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Antigen for the IFAT was derived from a strain of T. vivax isolated from a cow in the Department of Cordoba, Colombia. The strain was maintained via serial passage in hemoparasite-free calves. Inoculation of approximately 2 x 10^9 trypanosomes into splenectomized, triamcinolone acetonide-treated calves resulted in parasitemias suitable for IFAT antigen production at 66 to 69 hours post inoculation.

In field cases of trypanosomiasis, the IFAT was up to 21 times more effective in detecting T. vivax - positive cattle on the basis of antibody presence than the thick blood smear technique.

Results of the survey revealed the presence of T. vivax antibodies in cattle from the department of Boyaca, Cordoba, Meta, Sucre and Valle. No T. vivax IFAT antibodies were detected in serums obtained from cattle in the departments of Antioquia, Cauca, Caqueta, Cundinamarca, Tolima and Vichada.

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THE DEVELOPMENT OF AN INDIRECT FLUORESCENT ANTIBODY TEST FOR *TRYPANOSOMA VIVAX* IN COLOMBIA

A Thesis

by

KENNETH BRADLEY PLATT

Submitted to the Graduate College of
Texas A & M University in
partial fulfillment of the requirement for the degree of

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May 1974

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THE DEVELOPMENT OF AN INDIRECT FLUORESCENT ANTIBODY TEST FOR *TRYPANOSOMA VIVAX* IN COLOMBIA

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(Co-Chairman of Committee) (Co-Chairman of Committee)

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May 1974

ABSTRACT

The Development of an Indirect
Fluorescent Antibody Test for
Trypanosoma vivax in Colombia
(May 1974)

Kenneth Bradley Platt, B.S., D.V.M., Cornell University
Co-Chairmen of Advisory Committee: Dr. L. G. Adams
Dr. L. C. Grumbles

An indirect fluorescent antibody test (IFAT) for *Trypanosoma vivax* infections was developed for a survey involving over 2000 cattle distributed throughout 11 departments and territories in Colombia.

Antigen for the IFAT was derived from a strain of *T. vivax* isolated from a cow in the Department of Cordoba, Colombia. The strain was maintained via serial passage in hemoparasite-free calves. Inoculation of approximately 2×10^9 trypanosomes into splenectomized, triamcinolone acetonide-treated calves resulted in parasitemias suitable for IFAT antigen production at 66 to 69 hours post inoculation.

Thin blood smears were prepared from citrated *T. vivax* laden blood, air dried and fixed in acetone:methanol::60:40, -20 C for 30 minutes. Fixed antigen slides were stored dry at -35 and -70 C and used successfully

in the IFAT for 144 and 116 days respectively.

Indirect fluorescent antibody test serum titers of 1:100 or greater were observed at a mean time of 15.9 days post parasitemia (PP) with a range of 6 to 32 days. Maximum titers of 1:400 developed in 14 to 33 days PP in 8 calves. In one calf, 109 days PP were required before the maximum titer was attained. Serum titers persisted in calves up to 278 days PP. Maternal antibodies capable of causing a positive fluorescent reaction at 1:100 serum dilution persisted for 29 days after birth in 1 calf.

At serum dilutions of 1:100, the IFAT detected positive reactions in 80.4% of 133 serum samples obtained from 9 calves at various times during the course of *T. vivax* infections. At serum dilutions of 1:50, the percentage of positive reactions increased to 97%.

The IFAT was specific for *T. vivax*. No cross reactivity was observed between *T. vivax* and *Anaplasma marginale*, *Babesia argentina*, *Babesia bigemina*, *Eperythrozoon spp.* or *Trypanosoma theileri* at 1:50 serum dilutions. Suspicious reactions were occasionally observed when *Trypanosoma evansi*-positive serum diluted 1:50 and 1:100 was used in the IFAT for

T. vivax. No false positive reactions were observed when serums from 36 hemoparasite-free calves were tested. Results from the IFAT were repeated within plus or minus 1 dilution approximately 80% of the time using different antigen lots on the same and different days.

Samples obtained for the IFAT by eluting serum from dried blood-impregnated filter paper that had been stored at -20 C for 10 days or less, produced results nearly equal to those obtained by using conventional serum samples.

In field cases of trypanosomiasis, the IFAT was up to 21 times more effective in detecting *T. vivax*-positive cattle on the basis of antibody presence than the thick blood smear technique.

Results of the survey revealed the presence of *T. vivax* antibodies in cattle from the departments of Boyaca, Cordoba, Meta, Sucre and Valle. No *T. vivax* IFAT antibodies were detected in serums obtained from cattle in the departments of Antioquia, Cauca, Caqueta, Cundinamarca, Tolima and Vichada.

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INTRODUCTION

The presence of *Trypanosoma vivax* in the Western Hemisphere was first documented in 1919 and to date its presence has been confirmed in Panama, Venezuela, Surinam, French Guiana, Brazil and Colombia.[46]

An extensive review of governmental records and a survey employing non-seriological techniques of Colombian cattle was undertaken by Wells *et al.*[53] in 1968 to establish the current distribution and epidemiology of *T. vivax* in Colombia.

The development of the indirect fluorescent antibody test (IFAT) was undertaken to supplement the findings reported by Wells *et al.*[53] and to survey over 2,000 bovine serum samples collected from various parts of Colombia from October 1968 through October 1972. The test will also be indispensable for future surveys that will be required to establish the economic importance of this hemoparasite in Colombia.

The citations in this paper follow the style of the *American Journal of Veterinary Research*.

REVIEW OF LITERATURE

Morphological studies of African strains of *T. vivax* by Moore and Broom[24] in 1938 and by Fairbairn[18] in 1953 indicated this trypanosome ranged in length from 19.0 to 25.5 μ. Kubes[29] described a Venezuelan bovine strain of *T. vivax* with a mean length of 22 μ ranging from 17.6 to 28 μ. The most frequent lengths observed ranged from 21 to 23 μ.

Shaw and Lainson[46] made a thorough review of the literature concerning the distribution of *T. vivax* in South America. They compared the results of morphological studies by earlier workers to their own findings based on a strain of *T. vivax* isolated from an Indian water buffalo (*Bubali bubali*) near Belen, Brazil. The mean length of 22.77 μ with a range of 19.2 to 25.0 μ as determined by Shaw and Lainson was in close agreement with those reported by earlier workers ranging from 21.0 to 22.7 μ.[26,29,38,39]

Trypanosomes have been preserved by low temperature storage. The ease by which this has been accomplished has depended on the specie of trypanosome involved and the method employed. Herbert *et al.*[22] were able to preserve the infectivity of *Trypanosoma*

brucei for up to 200 days by rapid cooling in liquid nitrogen, i.e. from ambient temperature to -196 C in less than 3 seconds. When 7.5% glycerol or 20% dimethylsulfoxide were added as cryoprotectants, the viability of trypanosomes was completely lost after 83 and 14 days respectively. Polge and Soltys[40] stored *T. brucei* for 8 months in 5% and 10% glycerol at -79 C. They found that quick cooling of *T. brucei* in the presence of glycerol adversely affected the survival rate.

Cunningham *et al.*[11] reported blood stream forms of *T. vivax*, *Trypanosoma congolense*, *T. brucei*, *Trypanosoma rhodesiense* and *Trypanosoma cruzi* in heparinized blood containing 7.5% glycerol could be successfully stored at -80 C by controlling the rate of cooling. No data was presented concerning viability following thawing after specific intervals of storage. The method of preservation used by Cunningham *et al.* provided a cooling rate of 1.7 C per minute.[15]

Dar *et al.*[15] successfully preserved metacyclic forms of *T. vivax*, *T. brucei* and *T. congolense* in suspensions containing 8% glycerol by regulating the rate of cooling at 2 C per minute from ambient temperature to -40 C in the vapor phase of a liquid

nitrogen refrigerator. The rate of cooling was most critical for T. vivax. A cooling rate of 27.5 C per minute resulted in loss of viability of T. vivax in less than 3 weeks. A cooling rate of 2 C per minute did not result in any significant decrease in viability or infectivity. Trypanosoma brucei was the least affected by cooling rate. Cooling rates of 27.5, 6.0, 3.0 or 2.0 C did not significantly alter viability or infectivity. By using the same technique and employing a cooling rate of 2 C/minute, Dar[14,15] successfully preserved blood stream forms of T. vivax.

Blood stream forms of T. vivax were also preserved by Shaw[46] who stored infected blood containing 10% glycerol in ampoules at -72 C. No mention of anti-coagulant was made. MacLennan and Na'Isa[34] preserved the blood stream form of T. vivax by storage of infected, heparinized blood containing 10% glycerin in liquid nitrogen. No mention of cooling rate was made.

Desowitz and Watson[16] showed that it was possible to infect white rats with T. vivax. They noted that the intensity of the initial parasitemia in the rats was not completely related to the size of the inoculum. The stage of parasitemia in infected donor sheep also appeared to influence the initial parasitemia in rats.

It was subsequently shown that it was possible to maintain T. vivax in white rats by inoculating them with as little as 0.005 ml. of ovine serum within 24 hours of infecting the rats with T. vivax organisms.[17]

Trypanosoma theileri was isolated from the peripheral blood of cattle by inoculating infected blood into tubes containing modified blood agar medium.[27] Trypanosoma theileri organisms were maintained on a blood agar medium containing 20% ovine blood for up to 121 days without losing their ability to infect new medium.[52]

Clark and Benavides[8] were able to infect cattle with T. evansi via an infected vampire bat (Desmodus rotundus) and demonstrated that cattle served as a reservoir for T. evansi in ranana. Trypanosoma evansi readily infected rats and the pre-patent period appeared to be inversely proportional to the number of trypanosomes in the inoculum.[21]

A variety of methods exist for detecting trypanosomes in blood. The examination of wet blood mount preparations, thick and thin blood smears, and the inoculation of experimental animals with suspect blood have been labeled standard trypanosome detecting methods (STDM) by Wilson.[55] The examination of thick

blood smear preparations has been estimated to be 12 to 120 times more efficient than thin smear examination.[27] MacLennan[33] stated that thick blood smear examination will detect the presence of trypanosomes when these organisms are as scarce as 11/mm^3. By virtue of the larger amount of blood examined, thick blood smear examination is also more efficient than wet mount examination (WME).[27] Woo[58,59] developed the hematocrit centrifuge technique for trypanosome detection and its efficiency over stained blood smear techniques and WME has been confirmed.[42]

The first successful utilization of the IFAT for the detection of trypanosomal infections was made by Fife and Muschel[20] in the diagnosis of Chagas' disease. They utilized formalin-fixed trypanosomes in suspension as test antigen. Williams et al.[54] modified the technique of Fife and Muschel by using Trypanosoma rhodesiense and Trypanosoma gambiense blood forms and culture forms fixed on glass microscope slides. Kimber[28] further refined the IFAT for T. rhodesiense infections in humans by separating trypanosomes from infected mouse blood via differential centrifugation. He used the trypanosome-rich supernatant to prepare thin antigen preparations on glass slides. Wilson

et al.[57] adapted the test for the detection of antibodies in cattle infected with T. vivax, Trypanosoma brucei and Trypanosoma congolense.

Sadun et al.[43] used the method described by Anderson et al.[1] to extract serum with a vise-like devise from dried blood samples collected onto filter paper. Sadun et al. successfully used such samples obtained from trypanosome-positive humans in the IFAT as modified by Williams et al.[54]

Bailey et al.[5,6] modified the technique of Sadun et al.[43] by collecting whole blood onto Whatman #4 filter paper. Three-mm.-in-diameter discs were removed and placed into circular wells formed by nail varnish directly on glass antigen slides. Each well contained 0.025 ml. diluent. The resulting eluate was considered to be equivalent to a 1:50 serum dilution as a result of "weighing experiments." Serum samples collected in the form of dried blood retained their serologic activity for 14 days at 25 C and up to 2 months when stored at -20 C. Cunningham et al.[12] adapted the technique of Bailey et al.[6] for the detection of bovine antibodies to T. brucei subgroup and T. congolense group trypanosomes. They concluded that serums with titers of 1:80 would be detected.

Other workers[10,36,51,55] have successfully used this modification of the IFAT for bovine trypanosomiasis.

Antigen for the IFAT has been prepared by different methods. Trypanosoma rhodesiense was separated from heparinized rodent blood via differential centrifugation. The trypanosome-rich supernatant was then thinly spread over a glass slide and allowed to dry.[5,6,10,28,57] Yaeger[60] described the use of phytohemagglutinin in combination with differential centrifugation to separate Trypanosoma cruzi from mouse blood. Lanham[30] described the use of anion exchange resins to achieve the same purpose. Whole blood containing large numbers of trypanosomes was likewise evenly and thinly spread over glass slides and allowed to dry. [,10,12,36,51,54,55,57,61]

The potential exists for means to increase parasitemias in antigen donor animals. Callow and Parker[7] were able to induce a relapse of Babesia argentina with cortisone. Luckins[32] effectively used X-irridation and cortisone treatment of white rats to enhance T. brucei blood parasitemias. Unsworth[50] was able to increase the intensity of T. vivax parasitemias in mice by splenectomy.

Antigen preparations were heat fixed and stored

dry at -20 C for up to 6 months without losing activity.[4,5,57] Antigen has also been stored dry at -20 C and fixed with chemical agents after equilibrating to room temperature.[61]

Williams et al.[54] compared washed and unwashed culture forms of T. rhodesiense and T. gambiense to blood forms of these trypanosomes in the IFAT. He found blood forms yielded a brighter and more consistent fluorescent response.

Both physical and chemical methods of antigen fixation have been employed. Williams et al.[54] subjectively compared 5 and 10% formalin, 0.1% HCl and methanol to each other. They found that 5% formalin with rhodamine conjugated to bovine serum albumin added 1:20, gave the best results. Gentle heat has also been used with success.[4,5,10,12,28,36,55,56,57]

Zwart et al.[61] objectively compared the efficacy of 'heat', 0.3% acetic acid, methanol, 0.01N HCl, 0.03N HCl, 5% formalin and acetone as fixatives. Indirect fluorescent antibody titers obtained using the same serum on antigen fixed by each of the above agents were compared and the intensity of fluorescence noted. Acetone at room temperature for 5 minutes gave

the best results. Good results were obtained with 0.01N HCl and 0.03N HCl. Poor results were produced with methanol for 10 seconds. Five percent formalin and 0.3% acetic acid produced better results than "heat", but the intensity of fluorescence was less than that produced by acetone fixation.

Varying degrees of cross reactivity in the IFAT have been reported. Wilson et al.[57] showed that no significant difference in IFAT titers was observed when T. congolense and T. brucei-positive bovine serum was used with antigen prepared from 4 variants of T. congolense and 5 variants of T. brucei. Some cross reactivity did exist between trypanosome groups. Trypanosoma brucei-positive bovine serum exhibited titers of 1:160 when exposed to both homologous antigen and T. rhodesiense antigen. Titers ranging from 1:20 to 1:40 were observed when either T. congolense or T. vivax antigen was employed. Wilson and Cunningham[56] showed that titers to heterologous antigens "rarely exceeded" a +2 fluorescent response at serum dilutions of 1:40. Schindler[44] presented similar findings. Serums with homologous T. brucei IFAT titers of 1:5120 produced heterologous titers of 1:40 to 1:80 when T. vivax antigen was used. When serums of homologous

T. brucei titers of 1:2560 were tested, heterologous titers of 1:10 and 1:20 were observed.

Williams et al.[54] demonstrated cross reactivity when Trypanosoma lewisi antigen was subjected to both rabbit anti T. rhodesiense and T. gambiense serum. Zwart et al.[61] showed significant cross reactivity between T. congolense and T. brucei. When bovine serums with either a 1:640 T. congolense titer or a 1:2560 T. brucei were used in the IFAT test employing T. vivax antigen, positive fluorescent reactions were recorded at serum dilutions of 1:40. However, when bovine positive T. vivax serum with a titer of 1:2560 was used in conjunction with T. brucei and T. congolense antigen, heterologous titers of 1:1280 and 1:640 respectively were obtained.

Non-specific fluorescence has been a sporadic problem. Wilson et al.[57] tested serum from 101 known pathogenic trypanosome negative cattle with the IFAT without observing a false positive reaction. They did notice that a greater incidence of fluorescence occurred in serum dilutions of 1:10 and 1:20 in cattle positive for T. theileri. Bailey et al.[6] in testing humans assumed free of trypanosomes, detected 1 of 35, +4 reaction and 7 of 19, +3 reactions

to T. rhodesiense. Sadun et al.[43] recorded a +1 "doubtful" reaction in a human infected with leishmaniasis. However, in a second case of leishmaniasis, no doubtful reaction was observed. Williams et al.[54] detected no false positive reactions with serum from 49 known trypanosome negative rabbits when tested against T. gambiense and T. rhodesiense antigen.

