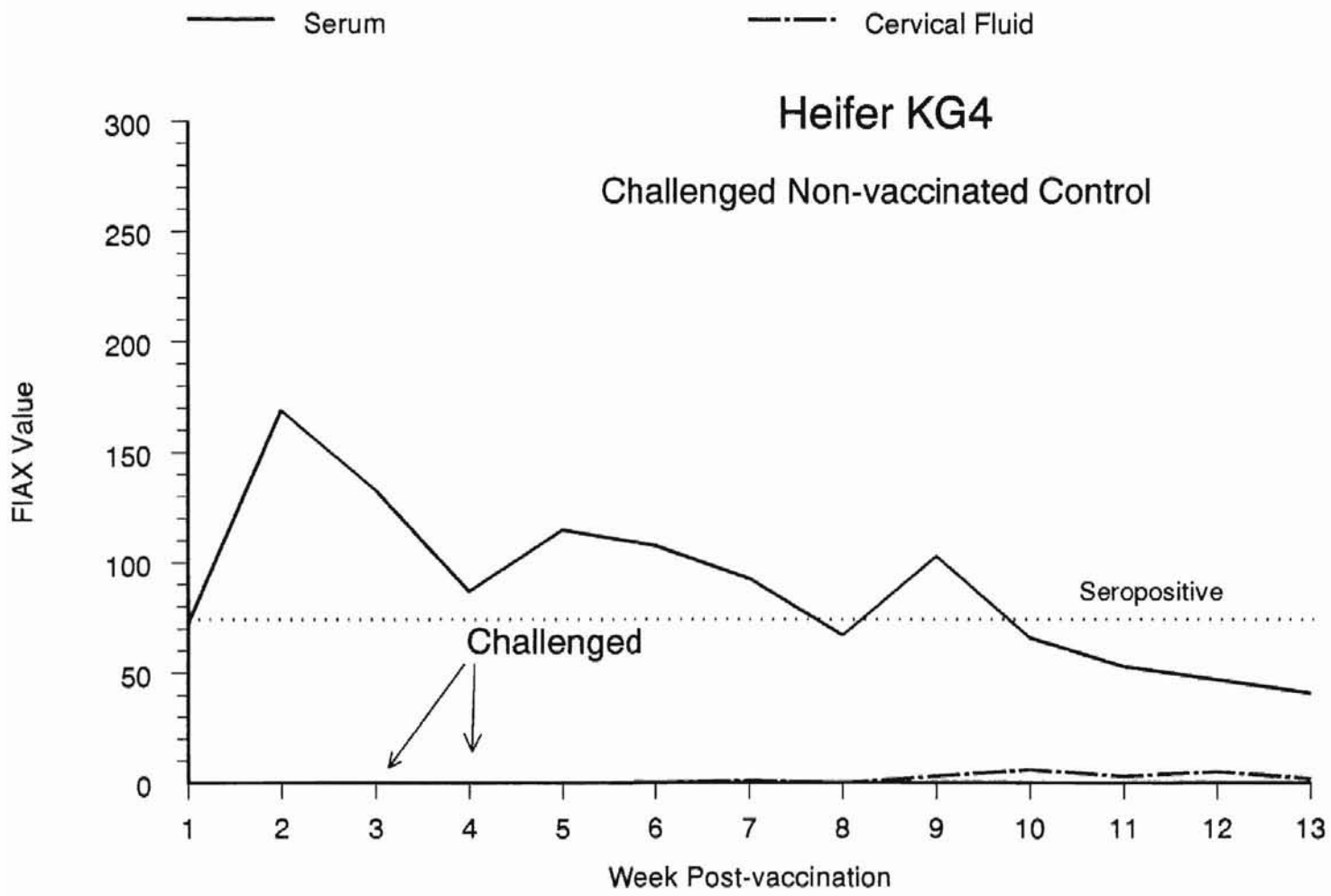

TABLE II

Tritrichomonas foetus **detected in direct smears of vaginal wash samples of cattle challenged with *T. foetus* after repeated vaccination**

Sample Time	Double Vaccinate (337)	Double Vaccinate (343)	Double Vaccinate (347)	Negative Control (355)
Week 1	challenge	challenge	challenge	challenge
Week 2	+	+	+	+
Week 3	+	-[a]	-	+
Week 4	-	-	-	+
Week 5	-	-	-	+
Week 6	-	-	-	-