To minimize the possibility of non-specific fluorescence, various base line dilutions have been adopted for the IFAT. Williams et al.[54] used 1:64, Sadun et al.[43] used 1:16, Zwart et al.[61] chose 1:160 and others have chosen 1:40.[12,15,36,51,55,57]

A principal difficulty of the IFAT has been differences in interpretation of the fluorescent end point.[55] On the basis of a fluorescent response scale ranging from negative to +4, some workers have chosen +2 as a positive reaction.[20,54,55,57] Others have chosen +3.[5,6,12,36] Compounding this problem is the variability of fluorescent intensity depending on the method of antigen fixation.[61]

Detectable IFAT titers occurred as early as 7 to 8 days post inoculation (PI).[10,45,56] However, a period as long as 49 days PI may be required.[56] Maximum titers of 1:2560 were reported by Zwart et al.[61] and

titers of 1:1280 were found by Wilson and Cunningham[56] to occur after 100 days PI. The average time for titers to decrease to a fluorescent grade of +2 at a serum dilution of 1:40 following self cure was 110 days. However, chemotherapeutic sterilization caused titers to decrease to +2 at a 1:40 serum dilution in 30 to 110 days.[56]

Wilson and Cunningham[56] categorized trypanosome-exposed cattle into 4 groups: group 1, early infection, characterized by a visiable blood parasitemia with a negative IFAT titer; group 2, established infection, possessing a visible parasitemia and an IFAT titer of 1:160 or greater; group 3, latent and cured infections, not exhibiting visible parasitemias but possessing IFAT titers of 1:80 or higher. If the titer remained constant or increased after 6 to 8 weeks, the infection was labeled latent and if a decrease in titer occurred, the cow was considered cured. Group 4 was composed of cattle with no titers or parasitemias. The above classification system was proposed to evaluate the immune status of herds and the effectiveness of chemotherapeutic programs.

The accuracy of the IFAT for detecting specific trypanosome antibodies in cattle has been verified.[10,

[12,55,56,57,61] Consequently the IFAT has been used effectively as an epidemiological tool in Africa. Wilson[55] conducted a survey in Ugandan cattle and compared STDM to the IFAT. In herds where both methods detected the presence of trypanosomes, the IFAT was up to 25 times more effective. In addition, the IFAT detected trypanosome-positive cattle in herds where STDM were ineffective. Other investigators have used the IFAT in field surveys with similar success.[36,51,61]

MATERIALS AND METHODS

Experimental Animals

Calves

Holstein-Friesian calves under 4 months old were obtained from the Bogota savanna which is considered to be free of ticks, *Anaplasma marginale*, *Babesia spp*. and *Trypanosoma spp*. The calves were transported to Palmira via truck and housed in a tick moated, screened stable for the duration of the study. They were maintained on the ration described in Table 1 at 3% of the body weight daily. Ground corn cob was used as roughage and fed at the rate of 1% of body weight daily. The calves were males with the exception of calves 1 and 2. At the time of inoculation the calves ranged in age from 6 to 10 months.

Prior to use each calf was examined and monitored for the presence of hemoparasites via stained blood smears, the complement fixation (CF) test,[2] rat inoculation and blood cultures, The CF test was used employing anaplasma CF antigen supplied by the U.S.D.A. and *Babesia argentina* and *Babesia bigemina* CF antigen prepared according to the method of Mahoney.[35] Two 5 ml. aliquots of citrated blood from each calf were inoculated intraperitoneally (IP) into 2 adult rats to

TABLE 1. Calf and Sheep Ration

Ground Corn	430 kg.
Cotton Seed or Soybean Meal	300 kg.
Wheat or Rice Bran	230 kg.
Iodized Salt	10 kg.
Bone Meal	10 kg.
Vitamin Premix*	10 kg.
Mineral Premix**	10 kg.

*Vitamin Premix

Ground Corn	10 kg.
Vitamin A-D Preparation (750,000 iu/gm Vit. A) (150,000 iu/gm Vit. D)	8 gm.

**Mineral Premix

Ground Corn	85.98 kg.
Copper Sulfate	1.95 kg.
Zinc Oxide	1.24 kg.
Iron Sulfate	7.47 kg.
Magnesium Sulfate	3.09 kg.
Cobalt Sulfate	0.20 kg.
Potassium Iodide	0.07 kg.

detect the presence of Trypanosoma evansi. Tail blood from the inoculated rats was examined twice weekly for 28 days.

Modified blood agar medium was prepared by adding 32.9 g of tryptose blood agar base[a] and 0.6 g dextrose[b] to 800 ml. of double glass distilled water and sterilized at 120 C for 15 minutes. The medium was cooled to 50 C and 200 ml. of defibrinated sterile sheep blood were added. Penicillin G and dihydrostreptomycin were added to the medium at the rate of 400 units per ml. and 0.5 mg. per ml. respectively. Nine ml. of citrated calf blood were drawn from individual calves, divided into 3 equal aliquots and cultured on modified blood agar at 27 C for 21 days to detect the presence of Trypanosoma theileri.

Sheep

Male Rambouillet crossbred sheep were obtained from the Ramulade cooperative which is situated at a tick free altitude in excess of 3,000 meters. The sheep were transported to Palmira via truck and maintained in a screened enclosure on a concrete pad. No evidence of

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[a]Difco Laboratory, Detroit, Michigan.

[b]Merck and Co., Darmstadt, West Germany.

ticks was observed during the course of the study. The sheep were maintained on the same diet and rate as were the calves with the exception that the copper concentration was reduced by one-half. Sheep were screened for the presence of hemoparasites via the CF test for anaplasmosis and stained blood smear preparations.

Rabbits and Rats

Adult male New Zealand white rabbits were obtained from the Centro Internacional de Agricultura Tropical (CIAT) colony. Adult and immature white rats (Charles River strain) were also obtained from the CIAT colony. Rabbits and rats were maintained on a diet of Purina Rabbit and Laboratory Animal Chow respectively.

Parameters

Relative parasitemia was determined by examining wet mount preparations one cell layer thick at x 400 magnification. The number of actively motile trypanosomes per field was determined and the following relative values were assigned: +1 equivalent to 1 trypanosome per 50 fields; +2 equivalent to 1 organism per 10 fields; +3 equivalent to more than 1 organism per 10 fields but less than 10 per 10 fields; +4 equivalent to 1 organism per field but less than 5 per field; and

+5 equivalent to at least 5 or more trypanosomes per field.

Thick blood smears were made by placing glass microscope slides over circular templates 18 mm. in diameter drawn on paper index cards. Blood was removed from a small ear vein via a heparinized capillary tube with an approximate volume of 85 ul. The capillary tube was filled completely and the entire contents deposited on the glass slide over the template area. Smears were allowed to air dry and stored until staining.

Thick smears were stained for 60 minutes in Giemsa stain[c] prepared in the following manner: 100 ml. of 0.15 M phosphate buffer pH 6.9 were added to 900 ml. of distilled water. One ml. of 10% Triton X-100[d] was added to the buffered water. Seven ml. of stock Giemsa were then added to 300 ml. of buffered water and thoroughly mixed. Although particulate matter was present, filtration was not required. Following colorization, the preparations were washed in buffered water, dried and examined.

One hundred fields of each thick preparation were

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[c]Gradwohl Labs, St. Louis, Mo.

[d]J. T. Baker Chemical Co., Phillipsburg, N.J.

examined with a Reichert Diapan microscope[e] using x 10 oculars and x 100 oil immersion objective.

The hematocrit centrifuge technique for the detection of trypanosomes utilizing blood capillary tubes was performed in accordance with the method described by Woo.[59]

The health of T. vivax-infected calves was monitored by recording relative parasitemia, rectal temperature and the hematocrit at various intervals throughout the study. Trypanosoma evansi-infected calves were similarly monitored without recording relative parasitemia. Trypanosoma evansi carrier status was confirmed by thick bloo smear examination and rat inoculation.

Experimental Organisms

Trypanosoma vivax

Morphology. Measurements of T. vivax organisms were made by first preparing thin blood smears utilizing sodium citrate as anticoagulant, fixing in reagent grade absolute methanol one minute, and staining in a Giemsa preparation. The infected blood for the smears was obtained from a calf during the last day of an ascending phase of a parasitemia 33 days following the first

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[e]Reichert, Vienna, Austria.

appearance of trypanosomes in the calf's blood. Standardized photographs of 100 thin form T. vivax organisms possessing relatively sharp posterior ends and undividing elongated nuclei were made at a magnification x 1620. Measurements were made after Hoare[23] with the aid of fine pointed calibers.

The strain of T. vivax used in this study was obtained from an infected calf in Tierra Alta, Cordoba, Colombia in November, 1971. It was identified by measurement, morphology, characteristic motility pattern in wet mount preparations, inability to readily infect white rats and its ability to produce a debilitating disease in calves.

Isolation and Purification. A sheep and calf were each inoculated intravenously with the blood of the infected calf at the Instituto Colombiano Agropecuario (ICA) station at Monteria, Córdoba. Sixty ml. of infected blood with a relative parasitemia of +5 were drawn into EDTA vacutainer tubes from both the sheep and calf. The infected sheep and calf blood were immediately hand carried via air travel to CIAT and inoculated IV upon arrival into T. vivax-maintenance calves 1 and 2 respectively.

Purification of the strain was attempted employing

a modification of the method described by Daley.[22] Fifty ml. of infected blood from calf 2 with a +5 relative parasitemia were collected into tubes containing EDTA and centrifuged 10 minutes at 300 x g in swinging buckets.[f] From a point approximately 3 mm. above the buffy layer, 0.2 ml. of trypanosome rich plasma were removed from each of 6 tubes, pooled and mixed with an equal part of Alsever's solution. This mixture containing 2.94×10^7 motile trypanosomes as counted via a hemocytometer was inoculated IV into a sheep.

To prevent the survival of Anaplasma marginale, the sheep was injected with alpha-ethoxyethylglyoxal dithiosemicarbazone (Gloxazone)[g] IV at the rate of 5 mg./kg. on the day of inoculation and on each of 3 consecutive days thereafter. The sheep was also injected with oxytetracycline HCl[h] IV at the rate of 12 mg./kg. on the day of inoculation and on each of 11 consecutive days thereafter.

Eight days following T. vivax inoculation, 50 ml. of infected sheep blood were processed as described above and yielded 1.12×10^7 motile trypanosomes which were

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[f]Centrifuge model GLC-1. Ivan Sorvall, Inc., Norwalk, Conn.

[g]Burroughs-Wellcome Co., Research Park Triangle, N.C.

[h]McKesson, Bogota, Colombia.

immediately injected IV into calf 3.

Immediately prior to T. vivax inoculation and for 4 consecutive days thereafter, calf 3 was injected with Gloxazone IV at the rate of 5 mg./kg. Calf 3 was also injected with oxytetracycline IV at the rate of 12 mg/kg. on the day of trypanosome inoculation and 8 consecutive days thereafter. Blood smear examinations were made during the course of drug treatment and throughout the duration of this study.

Maintenance and Storage. Maintenance of the strain was accomplished in the experimental calves described above. Trypanosoma vivax was syringe passed IV into new calves whenever the frequency of parasitemia declined or the survival of previously infected calves appeared threatened. Strain maintenance calves received IV inoculums varying in quantity from 10 to 30 ml. of infected blood with relative parasitemias of +5. Each calf was monitored throughout the course of this study for the presence of hemoparasites other than T. vivax via stained blood smears.

The strain was prepared and stored frozen at -70 C according to the method described by Cunningham et al.[11] with the exception of not rotating the sample during the

cooling phase.

Trypanosoma evansi

Trypanosoma evansi was isolated by Dr. S. Ayala of the Universidad de Valle from an infected vampire bat (Desmodus rotundus) captured in the Dagua valley approximately 45 km west of Cali, Colombia. Identification was made via morphology and the strain's ability to produce rapid massive parasitemias and death in infected rats and mice.

The strain was preserved by adding glycerol at the rate of 10% to infected rat blood in 3.5 ml. glass serum vials. Vials were placed in a polyurethane foam insulting jacket with outside dimensions of 2" x 2". The jacket containing vials was placed in the cooling compartment of a dry ice cabinet overnight. The following morning the vials were removed and lowered into the storage area. Other preservation techniques included rapid freezing of infected blood with glycerol concentrations of 7.5 and 10.0% and storage in liquid nitrogen.

Trypanosoma evansi was maintained in 2 calves. Calf A received 5.0×10^9 T. evansi organisms obtained from infected rats. Fifteen days post inoculation (PI),

200 ml. of blood from calf A were injected IV into calf B. Patent infections were demonstrated in each calf by injecting rats intraperitoneally with 3 ml. of blood from each calf at intervals up to 10 days. Thick blood smear, capillary tube and wet mount examinations were made on blood from *T. evansi* calf A 9 of the first 16 days PI.

Trypanosoma theileri

Trypanosoma theileri was isolated from a Zebu cow in the CIAT herd at Palmira by blood culture on modified blood agar medium. The strain was maintained by weekly subpassage to fresh medium and incubated at 27 C.

Indirect Fluorescent Antibody Test

Antigen Production

Trypanosoma vivax antigen. Antigen for the indirect fluorescent antibody test was prepared from the blood of rats, sheep and calves with high degrees of *T. vivax* parasitemias. Infected blood for antigen production was collected into a 2% sodium citrate solution at a ratio of 9 parts blood to 1 part citrate solution.

Rats were injected intraperitoneally (IP) with

3.0 to 6.0 ml. of trypanosome-infected bovine or rat blood taken during ascending parasitemias. Supplemental injections of 0.25 to 0.50 ml. of normal ovine serum were given to T. vivax inoculated rats.[17] Sheep were injected with infected bovine blood.

Trypanosoma vivax organisms used for IFAT antigen preparation were obtained from each of 2 calves designated T. vivax-calf A and T. vivax-calf B, hereafter referred to as calf A and calf B. Parasitemias were obtained by inoculating each calf IV with approximately 2.0×10^9 T. vivax organisms obtained from T. vivax-maintenance calves.

An attempt was made to suppress the immune response of each calf during antigen production by treatment with triamcinolone acetonide (Vetalog)[1]. Calf A was injected with 8 doses of Vetalog at a rate of 0.18 mg./kg. IM at 2 to 3 day intervals prior to inoculating the calf with T. vivax and on the day of inoculation. Calf B was injected with 0.33 mg./kg. Vetalog IM on each of 4 consecutive days prior to inoculation, on the day of inoculation and on the day after inoculation.

Blood from calves A and B was examined twice

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[1]E. R. Squibb and Sons, Inc., New Brunswick, N.J.

daily for the presence of trypanosomes following inoculation. At the point when a minimum of 10 trypanosomes per x 400 field in a wet mount preparation were observed, the parasitemias of both calves were monitored at 4 to 6 hour intervals by preparing stained standard thin blood smears and examining them under the x 100 objective with x 10 oculars. Blood was collected IV for antigen production when the average number of trypanosomes reached approximately 12 to 15 per x 1000 field on a thin blood smear preparation.

Trypanosoma evansi Antigen. Rats were inoculated IP with stabilate and wet mount examinations of tail blood were made on a daily basis. When levels of parasitemia were such that infected blood would yield 20 or more trypanosomes per x 400 field in a thin blood smear preparation, the rats were bled via cardiac puncture. Two percent sodium citrate was used as an anticoagulant at the rate of 1 part citrate to 9 parts blood.

Trypanosoma theileri Antigen. Fifth passage forms of T. theileri were grown on modified blood culture medium. The trypanosomes were harvested with a Pasteur pipette by collecting the supernatant

located adjacent to white shelf-like plaques of organisms and the plaques themselves. This material was mixed with an equal part of phosphate buffered saline (PBS) pH 7.4 and thoroughly suspended before further processing.

Antigen Processing, Fixation, Storage and Evaluation

Thin blood smears were made from T. vivax and T. evansi-infected blood in the following manner from all species of animals used for antigen production. Antigen slides consisted of frosted 72 mm. x 25 mm. x 1.2 mm. glass slides[j] previously cleaned in Alconox[k] detergent and stored in a solution consisting of equal parts of 95% ethanol and acetone. A drop of whole citrated blood 3 to 5 mm. in diameter containing trypanosomes was placed onto the end of a slide and evenly spread over the entire surface of the slide. The smear preparations were immediately dried with a fan, fixed as described in Table 2 and prepared for storage. Trypanosoma theileri antigen suspension

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[j]Scientific Products, Evanston, Ill.

[k]Alconox Inc., New York, N.Y.

TABLE 2. Agents and Methods Used in Fixation of Indirect Fluorescent Antibody Test Antigen

Agent	°C	Minutes
Acetone,* Methanol;* 60:40	-20	15
Acetone, Methanol: 60:40	-20	30
1% Buffered Formalin	25	5
5% Buffered Formalin	25	5
10% Buffered Formalin**	25	5
Heat	70	5
Heat	70	7.5
Heat	70	10
Stalheim's Method	25	17

*Analytical grade. Merck and Co., Darmstadt, West Germany

**Fisher Scientific, Fair Lawn, N.J.

smears were processed in an identical manner.

Fixed antigen preparations were packaged in aluminum foil and placed in air tight polyvinyl bags with a foil packet containing approximately 1 to 2 grams of anhydrous calcium chloride. The bags were manually sealed, removing as much air as possible and stored at -35 and -20 C until used.

Indirect fluorescent antibody test (IFAT) antigen preparations were evaluated using the following criteria: a) adherence of antigen material to the glass slide through processing: b) visibility of antigen material throughout processing; c) degree of background and extraneous fluorescence during the test and d) morphology of fixed trypanosomes.

Separation of *Trypanosoma vivax* from Erythrocytes and Serum Components

Trypanosome rich blood was passed through a DEAE cellulose[1] anion exchange column according to a modification of the method described by Lanham.[30]

The column was prepared by filling the outlet of a 50 ml., 2.6 cm. in diameter disposable syringe

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[1]Bio Rad Laboratories, Richmond, Calif.

with a 0.5 cm. thick layer of siliconized glass beads 2 to 3 mm. in diameter. Glass wool fibers were loosely packed over the surface of the beads and a second layer of beads approximately 0.25 cm. in depth was placed over the glass wool. A flow rate of 90 to 120 drops per minute was maintained by attaching an 18 gauge needle to the syringe outlet.

Fractions of 1.0 ml. were collected and all fractions containing trypanosomes were examined immunoelectrophorectically for serum protein components.[4] Fractions containing 25 or more trypanosomes per x 400 field under wet mount examination were retained for IFAT antigen preparations.

Attempts to separate bovine erythrocytes from trypanosomes with phytohemagglutin[m] according to the method described by Yaeger[60] and by differential centrifugation were made.

Additional Components and Preparative Procedures for Indirect Fluorescent Antibody Test

Phosphate buffered saline (PBS) was prepared by mixing equal volumes of physiological saline and

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[m]DIFCO Laboratory, Detroit, Mich.

0.15 M phosphate buffer pH 7.5. The pH of the resultant solution was adjusted to 7.5 by adding reagent grade 0.15 M KH_2PO_4 or 0.15 M Na_2HPO_4. Buffer was stored and used at 4 C.

Disposable Pasteur pipettes were modified by heating the ends and drawing them to a diameter that delivered a drop approximately the size of a drop delivered from a 25 gauge hypodermic needle.

Control positive and negative serums were diluted with buffer to the same extent as the lowest dilutions of unknown serums. Base dilutions of 1:50 and 1:100 were used for titration and survey testing respectively.

Conjugate was prepared by diluting rabbit anti-bovine globulin[n] conjugated to fluorescein isothiocyanate 1:65 with buffer. To this solution normal rabbit serum conjugated to rhodamine[o] was added at the rate of one part conjugated normal rabbit serum to 19 parts of diluted conjugated rabbit antibovine serum.

Antigen slide preparations were allowed to equilibrate at room temperature and then subdivided

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[n]The Sylvana Co., Milburn, N.J.

[o]Ibid.

with a diamond pencil into 3 horizontal rows of 5 circles each. Each area was approximately 6 mm. in diameter and was made directly on the antigen surface of the slide. Each area was further demarcated with a red wax pencil to serve both as a barrier against encroachment of test serums from adjacent test sites and to aid in site location during the reading of the test. The top row of circles was designated position numbers 1, 6, 7, 12, 13; the second row 2, 5, 8, 11, 14 and the third row 3, 4, 9, 10 and 15.

A Leitz ortholux binocular microscope[p] equipped with x 12 oculars and x 10, x 40, x 54 (oil) and x 100 (oil) objectives with a dark field oil condenser (D1.20) was used for the IFAT. Definitive readings were made with the x 54 oil objective. Ultraviolet light was supplied by an Osram high pressure mercury HBO-200 W lamp. A BG 12 3 mm. exciter filter and barrier filter (K 490) were routinely used.

Execution of Indirect Fluorescent Antibody Test

The IFAT was conducted in an identical manner for T. vivax, T. evansi and T. theileri antigen. The

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[p]Ernest Leitz (Canada) Ltd., Midland, Ontario

test proper was divided into 2 phases.

In phase I, positions 1, 2 and 3 received one drop delivered from modified pipettes of known positive serum, known negative serum and buffer respectively. The remaining 12 positions received bovine serums of unknown status. The serums were diluted 1:100 for use in screening procedures in 2 fold increments from 1:50 to 1:800 for antibody titer determinations. Care was taken to place each drop of test material directly in the center of each test position. The antigen slides were then placed in a humidity chamber and incubated at 37 C for 30 minutes. Following incubation, the slides were removed and washed in 3 consecutive buffer washes at 0-4 C for 5 minutes each. Gentle agitation of the wash solutions was maintained with a magnetic stirring bar and stirrer. The antigen slides were air dried under a fan following the third wash and checked for positions from which antigen had been removed during washing.

In phase II, all positions of the antigen slides received one drop of prepared conjugate delivered from a modified pipette. The slides were then incubated and washed as above. An additional wash of 10 seconds was made in mildly agitated distilled water.

The slides were then air dried, re-inspected for positions with missing antigen and examined in numerical order with the fluorescent microscope.

Reactions were given 1 of 3 different grades: negative, suspicious and positive. All suspicious reactions were re-tested and re-evaluated.

Evaluation of Indirect Fluorescent Antibody Test

Sensitivity

Sensitivity was determined by titrating available serums from calves that had been singularly infected with T. vivax for a pathogenesis study.[13] The strain was isolated from an infected cow near Neiva in the department of Huila, Colombia. Prior to titration the serums had been stored in excess of one year at -20 C. In addition IFAT titers were determined for serums collected over periods of time ranging from 36 to 278 days from 9 T. vivax strain maintenance calves.

Repeatability

Repeatability of IFAT results for any given serum sample using the same antigen lot and conjugate on the same day and different days was determined. Thirteen serum samples from known T. vivax positive calves were

collected, divided into 3 aliquots and stored at -20 C until used. On each of 3 consecutive days, dilutions were made of an aliquot from each sample and IFAT titers were determined in duplicate. Agreement of duplicate titer results was considered to occur when the titers were equal or not differing by more than 1 dilution. The probability of repeating titers of identical serum samples was expressed as the normal approximation to the mean at the 95% confidence interval[48] based on the total number of agreements per comparison.

Same day comparisons were made by comparing the duplicate titers of the 13 different serums. This procedure was repeated on 3 consecutive days. The total number of observations and agreements on all occasions was used in calculating the normal approximation to the mean.

The probability of repeating IFAT titers of a particular serum sample on different days was determined by comparing duplicate titers of each sample on a given day with the duplicate titers of the same sample determined on each of 2 other days. Comparisons were made in the following manner. The first titer determination of day 1 was compared to the first and second determination of both days 2 and 3. Subsequently, the

second titer determination of day 1 was similarly compared. The procedure was repeated with each IFAT titer determination of days 2 and 3.

Indirect fluorescent antibody titer repeatability for a given serum using 2 antigen lots was determined by randomly selecting 14 serum samples from known *T. vivax*-infected calves. Duplicate titers were determined for each serum sample using both *T. vivax* antigen lots. The first and second titer determinations of each sample obtained with antigen lot A were individually compared to the first and second titer determinations of the same samples using antigen lot B. The total number of agreements was determined and expressed as the normal approximation to the mean.

Specificity

Serums from normal calves and calves infected with *Anaplasma marginale*, *Babesia bigemina*, *Babesia argentina*, *Eperythrozoon spp.*, *Trypanosoma theileri* and *Trypanosoma evansi* were used in the IFAT for *T. vivax* to determine possible degrees of non-specificity and cross reactivity. With the exception of one *T. theileri* positive serum of Texas origin, all infected calf serums used were from calves singularly infected with the above

organisms.

Thirty-six non-infected calf serums were obtained from the Texas A & M serum bank at CIAT. Donor calves ranged from 4 to 8 months in age and originated from tick free altitudes in herds with no previous history of hemoparasite infections.

To reduce the possibility of previous hemoparasite contamination, serum from each calf was tested for the presence of complement fixing antibodies against A. marginale, B. bigemina and B. argentina. Thick and thin blood smear examinations were also made of blood collected from each calf. In addition subinoculations of pooled calf blood were also made into hemoparasite-free splenectomized calves.

Six A. marginale, 10 B. bigemina, 6 B. argentina and 6 Eperythrozoon spp. positive calf serums were obtained from the Texas A & M serum bank at CIAT. Complement fixation titers were determined for all samples with the exception of the serums from calves infected with Eperythrozoon spp.

Trypanosoma theileri infected adult cattle were identified by blood culture. Eight serums were obtained from infected cattle located at Angleton, Texas. Three serums were obtained from infected Zebu cattle on the CIAT farm at Palmira and 3 from Zebu cattle at the time

of slaughter in the municipal abbatoir of Cali. *Trypanosoma theileri*-infected serums were used in the IFAT utilizing *T. theileri* antigen to determine the homologous titer.

Trypanosoma evansi positive serum was obtained from the previously described *T. evansi*-infected calves A and B at various intervals throughout the course of infection. Homologous IFAT titers were determined for each sample using *T. evansi* antigen prepared from infected rat blood. Only serums with titers of 1:100 or greater were used in cross reactivity tests.

The above serums were diluted 1:50 and 1:100 and examined for non-specificity and cross reactivity in the IFAT utilizing 2 different lots of *T. vivax* IFAT antigen. Confirmation of these results was made by randomly mixing the above samples with known *T. vivax* positive serums. The test was then read and the results recorded.

Persistence of Maternal Antibody

Passive immunity studies were made using available serums obtained from calves born on the ICA station at Turipana, Córdoba, Colombia. Serums were obtained from the calves on the day of birth and on subsequent days at various intervals. Serum from the

dams of the calves was not available. Prior to IFAT examination, the bovine serums had been stored at -20 C in excess of 2 years. Trypanosoma vivax IFAT status was recorded as positive or negative based on a serum dilution of 1:100.

Use of Blood Saturated Filter Paper Discs as Field Samples in Indirect Fluorescent Antibody Test

Sample Preparation

Blood was collected onto Whatman #1 filter paper by allowing a single drop to fall approximately 5 cm. onto horizontally held filter paper. Following the complete absorption of the blood drop into the paper, the sample was vigorously waved in the air to hasten drying. The size of the sample was regulated by the number of blood drops collected. Source information was written in pencil on the unused portion of the filter paper. Dried collected samples were placed in polyvinyl bags containing desiccant and stored at -20 C until used. Samples to be tested were obtained by removing a disc 6 mm. in diameter with the aid of a paper punch from the center of each blood spot.

Determination of Dilution Factor

A sample set comprised of a filter paper blood sample and whole serum sample was obtained from the same syringe of blood obtained from each of 7 calves. The equivalent dilution that a 6 mm. in diameter disc would yield when placed in 0.1 ml. of pH 7.5 PBS was determined in triplicate by placing one 6 mm. in diameter disc in each of 3 wells in a disposable microtiter tray.[q] The addition of buffer to each well was facilitated by using a Biopette automatic pipette.[r] The tray was covered with a plastic sheet to prevent evaporation and placed in a refrigerator at 4 C overnight. The following morning each disc was swirled in its well with the aid of a small hypodermic needle to produce a homogeneous sample. The eluates from each sample set were removed and pooled.

The total protein content of each pooled eluate and whole serum sample was determined by measuring the optical density of the samples via a Beckman DB-G spectrophotometer[s] at 280 mn. The mean of duplicate

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[q]Cook Engineering, Alexandria, Va.

[r]Schwarz/Mann, Orangeburg, N.Y.

[s]Beckman Instrument Co., Fullerton, Calif.

readings was used for protein concentration determination employing a standard curve.

The percentage of albumin for each pooled eluate and whole serum sample was determined electrophoretically, separating the serum components on cellulose polyacetate membranes and preparing the membranes for densitometric analysis via a Gelscan automatic recording and integrating scanner[t] in accordance with the methods described in the Gelman manual.[3] The mean of 3 albumin determinations per sample was used for the albumin concentration calculations.

The albumin concentration of each pooled eluate and whole serum sample was derived by multiplying the total protein concentration of a sample by the percent albumin of that sample. The ratio of pooled eluate albumin concentration to whole serum albumin concentration was determined for each of the 7 sample sets. The dilution factor was derived by calculating the mean of the sum of the reciprocals of the above ratios. Eluates from field samples were prepared for use in the IFAT in the manner described above with the exception that the eluent was increased to 0.125 ml.

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[t]Gelman Instrument Co., Ann Arbor, Mich.

Comparison of Indirect Fluorescent Antibody Test to Thick Blood Smear Technique for Detecting *Trypanosoma vivax*-Infected Cattle

The IFAT positive results from 3 separate field surveys involving 471 cattle were compared to the results obtained by examining 100-x1000 fields of thick blood smears obtained from corresponding cattle for *T. vivax* organisms. The identities of all samples were coded to eliminate bias.

Comparison of Eluates from Blood Saturated Filter Paper Discs and Conventional Serum Samples in Indirect Fluorescent Antibody Test

Matching conventional serum and filter paper blood eluates were collected from the cattle of the 3 field surveys above. The blood paper samples from surveys I and II were stored at -20 C for 10 and 7 days respectively before eluate was produced. Similar samples from survey III were stored at 4 C for 73 days before use.

A comparison was made of the positive IFAT results derived from homologous eluate and conventional serum samples within each of the 3 surveys. Conventional IFAT serums were diluted 1:100. In addition, the percent agreement and disagreement between both sample types were calculated in surveys I and II. Agreements

were considered to be any of the following combinations of IFAT results from homologous eluate and conventional serum samples: positive/positive, negative/negative and positive/suspicious. Each category of agreements was tabulated separately and the total number of agreements calculated. Disagreements consisted of the positive/negative combinations. The identities of all samples were coded to eliminate bias.

Trypanosoma vivax Survey of Colombian Cattle Using Indirect Fluorescent Antibody Test

The IFAT for the detection of *T. vivax* positive cattle was conducted on 2,053 serum samples collected from Colombian cattle of all age groups from 11 different departments in Colombia. Four hundred and seventy-one of these samples were collected by the author and the remainder were obtained from the CIAT and Texas A & M serum bank. All samples were stored at -20 C until used. Dates of collection and IFAT results are presented in Table 15.

The location of each sample site was identified by a site number. Site numbers that were repeated represent more than one sampling as indicated by the date. Climate, altitude and average annual rainfall were given for each sample site.[3] Herd size was given

when the information was available. The herd sizes recorded may vary ± 10%. Sample description was included when known. Test serums were diluted 1:100 and graded positive, suspicious or negative. Suspicious reacting serums were re-tested and considered negative unless they exhibited bright intense fluorescence.

The differences in incidence of IFAT positive cattle within the sex and age classes were analyzed via the chi square test.

Photography

Photomicrographs of fluorescing trypanosomes were taken utilizing the Leitz orthomat automatic camera[u] supplied with Kodak Tri-X-Pan[v] ASA 400, black and white film used at an exposure index of 800. Photographs of fluorescing and non-fluorescing trypanosomes were taken automatically and at pre-determined exposures. Pre-determined exposures were ascertained by timing the exposure for a field of trypanosomes fluorescing at maximum intensity and terminating subsequent exposures of trypanosomes at lower intensities at the same exposure.

Standard photomicrographs of trypanosomes were

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[u]Ernst Leitz (Canada) Ltd., Midland, Ontario.
[v]Eastman Kodak, Rochester, N.Y.

taken automatically using a green filter and Kodak Panatomic X, ASA 32, black and white film exposed at an exposure index of 20.

Kodak Tri-X-Pan and Panatomic X films were developed in UFG developer[w] diluted 1:1 at 24 C for 6 minutes and 2½ minutes respectively. Three successive rinses in filtered tap water were used in place of a stop bath and the developed film was fixed in full strength Kodak rapid fixer for 3 minutes.

Prints were made on Kodak Kodabromide F-5 single weight paper with a Leitz Focomat 1C enlarger. Care was taken to ensure that the focal distance of the enlarger remained constant throughout the printing of photomicrographs of thin and thick blood smear preparations of T. vivax as well as during the printing of fluorescing T. vivax photomicrographs. The prints were developed in Kodak Dektol developer diluted 1:1 for 2 minutes at ambient temperature. The ambient temperature fluctuated between 20.4 and 26.6 C. A stop bath of 2.0% acetic acid for a minimum time of 2 minutes was employed, followed by fixation in Kodak rapid fixer diluted 1:1 for a minimum time of 10 minutes. Prints

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[w]Ethol Chemicals, Inc., Chicago, Ill.

were then placed in a shallow pan and washed in circulating tap water for a minimum of 6 hours. The washed prints were then dried with a glossy surface.

RESULTS

Parameters

Wet mount examination (WME) of blood provided a rapid means by which to evaluate the parasitemias of the T. vivax-maintenance calves (Figs. 1 through 9). Motility of the trypanosomes decreased with time following collection making their presence more difficult to detect. Thick blood smear examination and rat inoculation were the only methods that detected parasitemias in T. evansi-infected cattle. No T. evansi parasitemias were detected with the capillary tube method.

Packed cell volume (PCV) and rectal temperature recordings provided an adequate indication of the health of individual T. vivax-maintenance calves. Following the inoculation of the calves with T. vivax organisms, an average reduction of 49.98% occurred in the pre-inoculation PCV values. The average time required for this decrease to occur was 21 days post inoculation (PI), ranging from 14 to 34 days. At no time after these periods were lower PCV values observed. All T. vivax-maintenance calves, with the exception of calf 2 (Fig. 2), exhibited a slow gradual return

Fig. 1. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 1. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood.