[a]Negative samples were put in Diamond's media for culture

TABLE III

Tritrichomonas foetus **detected in direct smears of vaginal wash samples of cattle challenged with *T. foetus* after initial vaccination**

Sample Time	Single[a] Vaccinate (KG1)	Single Vaccinate (KG2)	Single Vaccinate (KG3)	Negative Control (KG4)
Week 1	challenge	challenge	challenge	challenge
Week 2	+	+	+	+
Week 3	+	-[b]	-	-
Week 4	+	-	-	-
Week 5	-	-	-	-

[a]Single vaccination means vaccinated once 2 weeks prior to challenge.
[b]Negative samples were put in Diamond's media for culture.

TABLE IV

Culture results for vaginal washings, checked at 24, 48, 72 or 96 hours, for cattle repeatedly vaccinated with *Tritrichomonas foetus* vaccine

Sample Time	Double Vaccinate (337) 24 48 72 96	Double Vaccinate (343) 24 48 72 96	Double Vaccinate (347) 24 48 72 96	Negative Control (355) 24 48 72 96
Week 3	[a]	- + -	+ [a]	
Week 4	- - +	- - +	- - +	[a]
Week 5	- - +	- - +	- - +	[a]
Week 6	- - - -	- - - +	- - - +	- - - +
Week 7	- - - -	- - - -	- - - +	[b] - - - +
Week 8	- - - - [b]	- - - +	- - - -	[b] - - - -
Week 9	CLEARED	- - +	- - +	- - +
Week 10	CLEARED	- - +	- - - +	- - - +
Week 11	CLEARED	- - +	- - +	- - +
Week 12	CLEARED	- - +	- - +	- - +
Week 13	CLEARED [b]	- - - +	- - +	- - +
Week 14	CLEARED	- - +	- - +	- - +

[a]Samples not cultured because of positive direct smear results.
[b]Samples contaminated with fungus.

TABLE V

Culture results for vaginal washings, checked at 24, 48, 72 or 96 hours, for cattle singly-vaccinated with *Tritrichomonas foetus* vaccine

Sample Time	Double Vaccinate (KG1) 24 48 72 96	Double Vaccinate (KG2) 24 48 72 96	Double Vaccinate (KG3) 24 48 72 96	Negative Control (KG4) 24 48 72 96
Week 3	[a]	- - +	- - +	- - +
Week 4	[a]	- - +	- - +	- - +
Week 5	- - - +	- - - +	- - - +	- - +
Week 6	- - +	- - +	- - +	- - +
Week 7	- - +	- - +	- - +	- - +
Week 8	- - +	- - +	- - +	- - +
Week 9	- - +	- - +	- - +	- - +

[a]Samples were not cultured because of positive direct smear results

DISCUSSION

The FIAX test was rapid, economical, easy to perform, and it was quickly and easily adapted for *T. foetus* tests using freeze-thawed whole-cell organisms as antigen. Other workers have used FIAX serology to detect IgG antibody to *Sarcocystis cruzi*[15], *Cytauxzoon felis*[12] and many other parasites[16]. This was the first FIAX test developed to fluorometrically detect IgG antibodies to *Tritrichomonas foetus* in serum and cervical exudates.

FIAX detected seroconversion in the Oklahoma State University (OSU) cattle within two weeks after giving the initial dose of the Fort Dodge *T. foetus* vaccine, and FIAX values increased for the next 5-6 weeks (Fig. 2). Similar response curves were found with reference sera obtained from an outside vaccine trial (Fig. 3). When FIAX values were regressed against previously determined reciprocal titers for the reference sera it was found that IFA titers were estimated as high as 7700 (Table 1).

Similar response curves and antibody titers were observed in both the in-house and Reno vaccine trials (see Chapter II). Seroconversion was apparent within 1 to 2 weeks following the initial inoculation (Fig 2). This was slightly earlier than could be detected using visual evaluation of slide IFA tests. The subjective visual estimates of antibody levels using IFA titers are inherently inaccurate at high titers because of the two-fold dilution schemes. Better titer estimates for comparative purposes would be obtained using more sensitive ELISA titers. The FIAX values could be converted to ELISA titers in the same manner as for the IFA titers herein reported.