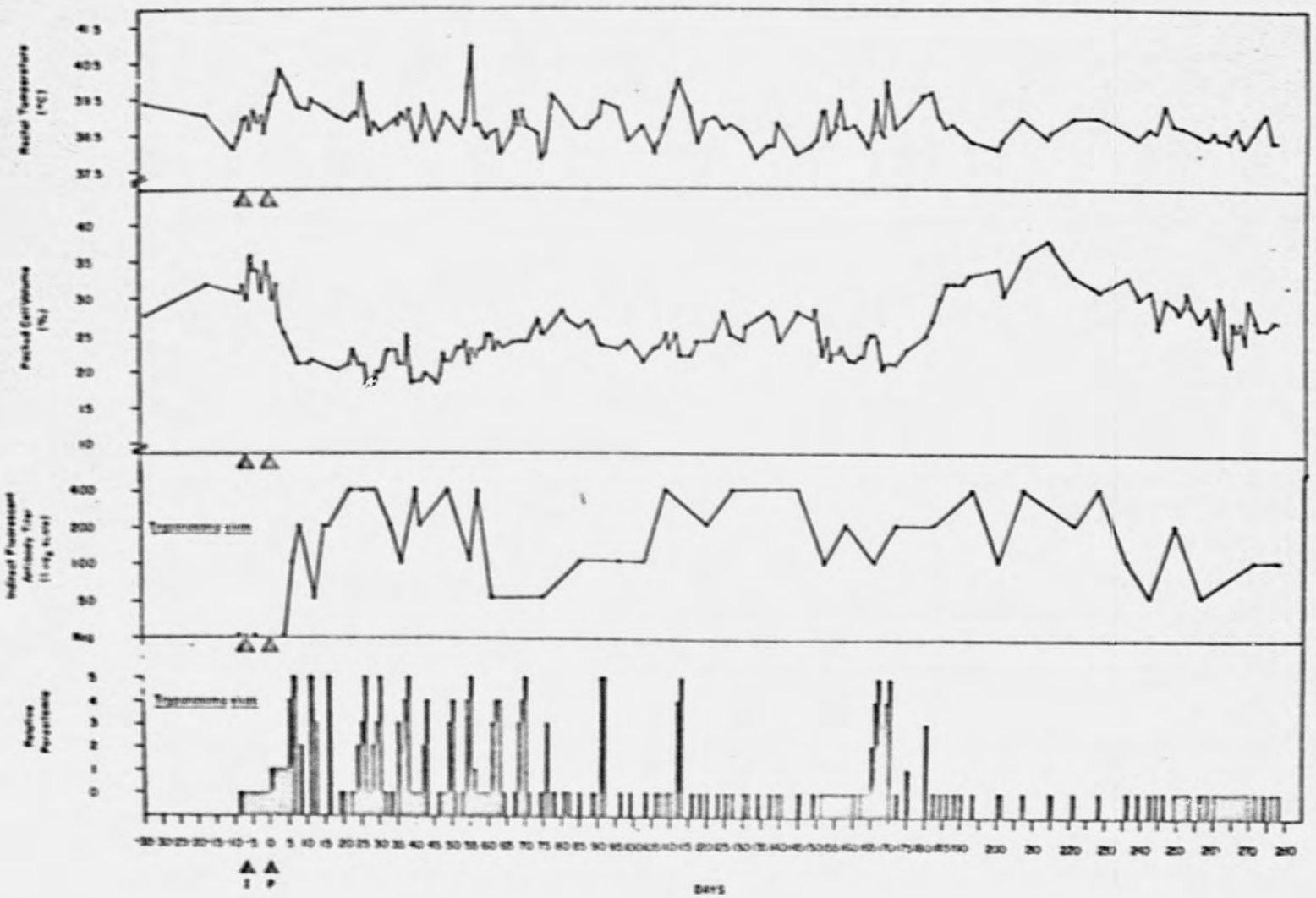

Rectal Temperature (°C)
Packed Cell Volume (%)
Indirect Fluorescent Antibody Titer
Trypanosoma vivax
Trypanosoma vivax
Relative Parasitemia
I
P
DAYS

Fig. 2. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 2. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood.

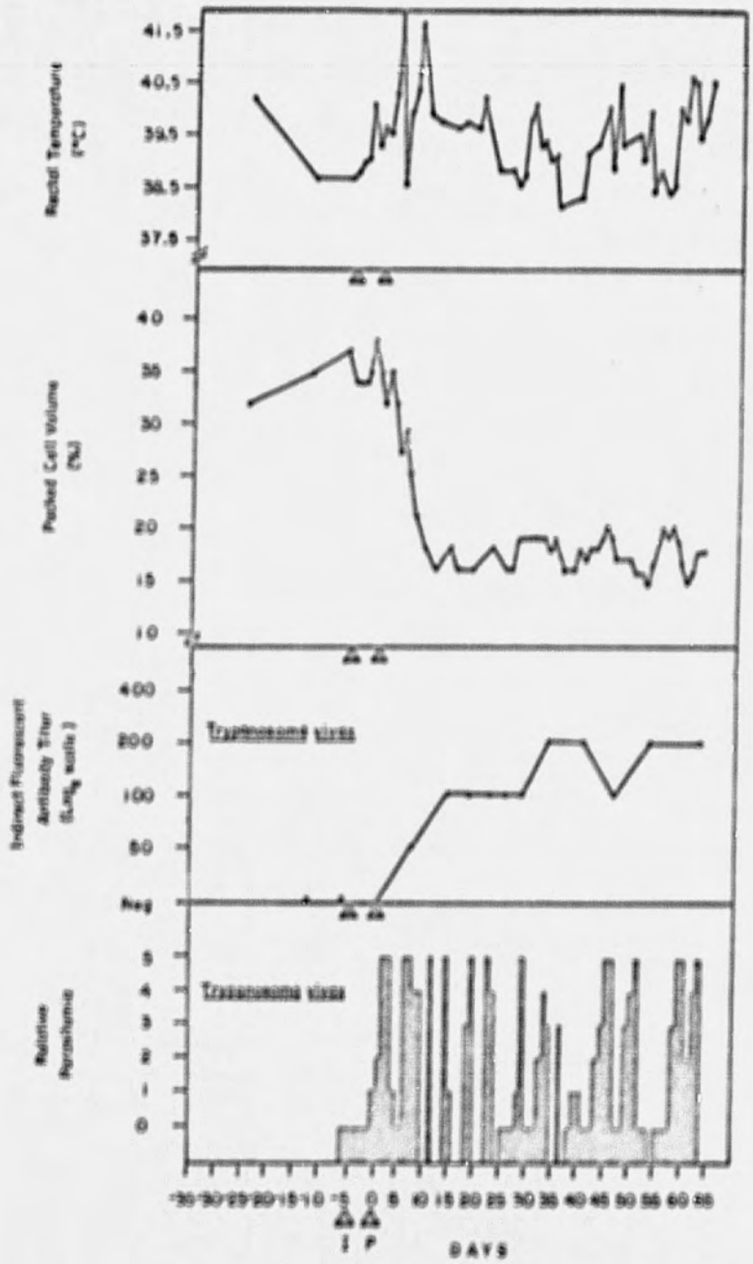

Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell Volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody Titer
400
200
100
50
Neg
Transmissible virus
Relative Parasitemia
5
4
3
2
1
0
Transmissible virus
-30 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65
I P
DAYS

Fig. 3. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 3. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood.

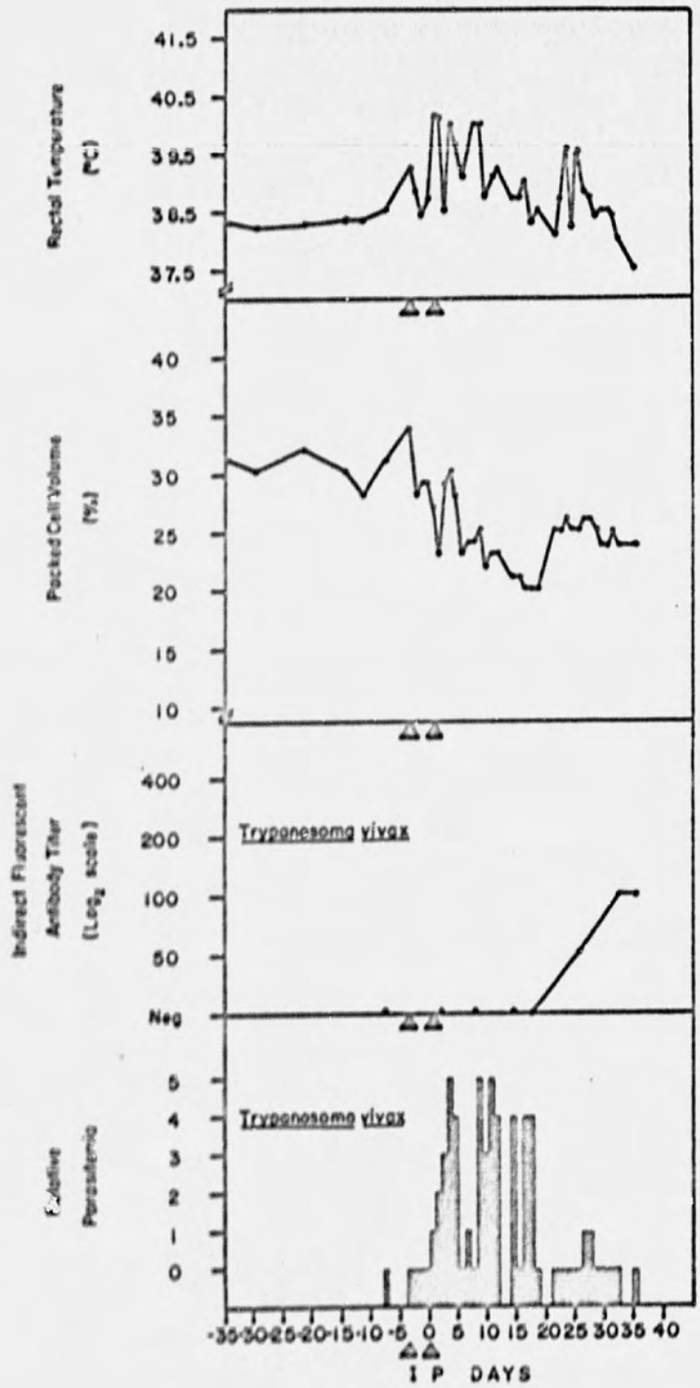

Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell Volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody Titer (Log2 scale)
400
200
100
50
Neg
Trypanosoma vivax
Relative Parasitemia
5
4
3
2
1
0
Trypanosoma vivax
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40
I P
DAYS

Fig. 4. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 4. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood.

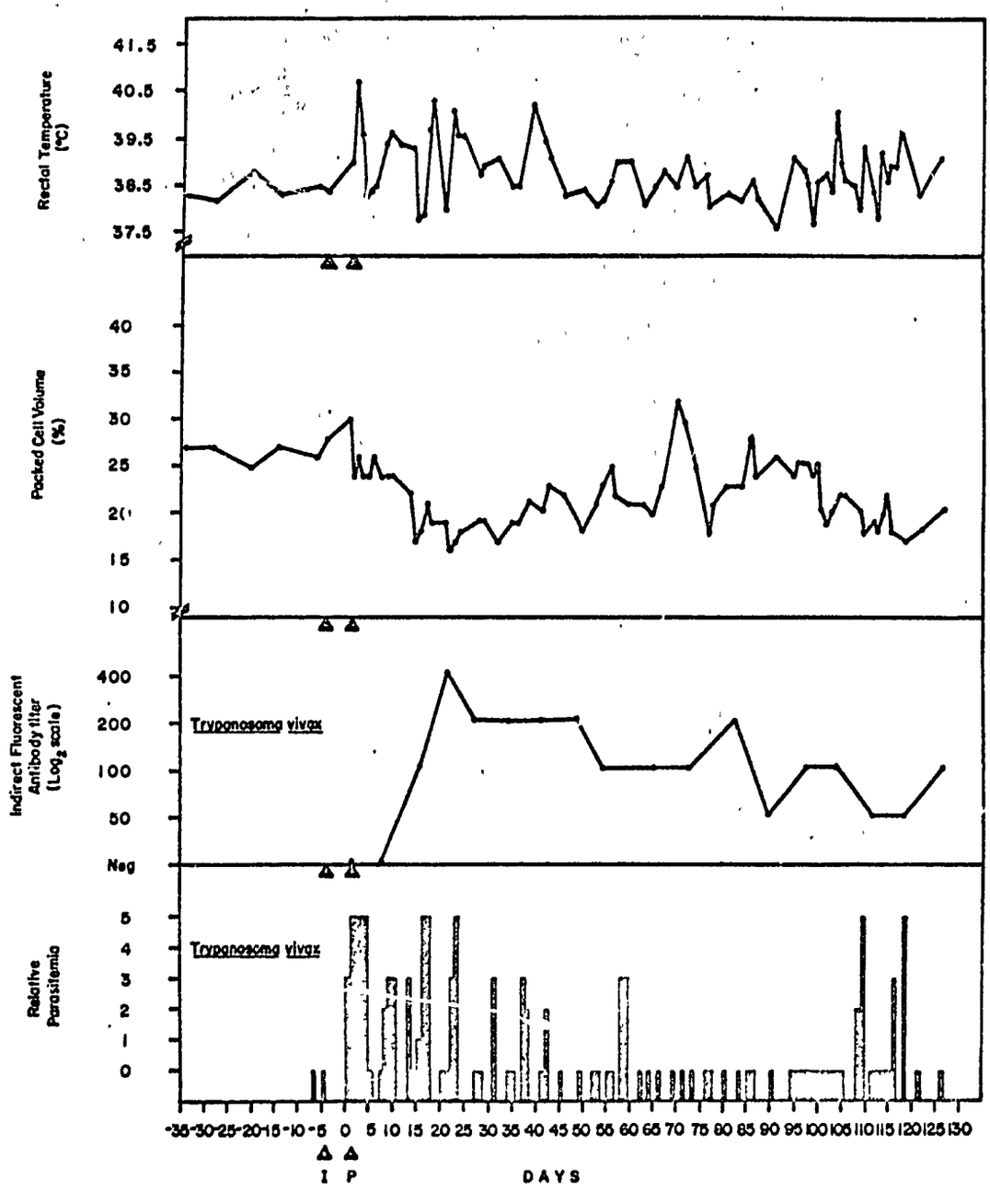

Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell Volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody titer (Log2 scale)
400
200
100
50
Neg
Trypanosoma vivax
Relative Parasitemia
5
4
3
2
1
0
Trypanosoma vivax
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130
I P
DAYS

Fig. 5. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 5. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood, S = suspicious IFAT reaction.

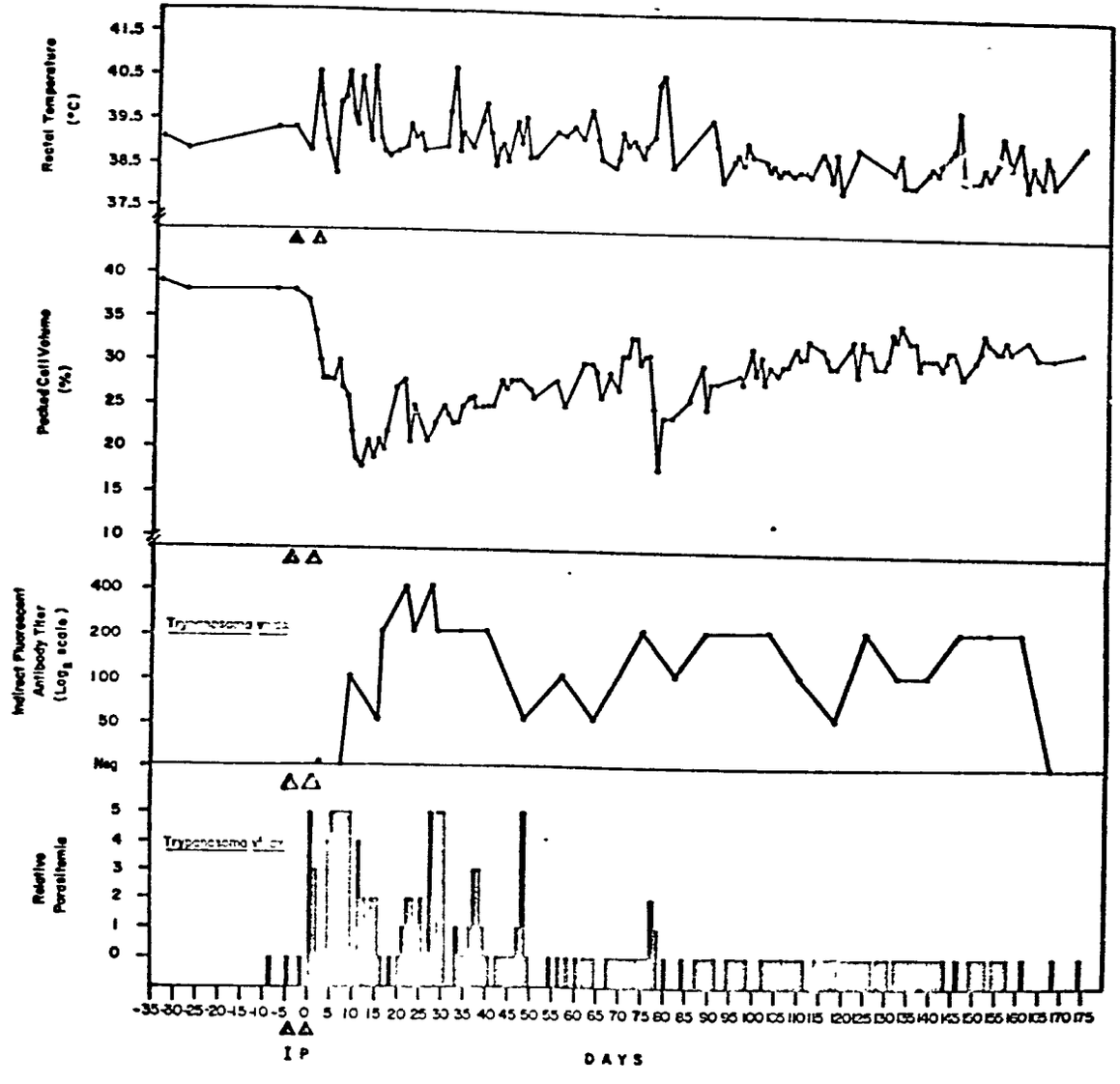

Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell Volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody Titer (Log2 scale)
400
200
100
50
Neg
Relative Parasitemia
5
4
3
2
1
0
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175
I P
DAYS

Fig. 6. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 6. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood.