Elevated IgG was apparent in one animal within one week of giving the vaccine, although not at a statistically significant level. However, IgG levels for all double-vaccinates increased significantly by the second week following initial administration

of the vaccine. At two weeks post-vaccination, the average estimated IFA titers had increased to approximately 2800. All three of the double-vaccinated OSU heifers developed titers that peaked between 7 and 10 weeks and fluctuated at high levels for up to 10 weeks. Response curves (Figs. 2 and 3) show the Fort Dodge vaccine was effective in stimulating strong IgG antibody responses in heifers.

This was the first time a FIAX test has been used to detect IgG antibody levels in serum and cervico-vaginal fluid in cattle challenged with *Tritrichomonas foetus* organisms. This study is also the first to compare the clearance of *T. foetus* in cattle challenged with the organism that have been vaccinated for two consecutive years against the clearance rate in cattle that have been vaccinated just once.

Seroconversion was detected in all single-vaccinated cattle within two weeks following vaccination with the Fort Dodge *T. foetus* vaccine. IgG antibody levels were detected in cervico-vaginal fluid between 4 and 5 weeks post-vaccination, at lower levels than in serum (Fig 4-11). There are no IgG level values in Figure 5 and 7 after week 14 due to loss of samples. Levels of IgG increased for two weeks following vaccination then began to decrease. IgG levels in three heifers decreased dramatically by weeks 6-8 of the study (Fig 5, 6 and 9). These same heifers showed IgG antibody increases in cervico-vaginal fluid in contrast to the decline of IgG antibody in the serum. This finding concurs with a study done by BonDurant *et al*[3] showing peak IgG_1 levels in cervical vaginal mucus was accompanied by a drop in serum levels due to "spillover" of IgG into vaginal secretions.

Anamnestic IgG responses occurred in twice-vaccinated cattle within one week of vaccination, and specific IgG elevated sooner than in the previous year. One heifer (Fig 4) had IgG levels that plateaued within two weeks after vaccination and remained

high for eight weeks then slowly declined. She cleared the *T. foetus* infection by week ten (Table 4). The other heifers (Fig 5 and 6) in this group had serum IgG levels that started to decline sharply by week four and five, and did not clear the infection (Table 4). This may suggest that an animal that does not produce a good antibody response to vaccine may become a carrier animal of the *T. foetus* organism and possibly infect herd bulls.

The single-vaccinate negative control (Fig. 11) had elevated serum IgG levels prior to challenge with *T. foetus*. This elevation in serum IgG might explain why *T. foetus* numbers decreased quickly in the cervico-vaginal fluid. This animal might have had previous exposure to *T. foetus*, and developed some immunity against the organism, although this is highly unlikely. A study done by Corbeil *et al*[9] demonstrated bovine immunoglobulin binding to *T. foetus* during growth in medium which contains normal bovine serum. This binding of immunoglobulin by *T. foetus* could be a problem in serologic assays in which *T. foetus* grown in bovine serum was used as the antigen, since anti-bovine Ig conjugates would react with this antigen preparation even if the test sample had no antibody. This cross-reactivity can also seen in ELISA tests, causing false positive results.[30] Yule *et al*[32] observed cross-reactivity between *T. foetus* and bovine cervical mucus. The other sera in this study (Fig. 4-10) did not demonstrate this problem of cross-reactivity. This researcher thinks this animal had a peculiar immune system, and cross-reactivity might be a possibility. A study done by Skirrow and BonDurant[28] showed that natural antibodies to *T. foetus* can be responsible for preinfection reactivity against *T. foetus*. Trauma, contamination and inflammation from collection of cervico-vaginal mucus may stimulate both local production of cross-reactive antibodies and infiltration of systemic antibodies into the reproductive tract.