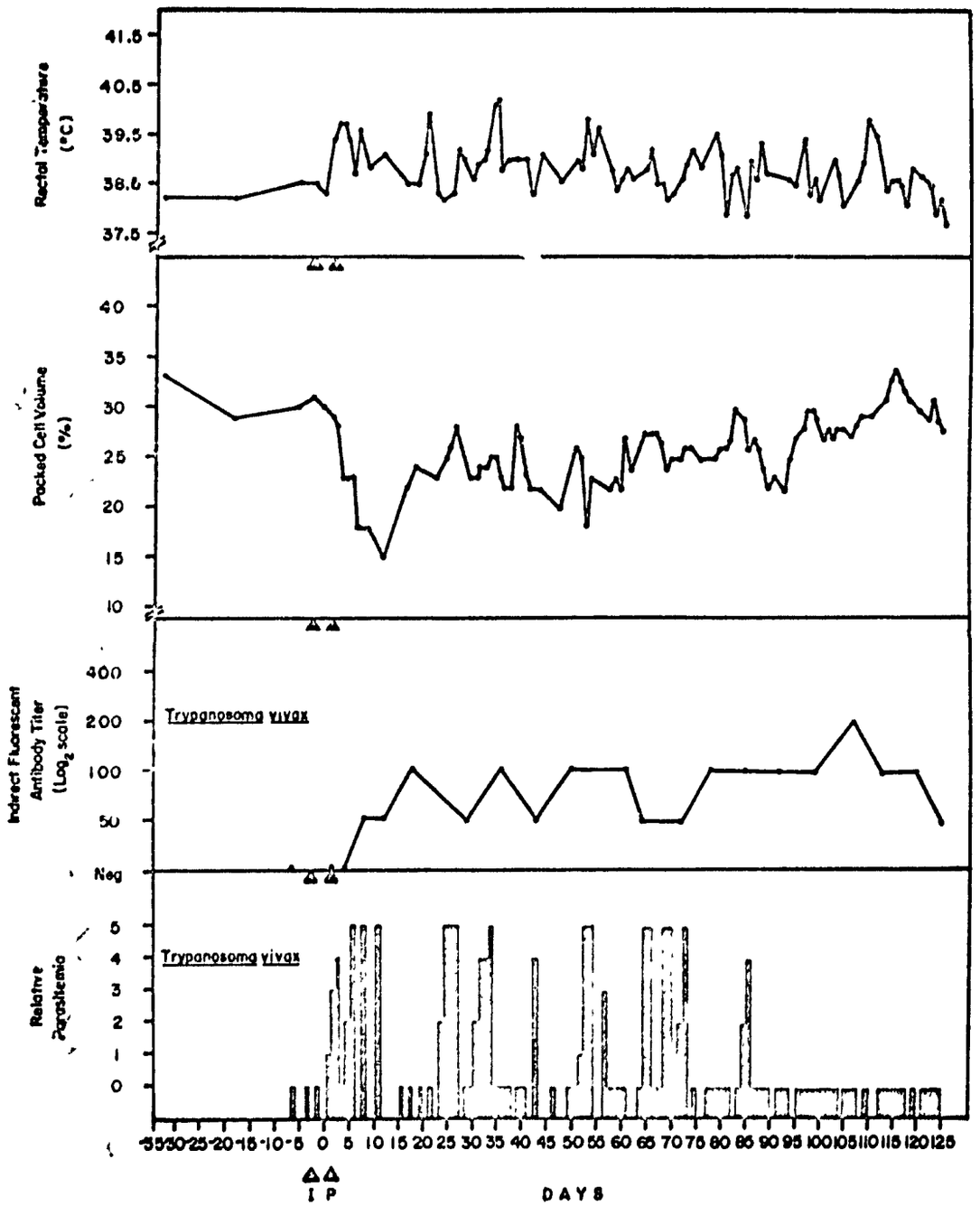

Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell Volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody Titer (Log_2 scale)
400
200
100
50
Neg
Trypanosoma vivax
Relative Parasitemia
5
4
3
2
1
0
Trypanosoma vivax
-35 30 25 20 15 10 5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125
I P
DAYS

Fig. 7. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 7. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood.

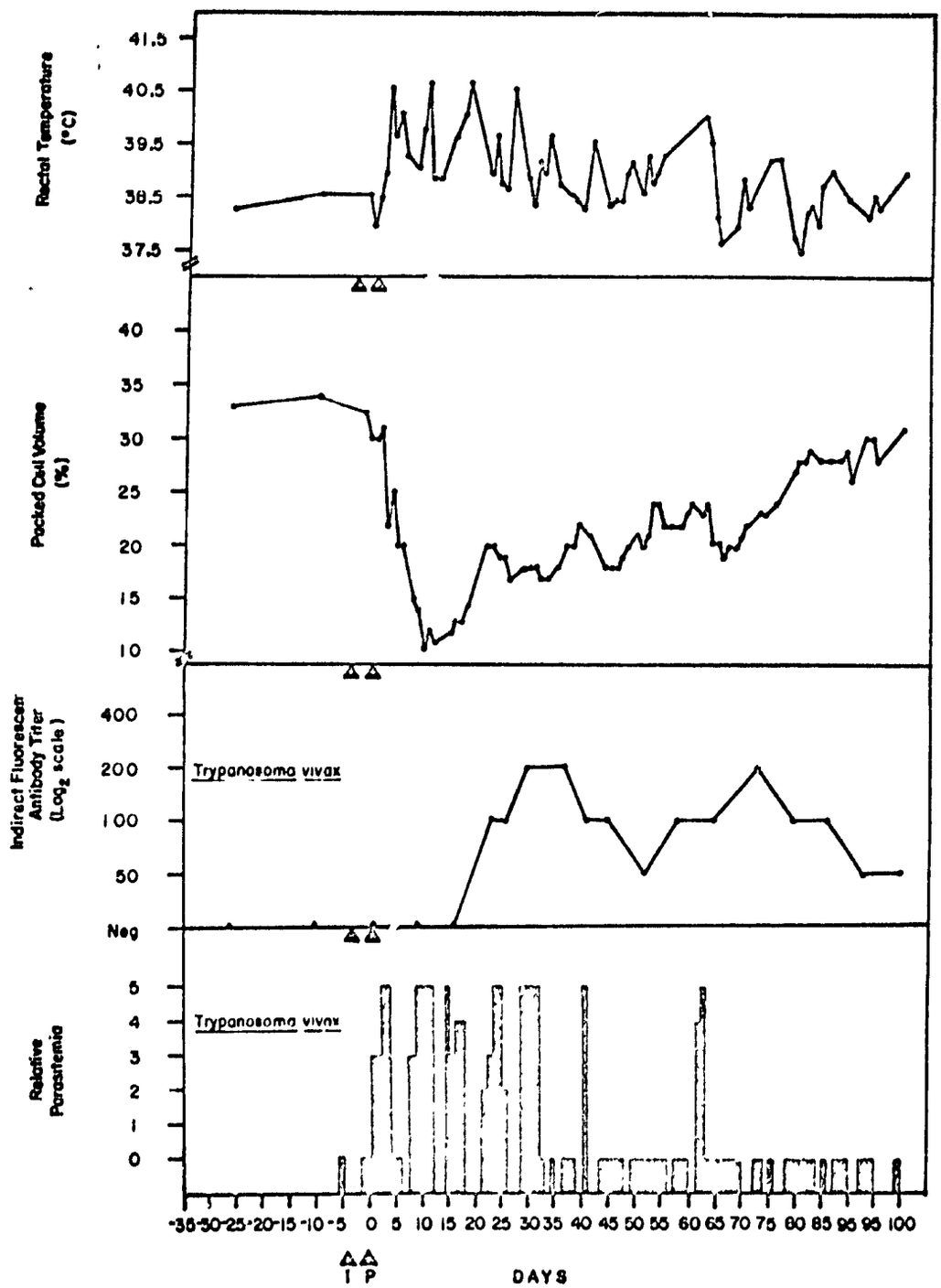

Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell Volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody Titer (Log2 scale)
400
200
100
50
Neg
Trypanosoma vivax
Relative Parasitemia
5
4
3
2
1
0
Trypanosoma vivax
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 95 95 100
I P
DAYS

Fig. 8. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 8. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood.

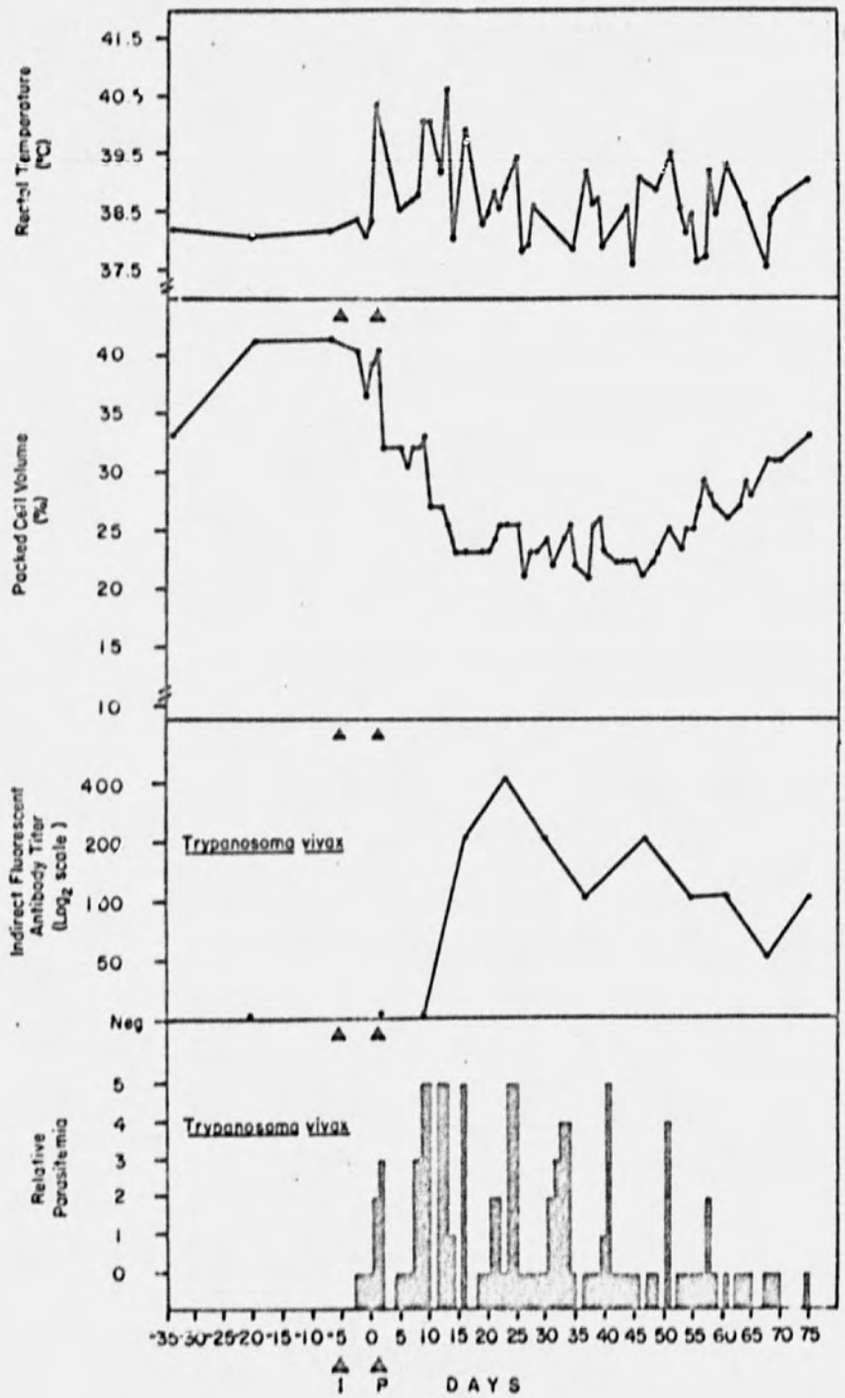
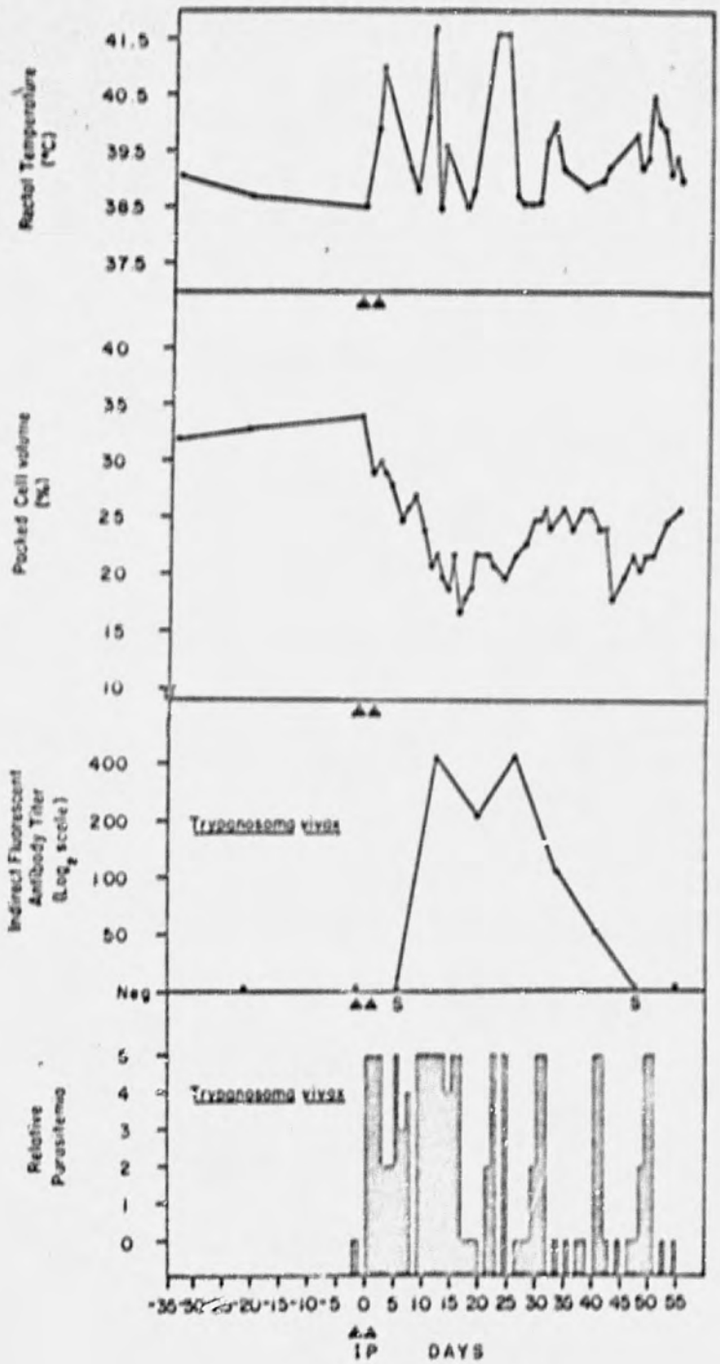

Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell Volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody Titer (Log_2 scale)
400
200
100
50
Neg
Trypanosoma vivax
Relative Parasitemia
5
4
3
2
1
0
Trypanosoma vivax
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
I P
DAYS

Fig. 9. Clinical, serologic and hematologic data of *Trypanosoma vivax* maintenance calf 9. I = Day of inoculation, P = day of first detectable parasitemia in peripheral blood, S = suspicious IFAT reaction.


Rectal Temperature (°C)
41.5
40.5
39.5
38.5
37.5
Packed Cell volume (%)
40
35
30
25
20
15
10
Indirect Fluorescent Antibody Titer (Log_2 scale)
400
200
100
50
Neg
Trypanosoma vivax
S
S
Relative Parasitemia
5
4
3
2
1
0
Trypanosoma vivax
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55
IP
DAYS

toward normal PCV values.

Some marked temporary periodic decreases did occur in PCV values during this upward trend. Calf 4 (Fig. 4) had a decrease in PCV from 25 on day 99 to 20 on day 107 and on day 108 a +2 parasitemia was observed. The PCV value of calf 5 (Fig. 5) decreased from 31 on day 75 to 25 on day 76 and on day 77 a +2 parasitemia was observed.

No consistent relationship was observed between the degree of parasitemia and rectal temperature. The highest rectal temperatures were observed during the initial phases of infection.

Experimental Organisms

Trypanosoma vivax

The morphological characteristics of the strain of T. vivax studied varied depending on the stage of parasitemia. Early and ascending phases of parasitemias were characterized by uniform slender types with tapering posterior ends and distinct sub-terminal kinetoplasts. The undividing nucleus of these forms was elongated and took up the entire width of the organism.

In stable and descending phases of parasitemias, the narrow form as well as intermediate and chronic forms were present. The typical chronic form was

characterized by a diminishing tapering width from the posterior to anterior end. The posterior end was large and blunt with a sub-terminal to terminal kinetoplast. The undividing nucleus was nearly spherical and did not take up the entire width of the organism.

Morphological features of acute, intermediate and chronic forms of T. vivax obtained from a calf one day after the parasitemia had reached its peak are shown in Fig. 10. The appearance of T. vivax in thick blood smear preparations is illustrated in Fig. 11.

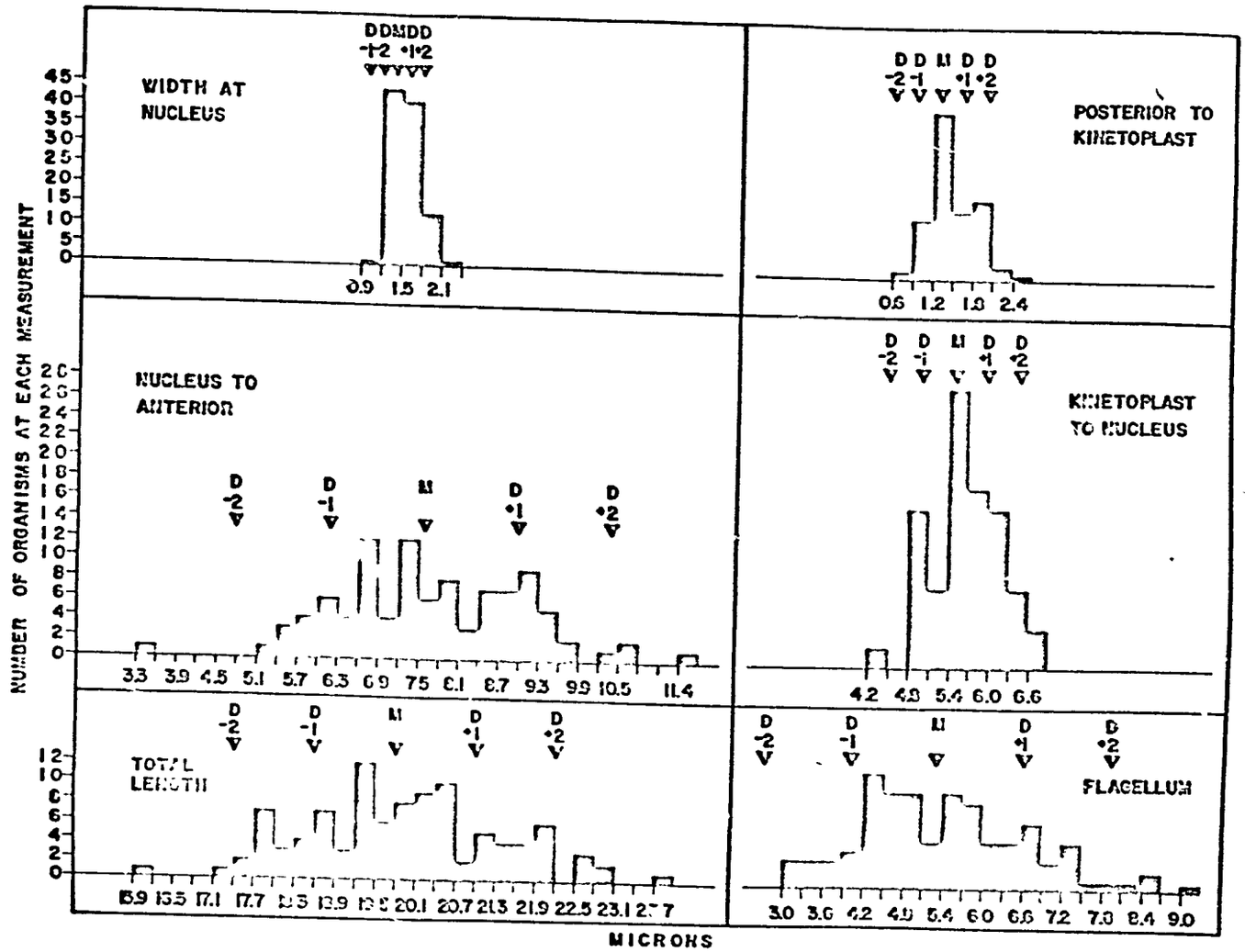
The mean length of 100 acute form T. vivax organisms in thin blood smear was 19.8 ± 1.50 microns. Other mean measurements were as follows: posterior extremity to mid-kinetoplast (PK), 1.35 ± 0.35 microns; mid-kinetoplast to mid-nucleus (KN), 5.53 ± 0.53 microns; mid-nucleus to anterior extremity (NA), 7.58 ± 1.36 microns; free flagellum (F), 7.58 ± 1.29 microns. The width at the nucleus (W) measured 1.41 ± 0.22 microns. Figure 12 presents the measurement distributions of the above. The coefficients of variability of the above anatomical measurements were: length, 0.76; PK, 0.259; KN, 0.096; NA, 0.179; F, 0.170; W, 0.156.

Thick and thin blood smear examinations for T. vivax-maintenance and antigen source calves were

Fig. 10. Various forms of Trypanosoma vivax organisms as seen in thin bovine blood smears prepared one day after the parasitemia had reached its peak. A = Acute forms used for measurement determination, I = intermediate form, C = chronic form. Giemsa; x 1800.

Fig. 11. Trypanosoma vivax as seen in a thick smear preparation. Giemsa; x 1800.

Fig. 12. Measurement distribution of 100 *Trypanosoma vivax* organisms taken from an ascending parasitemia in an experimental calf. D = Standard deviation, M = mean.


WIDTH AT NUCLEUS
POSTERIOR TO KINETOPLAST
NUCLEUS TO ANTERIOR
KINETOPLAST TO NUCLEUS
TOTAL LENGTH
FLAGELLUM
NUMBER OF ORGANISMS AT EACH MEASUREMENT
MICRONS

negative for Anaplasma marginale, Babesia bigemina and Babesia argentina throughout the course of the study.

Parasitemias in T. vivax maintenance calves, as determined by WME, were generally frequent in occurrence during the initial weeks following infection. During the first 60 days post parasitemia (PP), 5 calves which were on experiment for periods of time ranging from 99 to 278 days PP (Figs. 4, 7, 8, 9 and 10) exhibited circulating trypanosomes in 53.7% of 218 WME. From day 61 PP until experiment termination, 10.7% of 280 WME of blood from these calves revealed trypanosomes. Long term low temperature storage of T. vivax was unsuccesful as determined by stabilate examination after 5 months of storage.

Trypanosoma evansi

Time required for parasitemias to develop in rats injected with infected blood or stabilates appeared to be directly related to the number of organisms in the inoculating dose. Rats inoculated with 0.3 ml. of blood containing an average of 1 trypanosome per x 400 wet mount field developed parasitemias within 1 day. Visible parasitemias required 10 to 17 days to develop in rats inoculated with 3 ml. of blood from T. evansi

infected cattle in which trypanosomes were not observed by WME.

Storage of T. evansi in dry ice and liquid nitrogen was successful. Stabilates were viable and infective after 8 and 10 months storage respectively.

Patent infections of T. evansi were found to persist in T. evansi-infected calves A and B, 143 and 128 days respectively via rat inoculation. No signs of illness were observed in either calf during these periods.

Trypanosoma theileri

Maintenance of T. theileri in blood culture media was successful. The strain used for indirect fluorescent antibody test (IFAT) antigen production was maintained for 6 weeks before being discarded.

Indirect Fluorescent Antibody Test

Antigen Production

Twenty-three of 30 (70.6%) rats were successfully infected with T. vivax by inoculating them with infected bovine blood. Four of these 23 (17.4%) rats had parasitemias suitable for IFAT antigen production. Fourteen of 23 (60.9%) first rat to rat passages were successful

but only 3 of 14 (21.4%) rats had parasitemias suitable for antigen production. Three of 5 (60%) second rat to rat passages, 3 of 3 (100%) third rat to rat passages and 2 of 2 (100%) fourth rat to rat passages were successful but did not have suitable parasitemias for antigen production.

Attempts to establish a fifth rat to rat passage were unsuccessful. Of the 3 rats with T. vivax parasitemias suitable for antigen production, only 1 possessed a blood trypanosome concentration capable of yielding an antigen preparation containing more than 20 trypanosomes per x 540 field.

Sheep infected with T. vivax developed low levels of parasitemias which were usable for IFAT antigen but undesirable due to the small number of trypanosomes in the microscope field.

The most satisfactory source of T. vivax IFAT antigen was the calf. Trypanosoma vivax-antigen calf A had a +3 relative parasitemia at 42 hours PI. At 64 hours PI, an average of 6 trypanosomes per thin stained field was recorded per x 100 oil-immersion lens with x 10 oculars. At 66 hours PI, the average number of trypanosomes per field increased to 15 and IFAT antigen was prepared. Antigen production was feasible up to

156 hours PI at which time the condition of the calf required euthanasia. However, varying degrees of agglutinated and individual trypanosomes which fluoresced non-specifically were observed with increasing frequency after production of the first antigen batch rendering subsequent batches undesirable but usable (Fig. 13).

Trypanosoma vivax-antigen calf B had a +5 relative parasitemia at 41 hours PI. A parasitemia of 5 trypanosomes per thin field was recorded at 64 hours PI. At 69 hours PI, the average number of trypanosomes increased to 12 per field and antigen was prepared. The parasitemia continued to rise to greater than 30 trypanosomes per field by 93 hours PI at which time considerable agglutination of trypanosomes with non-specific fluorescence was present. By 118 hours PI, the parasitemia had decreased to 11 trypanosomes per field with agglutination present and the calf was euthanatized. Figure 13 illustrates non-specific fluorescence of agglutinated T. vivax organisms.

Separation of bovine erythrocytes from T. vivax using phytohemagglutin and also differential centrifugation was unsatisfactory. The concentration of trypanosomes in eluates from DEAE-cellulose columns was

Fig. 13. Fluorescing agglutinated *Trypanosoma vivax* organisms subjected to negative *T. vivax* calf serum as seen in the indirect fluorescent antibody test. Dark field. x 800.

sufficient for suitable antigen production. However, all fractions of the eluate containing trypanosomes were found to contain bovine serum globulin components.

Trypanosoma evansi parasitemias suitable for IFAT antigen production were observed in rats 24 to 48 hours PI. In ord r to obtain *T. theileri* suspensions suitable for IFAT antigen production, the contents of 6, one-week old cultures (containing approximately 1.5 ml. of liquid phase each) were concentrated into 1 ml. of PBS.

Antigen Processing, Fixation, Storage and Evaluation

Fixation of bovine origin *T. vivax* IFAT antigen with absolute acetone:methanol::60:40 at -20 C for 30 minutes gave the best results. Adherence of antigen to the slide during staining was excellent. Erythrocytes maintained their color giving good visibility of the antigen during staining and the microscopic field was clear with a minimum of background fluorescence. The morphological features of the trypanosomes were excellent. When fixation time was decreased to 15 minutes the results were equally good except that adherence to the glass slides was not as consistent.

Heat fixation produced an undesirable film over the field and disrupted erythrocytes and trypanosomes.

Staining decolorized the erythrocytes making visualization of antigen during staining difficult. Excessive background fluorescence interferred with the interpretation of suspicious reactions.

Buffered formalin at all concentrations gave results similar to those of heat fixation. Background fluorescence was more of a problem than that observed in heat fixed preparations especially at a formalin concentration of 10%.

Fixation according to the method described by Stalheim[23] produced good adherence. However, erythrocytes were decolorized during staining and the field was consistently covered by a thin opaque film.

Trypanosoma vivax-antigen lots A and B were used successfully in the IFAT after storage at -35 C and -70 C for 144 and 116 days respectively.

Trypanosoma evansi IFAT antigen fixed in acetone: methanol::60:40 for 15 and 30 minutes at -20 C exhibited the same qualities as T. vivax IFAT antigen fixed in an identical manner for 30 minutes at -20 C. Non-specific and background fluorescence did not occur.

Trypanosoma theileri IFAT antigen did not adhere well to glass slides when the organisms were suspended in phosphate buffered saline (PBS) alone. However,

adherence was markedly improved when equal parts of trypanosome suspension and rat blood were mixed. Autofluorescence occurred consistently.

Execution of the Indirect Fluorescent Antibody Test

Test positions on the antigen slides were examined in numerical order. Reactions were given 1 of 3 different grades: negative, suspicious and positive. All suspicious reactions were re-tested. Figure 14 is typical of a negative serum reaction. Figure 15 represents the minimum degree of fluorescence considered suspicious. Figure 16 represents the minimum degree of fluorescence considered positive. Figure 17 illustrates maximum fluorescence.

Evaluation of Indirect Fluorescent Antibody Test

Sensitivity

Figure 18 illustrates IFAT serum titers of 100 or greater which were observed 7 days PP in calves singularly infected with a Huila strain of T. vivax. Of 7 calves sampled at this time, 2 were suspicious at serum dilutions of 1:50, 2 had titers of 1:50 and 3 had titers of 1:100 or greater. A geometric mean titer of 1:164 was calculated from the results of 5 samples

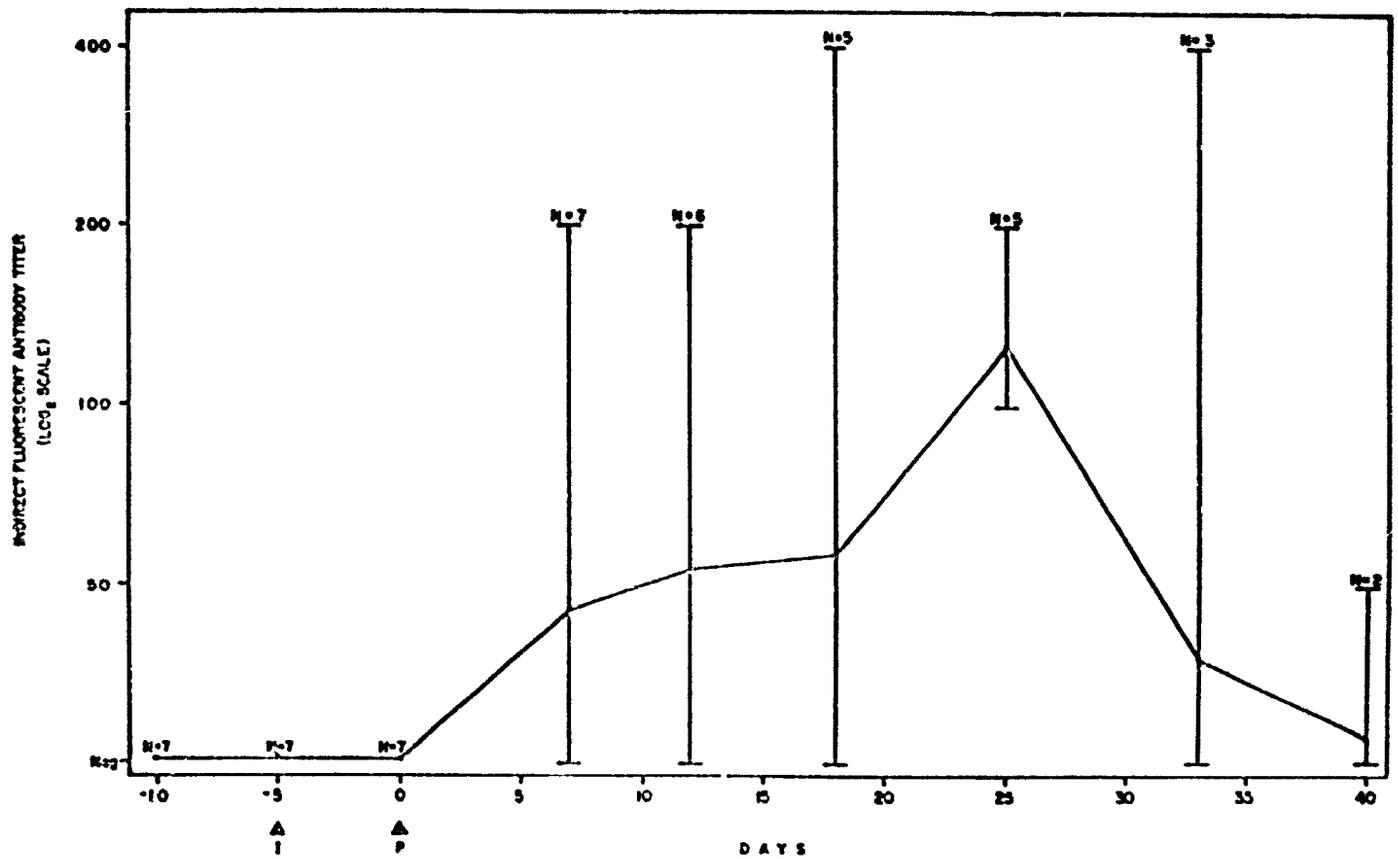
Fig. 14. Trypanosoma vivax organisms as seen in negative antibody test reactions. Dark field. x 800

Fig. 15. Trypanosoma vivax organisms fluorescing at the minimum suspicious level as seen in the indirect fluorescent antibody test. Dark field. x 800.