The twice-vaccinated negative control (Fig. 7) exhibited a seropositive response following challenge with *T. foetus*. Serum IgG levels are not elevated as high as the twice-vaccinated animals (Fig. 4-6), but the characteristics of the graphs are similar in pattern. Both vaccinated and unvaccinated animals exhibit a seropositive response following challenge, and IgG can be detected in the cervico-vaginal mucus. This challenges the "spillover" theory of serum IgG passing from the systemic circulation over into the reproductive tract. The elevated serum IgG in the twice-vaccinated negative control is due to a local infection of the cervico-vaginal area with *T. foetus*. It is unclear with the twice-vaccinated animals whether the elevation of cervico-vaginal mucus IgG was due to a "spillover" from serum or a local immune response in the reproductive tract. To clear up this issue, a negative control animal that is not challenged with *T. foetus* could be utilized and compared to the challenged negative control.

The double and single-vaccinate direct smear results (Table II and III) are similar with respect to almost all cervical mucus samples were cultured by week 3 after challenge with *T. foetus*. All double-vaccinate animals (Table IV) except one remained culture positive for *T. foetus* up to week 14. All single-vaccinate animals (Table V) were still culture positive by week 9. This suggests that direct smear results might not be reliable beyond 2-3 weeks for detection of parasites.

A main aspect of this study was to determine if the twice-vaccinated cattle cleared a *T. foetus* infection more quickly than single-vaccinated cattle. Organisms initially localize in the vagina, and later move to the uterus and oviduct, but their presence there does not prevent conception. However, the secretions in these organs continue to contain parasites. Some cows develop an active immunity and may conceive and carry a calf to term following three to five heat cycles after an abortion. This

immunity, however, is not permanent or "solid" and the cow can be subject to reinfection during later breeding periods.[19]

There were six vaccinated cattle in this study, and only one cleared infection by ten weeks into the experiment (Tables 4 and 5). The only animal that cleared the infection was double-vaccinated. The lack of clearance in other heifers indicates *T. foetus* vaccine does not elicit a protective immune response in cattle, however the twice-vaccinated heifer that cleared infection appeared to be protected. This suggests that vaccine response may be related to an animal's individual immune response. A study done by BonDurant *et al*[3] featured a surface antigen of *T. foetus* as the basis for a vaccine. The *T. foetus* surface antigen reacted with a monoclonal antibody which immobilized and mediated complement killing of the organism and prevented adherence to vaginal epithelial cells. This indicates that the recognized epitopes are accessible by antibody in the living organism. The latter demonstrated clearance of *T. foetus* from 75% of vaccinated heifers by week six in contrast to ten weeks for non-vaccinated controls.

Another possible explanation for the ineffectiveness of this *T. foetus* vaccine could be the severity of challenge with *T. foetus* organisms. Previous challenge studies[5,22,28,29] show a range between 7×10^6 to 2×10^7 organisms per milliliter covering both bulls and heifers. The utilization of 1×10^7 organisms per milliliter in this experiment, although in agreement with previous studies, could have overwhelmed this *T. foetus* vaccine. We can probably assume this challenge would be more severe than a natural infection. It would be interesting to use this vaccine in a natural field infection and compare the results with this challenge study.

The BonDurant *et al*[3] study suggests that vaccination is not likely to completely

prevent colonization with *T. foetus*. Infertility is prevented by protection from cervicitis, endometritis, placentitis and/or embryonic loss. Another study done by Kvasnicka *et al*[22] using a polyvalent vaccine containing *T. foetus*, *Campylobacter fetus*, and *Leptospira* sp. showed that immunization lowered the incidence of infection and reduced the duration of infection. Poly-valent vaccination also demonstrated higher calving rates than non-vaccinated controls, perhaps by protection against agents other than trichomonads.

The OSU study did not demonstrate enhanced clearance rate as with the BonDurant[3] experiment. This may be due to the difference in the *T. foetus* antigen and/or adjuvant used. This is the first study using cattle vaccinated with commercial vaccine for two consecutive years. The double-vaccinated cattle did not clear infection faster than the single- vaccinated animals. This raises doubt that this particular *T. foetus* vaccine works well in the field. Further study is ongoing with different vaccine preparations, but it is evident that current vaccines are not adequately effective.

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VITA

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Master of Science

Thesis: FLUOROMETRIC MEASUREMENT OF IgG ANTIBODY RESPONSES IN SERUM AND CERVICO-VAGINAL MUCUS IN CATTLE VACCINATED WITH *TRITRICHOMONAS FOETUS*

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