Fig. 16. Trypanosoma vivax organisms fluorescing at the minimum positive level as seen in the indirect fluorescent antibody test. Dark field. x 800

Fig. 17. Maximum positive fluorescence of Trypanosoma vivax organisms as seen in the indirect fluorescent antibody test. Dark field. x 800

Fig. 18. Geometric means and ranges of indirect fluorescent antibody test *Trypanosoma vivax* titers of serums collected from calves used for a sequential pathogenesis study of *T. vivax*. N = Number of calf serum samples available, I = day of inoculation, P = day of first detectable parasitemia in peripheral blood.


INDIRECT FLUORESCENT ANTIBODY TITER
(LOG2 SCALE)
400
200
100
50
N=2
N=7
N=7
N=7
N=7
N=6
N=5
N=5
N=3
N=2
-10
-5
0
5
10
15
20
25
30
35
40
I
P
DAYS

observed on day 25 PP.

The 164 IFAT T. vivax titer determinations of serums collected from the T. vivax-maintenance calves as seen in Figs. 4 through 12 were made on serum samples collected at an average interval of 6.3 days. The shortest and longest time period before a titer of 1:100 or greater was observed was 6 and 32 days PP respectively with a mean of 15.9 days PP. The highest titer observed throughout the course of this study was 1:400. Maximum titers developed with one exception, in 14 to 33 days PP, averaging 23.4 days PP. Calf 6 (Fig. 6) showed its maximum titer on day 109 PP.

Of the 133 serum samples obtained from the maintenance calves after the first titer of 1:100 was observed in each calf, 86 of 107 (80.4%) samples had titers of 1:100 or greater. Twenty-three (17.3%) titers were 1:50. Of these 23 titers, 18 (78.3%) were suspicious at serum dilutions of 1:100. Three (2.3%) of the 133 serums titered were negative, 2 of which gave suspicious reactions at serum dilutions of 1:50.

Titers for T. vivax were detected by the IFAT for up to 278 days PP. Sudden decreases were observed. Calf 9 exhibited a rapid decline in IFAT titer from a

high of 1:400 on day 26 PP to negative on day 47 PP in the presence of circulating trypanosomes. A dramatic rapid drop in titer was observed in calf 5 but in the absence of a visible parasitemia.

Repeatability

Limited repeatability studies indicated that the probability of repeating titers within ± 1 dilution was: a) 82.1 ± 11.8% using the same antigen on the same day (Table 3); b) 77.9 ± 4.5 % using the same antigen on different days (Table 3); c) 87.5 ± 8.8% using different antigen lots on the same day (Table 4).

Specificity

No cross reactivity was observed with serums collected from calves infected with A. marginale, Eperythrozoon spp., Babesia spp. or T. theileri (Tables 5 through 9). Slight evidence of cross reactivity was observed between T. vivax antigen and T. evansi positive serums, however, it was not sufficient to interfere with the IFAT for T. vivax (Table 10).

Persistence of Maternal Antibodies

Data summarized in Table 11 indicate that IFAT

TABLE 3. Probability of Repeating Indirect Fluorescent Antibody Test Titers on Same Day and Different Days Using Same Antigen Lot

Sample Number	Day 1 Titer Determinations		Day 2 Titer Determinations		Day 3 Titer Determinations	
	First	Second	First	Second	First	Second
1	200	100	100	100	200	50
2	200	200	100	100	100	100
3	100	200	200	200	50	50
4	100	50	100	100	200	100
5	400	100	400	200	50	50
6	100	50	200	50	50	100
7	200	100	100	200	50	50
8	200	200	200	50	200	200
9	200	100	200	200	800	400
10	100	100	100	100	200	200
11	200	50	200	100	100	400
12	200	50	100	50	100	50
13	100	50	100	50	50	50

Same Day Agreements[a]

Comparisons	Number Agreements	Number Observations
Day 1	10	13
Day 2	11	13
Day 3	11	13

Total Agreements = 32
Total Observations = 39
Probability of repeating IFAT titer = 82.1 ± 11.8%[b]

[a]Same day agreements equal the number of duplicate IFAT titers that are identical or do not differ by more than 1 dilution for a given day.

[b]Represents the normal approximation to the mean at the 95% confidence interval.

TABLE 3. Continued

Between Day Agreements[a]		
Comparisons	Number Agreements	Number Observations
Day 1	86	104
Day 2	82	104
Day 3	75	104

Total Agreements = 243
Total Observations = 312
Probability of repeating IFAT titer on different days = 77.9 ± 4.5%[b]

[a]Between day agreements equal number of IFAT titers that are identical or do not differ by more than 1 dilution when duplicate IFAT titers of homologous serum samples of 1 day are compared in turn to duplicate titers of same sample determined on each of 2 different days.

[b]Represents the normal approximation to the mean at the 95% confidence interval.

TABLE 4. Probability of Repeating IFAT Titers Using Different Antigen Lots

Sample Number	Antigen A Titer Determinations		Antigen B Titer Determinations	
	First	Second	First	Second
1	50	100	100	50
2	200	200	400	400
3	200	200	400	400
4	400	400	400	400
5	100	100	100	100
6	50	50	100	200
7	200	100	200	200
8	50	100	200	200
9	400	200	400	400
10	400	400	400	400
11	100	100	100	200
12	50	50	100	200
13	100	200	200	400
14	200	200	400	400

Agreements[a]

Comparisons	Number Agreements	Number Observations
F.D. Antigen A to F.D. Antigen B	13	14
F.D. Antigen A to S.D. Antigen B	10	14
S.D. Antigen A to F.D. Antigen B	14	14
F.D. Antigen A to S.D. Antigen B	12	14

TABLE 4. Continued

Total Agreements = 49

Total Observations = 56

Probability of repeating IFAT titers using different antigen = 87.5 ± 8.6%[b]

F.D. = First Determination
S.D. = Second Determination

[a]Agreements equal the number of IFAT titers that are identical or do not differ by more than 1 dilution when duplicate titers of homologous samples, determined using antigen A, are compared in turn to duplicate titers of the same samples determined by using antigen B.

[b]Represents the normal approximation to the mean at the 95% confidence interval.

TABLE 5. Indirect Fluorescent Antibody Test Results Obtained from Exposure of *Trypanosoma vivax* Antigen to Serum Collected from Calves Infected with *Anaplasma marginale*

Calf Number	*A. marginale* CF Serum Titer	*T. vivax* Antigen Lot A		*T. vivax* Antigen Lot B	
		A. marginale-positive Serum Dilutions			
		1:50	1:100	1:50	1:100
304	160	N	N	N	N
315	160	N	N	N	N
0270	80	N	N	N	N
0770	80	N	N	N	N
1070	80	N	N	N	N
1270	80	N	N	N	N

N = negative

TABLE 6. Indirect Fluorescent Antibody Test Results Obtained from Exposure of *Trypanosoma vivax* Antigen to Serum Collected from Calves Infected with *Eperythrozoon spp*.

Calf Number	*T. vivax* Antigen Lot A		*T. vivax* Antigen Lot B	
	Eperythrozoon-positive Serum Dilutions			
	1:50	1:100	1:50	1:100
53	N	N	N	N
217	N	N	N	N
228	N	N	N	N
235	N	N	N	N
247	N	N	N	N
295	N	N	N	N

N = negative

TABLE 7. Indirect Fluorescent Antibody Test Results Obtained from Exposure of *Trypanosoma vivax* Antigen to Serum Collected from Calves Infected with *Babesia argentina*

Calf Number	*B. argentina* CF Serum Titer	*T. vivax* Antigen Lot A		*T. vivax* Antigen Lot B	
		B. argentina-positive Serum Dilutions			
		1:50	1:100	1:50	1:100
318	80	N	N	N	N
317	40	N	N	N	N
770	40	N	N	N	N
1370	40	N	N	N	N
1570	40	N	N	N	N
286	20	N	N	N	N

N = negative

TABLE 8. Indirect Fluorescent Antibody Test Results Obtained from Exposure of *Trypanosoma vivax* Antigen to Serum Collected from Calves Infected with *Babesia bigemina*

Calf Number	*B. bigemina* CF Serum Titer	*T. vivax* Antigen Lot A		*T. vivax* Antigen Lot B	
		B. bigemina-positive Serum Dilutions			
		1:50	1:100	1:50	1:100
6	640	N	N	N	N
274	640	N	N	N	N
218	320	N	N	N	N
240	320	N	N	N	N
249	320	N	N	N	N
290	320	N	N	N	N
7	160	N	N	N	N
68	160	N	N	N	N
236	80	N	N	N	N
238	80	N	N	N	N

N = negative

TABLE 9. Indirect Fluorescent Antibody Test Results Obtained from Exposure of *Trypanosoma vivax* Antigen to Serum Collected from Cattle Infected with *Trypanosoma theileri*

Cow Number	Origin of Cow	*T. vivax* Antigen Lot A		*T. vivax* Antigen Lot B	
		T. theileri-positive Serum Dilutions			
		1:50	1:100	1:50	1:100
B55	Texas, U.S.	N	N	N	N
H100	Texas, U.S.	N	N	N	N
289	Texas, U.S.	N	N	N	N
299	Texas, U.S.	N	N	N	N
5583	Texas, U.S.	N	N	N	N
627[a]	Texas, U.S.	N	N	N	N
650	Texas, U.S.	N	N	N	N
726	Texas, U.S.	N	N	N	N
25	Valle, Colombia	N	N	N	N
053	Valle, Colombia	N	N	N	N
81	Valle, Colombia	N	N	N	N
307	Valle, Colombia	N	N	N	N
314	Valle, Colombia	N	N	N	N
394	Valle, Colombia	N	N	N	N

N = negative

[a]Cow 627 was positive for *Anaplasma marginale*

TABLE 10. Indirect Fluorescent Antibody Test Results Obtained from Exposure of *Trypanosoma vivax* Antigen to Serum Collected from 2 Calves Infected with *Trypanosoma evansi*

T. evansi-positive Serum Description		*T. vivax* Antigen Lot A		*T. vivax* Antigen Lot B	
		T. evansi-positive Serum Dilutions			
Days Post Inoculation	*T. evansi* IFAT Titer	1:50	1:100	1:50	1:100
		Calf 1			
67	100	S	N	N	N
88	100	N	N	N	N
91	200	S	N	N	N
102	200	N	N	N	N
108	200	N	N	S	S
116	200	N	N	N	N
122	100	N	N	N	N
130	200	N	N	S	N
143	100	N	N	N	N
150	200	N	N	N	N
		Calf 2			
47	100	N	N	N	N
55	400	N	N	N	N
60	400	N	N	N	N
65	100	S	N	N	N
70	200	N	N	N	N
77	100	N	N	N	N
97	200	N	N	N	N
104	100	N	N	N	N
124	200	N	N	N	N
138	100	N	N	N	N
145	100	N	N	N	N
152	100	N	N	N	N
159	100	N	N	N	N
160	100	N	N	N	N

N = negative
S = suspicious

TABLE 11. Maternal Antibody Study of 5 Calves Positive at 1:100 Dilution for *Trypanosoma vivax* at Birth as Determined by Indirect Fluorescent Antibody Test

Calf Number	Birth Date	Date and IFAT Results		
		4/10	5/24	9/04
9182	3/17	P	N	N
9220	3/26	P	-	N
9222	3/26	P	-	N
9229	3/26	P	N	-
9233	3/26	P	-	N

P = positive
N = negative
- = serum sample not available

T. vivax titers of 1:100 may persist as long as 24 days in newborn calves.

Use of Blood Saturated Filter Paper Discs as Field Samples in Indirect Fluorescent Antibody Test

Determination of albumin ratios in sample sets indicated that an equivalent serum dilution factor of 67.6 ± 11.1 resulted when 6 mm. filter-paper disc-blood samples were placed in 0.1 ml. of diluent (Table 12).

Table 13 summarizes the results of 3 field surveys in which the IFAT and thick blood smear technique (TBST) for the detection of *T. vivax*-infected cattle were compared. Using conventional serum samples, the IFAT was 3 and 21.6 times more effective than the TBST in surveys II and III respectively in detecting exposure of cattle to *T. vivax*. In survey I, only the IFAT detected exposed cattle. Results were similar when eluates from dried blood samples were used in the IFAT.

A high degree of agreement existed between IFAT results obtained from conventional serum and filter paper blood eluted samples. Table 13 shows complete agreement between positive IFAT results for both

TABLE 12. Results of Dilution Determinations in Filter Paper Eluate[a] and Conventional Serum Samples

Sample Number	Eluted Protein mg/ml	Eluted Albumin %	Eluted Albumin mg/ml	Serum Protein mg/ml	Serum Albumin %	Serum Albumin mg/ml	Ratio mg/ml Eluted Alb./ Serum Alb. mg/ml
1	14.0	4.8	0.67	132.7	29.0	38.5	1:57.5
2	10.0	6.0	0.60	130.0	22.4	29.1	1:48.5
3	10.2	5.7	0.58	140.7	31.6	44.6	1:76.9
4	13.9	4.7	0.65	102.5	41.6	42.6	1:65.5
5	9.8	5.6	0.55	92.5	44.7	41.3	1:75.1
6	10.5	6.5	0.68	122.6	43.5	53.3	1:78.4
7	8.7	5.5	0.48	120.6	27.6	34.1	1:71.0
						Mean	= 1:67.6[b]

[a]6 mm. dried blood saturated filter paper disc eluted in 0.1 ml. of diluent

[b]Standard deviation of the reciprocal of the mean = ± 11.1

TABLE 13. Comparison of Indirect Fluorescent Antibody Test Using Conventional Serum and Filter Paper Blood Eluate Samples to Thick Blood Smear Technique for Detection of *Trypanosoma vivax* in Cattle

	Survey					
	I		II		III	
	No.	%	No.	%	No.	%
Total cattle sampled	204	-	81	-	184	-
IFAT positive (conventional serum sample)	24	11.7	9	11.1	20	10.8
IFAT positive (eluate sample)	28	13.7	9	11.1	5	2.7
T. vivax positive thick smears	0	0.0	3	3.7	1	0.5

sample types in survey II. Results from survey I differ by only 2%. The dried blood samples of surveys I and II, from which eluates were obtained, were stored at -20 C for less than 10 days prior to use. Survey III indicated that conventional serum samples were 4 times more sensitive than blood saturated filter paper eluted samples which were stored at 4 C for 73 days prior to use. Table 14 shows a 98.5 and 100% agreement of IFAT results in surveys I and II respectively between eluted and conventional serum samples.

Trypanosoma vivax Survey of Colombian Cattle Using Indirect Fluorescent Antibody Test

The IFAT revealed the presence of *T. vivax* antibodies in the serums of cattle from the departments of Boyacá, Córdoba, Meta, Huerc and Valle. No *T. vivax* positive serums were found in cattle from Antioquia, Cauca, Caquetá, Cundinamarca, Tolima and Vichada. Cattle with *T. vivax* antibodies were found at altitudes of less than 1500 meters in temperate and hot climates with average annual rainfalls ranging from 1 to 3 meters. Holstein, Criollo, Zebu and crossbred cattle all were found to possess antibodies against *T. vivax*. Chi square analysis of the data revealed

TABLE 14. Comparison of Agreement and Disagreement of Indirect Fluorescent Antibody Test Results Obtained from Conventional Serum and Filter Paper Blood Eluate Samples

	Survey			
	I		II	
	No.	%	No.	%
Sample size	204	-	81	-
Agreement combinations				
(a) positive/positive	18	8.8	7	8.6
(b) negative/negative	169	82.8	72	88.9
(c) positive/suspicious	14	6.9	2	2.5
Total agreements	201	98.5	81	100.0
Total disagreements	3	1.5	0	0.0

that there was no significant difference between the proportion of female and male cattle with *T. vivax* antibodies. Older cattle were found more likely to have *T. vivax* antibodies than younger cattle. These data are summarized in Tables 15 and 16.

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TABLE 25. COEFFICIENTS, A SURVEY OF INTERNATIONAL DEVICE IN ORGANICAL GATTLE WITH THE INDIRECT TRANSMISSIBLE ARTERIOSIS (1965)

OPERATION[1]	DATE	NO. OF CASES	ANESTHESIOLOGY	CLEANED[2]	ANTISEPSIS IN INFECTION	ANNUAL MEAN TEMPERATURE IN INFECTION	MEAN AGE[3]	CARTILAGE LESIONS NO. %	CARTILAGE DISCOLORATION AGE IN MONTHS 0–16	17–48	≥49	0–16	17–48	≥49	CARTILAGE DISCOLORATION AGE IN MONTHS 0–16	17–48	≥49	TOTAL NO.	TOTAL %
CARDIOTIA	1967/10	30	PHENOBARBITAL	NONE	0.2%0.5	0.0%0.5	100	5	16.7	100.0	0.0	0.0	0.0	100.0	—	—	—	—	—
CARDIOTIA	1967/10	31	PHENOBARBITAL	NONE	0.2%0.5	0.0%0.5	60	3	5.7	100.0	0.0	33.3	66.7	0.0	—	—	—	—	—
CARDIOTIA	1967/10	32	PHENOBARBITAL	NONE	0.2%0.5	0.0%0.5	11	1	100.0	100.0	0.0	0.0	0.0	100.0	—	—	—	—	—
CARDIOTIA	1967/10	33	PHENOBARBITAL	NONE	0.2%0.5	0.0%0.5	36	6	16.7	100.0	0.0	0.0	0.0	100.0	—	—	—	—	—
CARDIOTIA	1967/10	34	PHENOBARBITAL	NONE	0.2%0.5	0.0%0.5	50	5	10.0	80.0	20.0	0.0	40.0	60.0	—	—	—	—	—
CARDIOTIA	1967/10	35	PHENOBARBITAL	NONE	0.2%0.5	0.0%0.5	116	26	22.4	50.0	50.0	20.0	13.3	66.7	—	—	—	—	—
CARDIOTIA	1967/10	36	HALOTHANE	NONE	0.2%0.5	>0.5	100	11	11.0	81.8	18.2	0.0	36.4	63.6	—	—	—	—	—
CARDIOTIA	1967/10	37	HALOTHANE	NONE	0.2%0.5	>0.5	75	10	13.3	80.0	18.2	9.0	18.2	72.8	—	—	—	—	—
CARDIOTIA	1967/10	38	HALOTHANE	NONE	0.2%0.5	>0.5	110	20	18.2	65.0	35.0	20.0	20.0	60.0	—	—	—	—	—
CARDIOTIA	1967/10	39	METHOXYFLURANE	NONE	0.2%0.5	0.0%0.5	19	5	26.3	80.0	20.0	0.0	0.0	100.0	—	—	—	—	—
CARDIOTIA	1967/10	40	METHOXYFLURANE	NONE	0.2%0.5	0.0%0.5	9	3	33.3	0.0	100.0	0.0	33.3	66.6	—	—	—	—	—
CARDIOTIA	1967/10	41	METHOXYFLURANE	NONE	0.2%0.5	0.0%0.5	7	4	57.1	100.0	0.0	25.0	0.0	75.0	—	—	—	—	—
CARDIOTIA	1967/10	42	METHOXYFLURANE	NONE	0.2%0.5	0.0%0.5	13	3	23.1	100.0	0.0	0.0	0.0	100.0	—	—	—	—	—
CARDIOTIA	1967/10	43	METHOXYFLURANE	NONE	0.2%0.5	0.0%0.5	37	4	10.8	100.0	0.0	0.0	25.0	75.0	—	—	—	—	—
CARDIOTIA	1967/10	44	METHOXYFLURANE	NONE	0.2%0.5	0.0%0.5	100	5	5.0	0.0	100.0	0.0	60.0	40.0	—	—	—	—	—

[1] CARDIAC PROSTHETICAL CARDIOSTEATOTIC DEVICES

[2] CARDIAC TONE TO CARDIAC STATE

[3] ANNUAL MEAN TEMPERATURE, NONE <25°C (77°F), TONE, 0 100.25°C (77°F), ONLY >25°C (77°F)

[4] DEFERRED DEFECTS ONE

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TABLE 56. CONTINUED. A SURVEY OF [illegible] IN GOVERNMENT SERVICE IN GOVERNMENT SERVICE WITH THE UNIVERSITY TRAINING AND EXPERIENCE TEST

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[1] [illegible] PROVINCIAL ADMINISTRATIVE DIVISION

[2] [illegible] ZONE OR SAMPLE SITE

[3] ANNUAL [illegible] TEMPERATURE. [illegible] [illegible], [illegible] [illegible], [illegible] [illegible]

[4] ESTIMATED [illegible] [illegible]

TABLE 16. Results of Chi Square Analysis of Sex and Age Class Distribution of *Trypanosoma vivax* IFAT Positive Cattle Presented in Table 15

Comparison	Chi Square Values					
	Site 2	Site 3	Site 45 (10/68)	Site 45 (4/72)	Site 52	Site 66
Age[a]						
Low vs Middle * High	4.40**	0.56	4.01**	4.18**	NA	NA
Low vs Middle	2.00	0.00	2.11	0.10	NA	NA
Low vs High	5.88***	1.36	4.43**	4.37**	NA	NA
Middle vs High	2.04	3.88**	0.57	0.64	.93	2.29
Sex	3.22*[b]	0.03	2.26	NA	.27	NA

* Significant at $P \leq .10 \geq .05$, df = 1

** Significant at $P \leq .05 \geq .025$, df = 1

*** Significant at $P \leq .025 \geq .010$, df = 1

[a]Low = cattle 6 months of age and younger
Middle = cattle 7 to 18 months of age
High = cattle older than 18 months

[b]When incidence between sexes of *T. vivax* IFAT positive serums was compared in all cattle from sites 2, 3, 45 (10/68) and 52, x^2 was not significant

NA = not applicable

DISCUSSION

Parameters

Care was taken to examine blood samples via wet mount preparation within 30 minutes of their collection to insure adequate motility. Lack of motility has been cited as a hindrance to effective wet mount examination (WME).[27] The apparent greater effectiveness of the thick blood smear technique (TBST) over the capillary tube method (CTM) in detecting T. evansi parasitemias in calves was attributed to the small number of observations made using these methods. The efficacy of TBST over WME has been established.[27] Rat inoculations were routinely used to monitor T. evansi in 2 calves because of the method's ease and greater effectiveness in detecting sub-patent infections than either the TBST or WME.

A pathogenesis study of the Huila strain of T. vivax[13] indicated that packed cell volumes (PCV) values underwent a steady decline to a point where death appeared imminent. The PCV values at this point ranged from 16 to 20%. This trend is opposite to the trend observed in the T. vivax-mainterance calves of this study. The difference may be due to strain variation. The PCV values of calf 2 (Fig. 2) may have ultimately

been seen to increase had the calf been followed for a longer period of time. The periodic decreases preceding the reappearance of circulating trypanosomes may have been due to an induced expansion of the plasma volume.[9] It was concluded that once an upward trend in PCV occurred, calves infected with the Monteria strain of T. vivax would survive.

The variable rectal temperature values in response to T. vivax parasitemias precluded the use of this parameter as a suitable monitoring system. Inconsistency between degrees of T. vivax parasitemias and rectal temperature has been noted.[13,19,25,39,46]

Experimental Organisms

The mean length of 19.8 microns falls within the ranges but is less than the means of African strains of T. vivax as recorded by Hoare and Broom[24] and Fairbairn.[18] The mean length also is within the ranges recorded for South American strains but is less than the reported means.[26,29,38,39,46]

Variation between the means reported by the above workers may have been due to strain differences, the method of sample collection, smear preparation, staining, measurement techniques and the form of the trypanosome

as well as the homogeneity of the population measured. As indicated by the author, the morphological characteristics of T. vivax appear to be influenced by the stage of parasitemia. A similar observation was made by Wells et al.[53] No mention of the parasitemic stage at the time of collection was made in the above papers.

In the present study total length, mid-kinetoplast to mid-nucleus (KN) and width at nucleus (WN) measurements of T. vivax were the least variable based on the coefficients of variability. Measurements of mid-nucleus to anterior end (NA) and free flagellum (F) varied due to difficulty in determining the exact point where the F began. The high coefficient of variability of posterior end to mid-kinetoplast (PK) was attributed to its small size. The coefficients of variability of identical anatomical measurements, with the exception of WN, made by Shaw and Lainson[46] agree closely with the results of this study.

From the above results, the author thinks that in addition to total length, KN should also be routinely determined for each new strain of T. vivax isolated in Colombia. Such information may aid in strain identification in epidemiological studies. Homogeneity of the population to be measured is an absolute requirement.

Therefore, trypanosomes should be collected during the rapidly ascending phase of a parasitemia. At this time large numbers of organisms from a homogeneous population are available. Techniques of smear preparation, fixation, staining and measurement should be standardized.

The observed frequency occurrence of T. vivax parasitemias in maintenance calves was similar to patterns observed by others.[29,46] The lack of success in low temperature preservation of T. vivax may have been due to intrinsic qualities of the Monteria strain. The possibility also exists that the modification of the method of Cunningham et al.[11] altered the critical rate of cooling required for the successful cryopreservation of T. vivax.[15] Admittedly, cryopreservation may have been successful for a period less than 5 months but was not detected.

Development of T. evansi parasitemias in rats inoculated with infected blood or stabilates followed a characteristic pattern.[21] Successful cryopreservation of T. evansi was expected because of the close phylogenetic relationship to T. brucei[31] and the ease with which the latter has been preserved.[11,15,22,40] Trypanosoma evansi is present in the eastern and western hemispheres and has been reported to produce clinical

disease in cattle.[31] Observations from the author's study and the results of Clark and Benavides[8] and the study by Valero as cited by Wells *et al.*[53] tend to indicate that bovine *T. evansi* infection is not a clinical problem in South America.

Indirect Fluorescent Antibody Test

The limited success in obtaining *T. vivax* parasitemias in white rats parallels the initial successes reported by Desowitz and Watson[16,17] in their preliminary studies designed to adapt *T. vivax* to rodents. The high degree of success achieved in the cow to rat and first rat to rat passages apparently were due to the large inocula used. Greater success with subsequent rat to rat passages may have been obtained if amounts of ovine serum as large as 3.0 ml. were given to all rats.[17]

Failure of *T. vivax* infections in sheep to produce high and persistent levels of parasitemias has been observed.[17,46] Parasitemias may have been increased if the sheep had been immunologically suppressed. Due to the difficulty in consistently producing suitable *T. vivax* parasitemias in the rat and the sheep, indirect fluorescent antibody test (IFAT) antigen from these sources was not compared to bovine derived *T. vivax*

IFAT antigen.

Immunosuppressive techniques have been used effectively in increasing hemoparasite parasitemias.[7, 32,50] Because no controls were employed when antigen was produced in T. vivax-antigen calves A and B, definitive conclusions cannot be made regarding the efficacy of the immunosuppressive techniques used in this study. However, subjective observations between T. vivax-maintenance calves and antigen calves indicated that the above techniques were useful.

The presence of non-specifically fluorescing trypanosomes was apparently due to tagged anti-bovine globulin combining with bovine serum agglutinins that had attached to the surface of the trypanosomes. Non-specific fluorescence observed by others at low serum dilutions[42,54,57] may have been due to low levels of agglutinins induced by other species of trypanosomes, immune adherence antibody,[37,41] normal opsonins or other non-specific serum-antigen interactions. Because non-specific fluorescing trypanosomes are a potential source of error in the IFAT, test antigen should only be prepared from T. vivax-infected bovine blood not containing agglutinated trypanosomes.

Efforts to obtain euglobulin-free trypanosome rich

fractions from DEAE-cellulose columns were not pursued due to the success obtained with bovine blood-borne antigen.

Results from the use of various fixatives in this study agree closely with other comparative studies.[54,61] The thin hazy film covering antigen fixed via heat, formalin and Stalheim's method was thought to be denatured protein and or lipid material. The presence of this film was thought to have contributed to the excessive background fluorescence observed when the preceding fixation agents were used. This film may also have contributed to the lower IFAT titers observed by Zwart[61] when he compared heat fixation to various chemical fixatives.

The excellent results obtained in this study by using acetone:methanol::60:40 were attributed to the ability of acetone to extract lipid material. In addition the well defined morphological characteristics of the fixed trypanosomes and the retention of hemoglobin by erythrocytes indicated that fixation was not too severe. In contrast fixation via heat, formalin and Stalheim's method[47] were too severe as evidenced by dehemoglobinization of erythrocytes and the lack of distinctness of the margins of trypanosomes.

The time required for fixation of bovine IFAT *T. vivax* antigen appeared in part to be related to the thickness of the blood smears and the low temperature of fixation. *Trypanosoma evansi* IFAT antigen slides were characterized by thinner blood smears due to the massive parasitemias obtained in rats. There was no appreciable difference between fixation at 15 or 30 minutes.

Although IFAT *T. vivax* antigen lot A was stable for a minimum of 144 days, slight autofluorescence began to develop at this time. Other workers have reported successful storage of IFAT antigen at -20 C for 6 months.[6,57] During the period that lot A was stored, several electrical power disruptions occurred resulting in the fluctuation of the internal temperatures of storage freezers. It is possible such temperature variation may have hastened the onset of autofluorescence.

Trypanosoma evansi IFAT antigen was used within 2 weeks of production. Although no autofluorescence was observed, it is likely that had *T. evansi* antigen been stored for prolonged periods of time, autofluorescence would have developed.

Autofluorescence which characterized *T. theileri*

IFAT antigen, precluded its use to ascertain T. theileri IFAT titers in bovine serums used in specificity tests. Storage was not felt to be a contributing cause of the autofluorescence because the antigen was used the same day as processing.

Differences in fluorescent characteristics between culture and blood forms of trypanosomes have been demonstrated.[54] Only crithidia-like forms of T. theileri were present in modified blood agar media used in this study. The use of bovine leucocyte cultures as growth medium appears to favor the development of trypomastigote forms of T. theileri.[52] Indirect fluorescent antibody test antigen derived from leucocyte cultures may not autofluoresce, making it possible to determine T. theileri IFAT serum titers.

The positive/negative fluorescent grading system was found to be easier to interpret than the 5 grade numerical system. This was especially apparent when deciding if a particular reaction was a strong +2 or a weak +3. In using the positive/negative system, any reaction requiring hesitation for grading was considered suspicious and re-tested. Two consecutive suspicious reactions were classified negative.

The adoption of the positive-negative system with

photographic and point references would help eliminate confusion and promote a trend toward standardization. In the past both *2[20,54,55,57] and *3[6,12,54] have been cited as positive fluorescent grades. Compounding the problem is the fact that various methods of fixation affect the intensity of fluorescence.[61] Hence, what would be judged *3 by one worker, could be called *2 by another.

Rhodamine conjugated to normal rabbit serum was not essential to the IFAT, however, it caused a darker background and suppressed the non-specific fluorescence of platelets. The use of rhodamine was continued throughout the study because of the preference for a darker background.

Care was taken to place test serum and conjugate in the center of each numbered position on the antigen slide. Without such precautions it would be possible for only a portion of the antigen previously exposed to test serum, to receive conjugate. Consequently, false positive or questionable reactions would occur.

Test antigen positions were easily located with the aid of the red wax lines and diamond etched circles. The entire area containing antigen was scanned and the reading based on a minimum of 90% fluorescing free

non-agglutinated trypanosomes. Uneven degrees of fluorescence occasionally would be seen and were attributed to faulty staining technique or excessively thick preparations. Readings did not take into account the fluorescing organisms at the periphery of the antigen positions due to the occasional presence of unremoved excess conjugate.

It was necessary to occasionally adjust the oil condenser especially toward the end of a particular slide because the quantity of oil decreased and reduced the intensity of ultraviolet light. Reactions that were judged suspicious were frequently positive following condenser readjustment.

Two hundred and seventy readings involving 18 slides were routinely made over a 90 to 120 minute period. When the number of readings was increased to 360, eye fatigue occurred which affected judgment on borderline decisions.

Evaluation of Indirect Fluorescent Antibody Test

The IFAT sensitivity results closely agree with those of other workers[10,45,56] with regard to the first occurrence of detectable IFAT titers. However, there is a marked difference in the times of maximum IFAT titer

occurrence in cattle infected with African trypanosomes[56] and in cattle used in this study.

A possible explanation is the differences in the grades of fluorescence required for positive readings. When a lower grade of fluorescence was used to signify positive reactions in preliminary studies, titers of 1:800 were consistently observed in a cow 62 days post inoculation (PI). A maximum titer of 1:1600 was detected on day 137 PI. Had the strict criteria for positive grade fluorescence been decreased in the author's study, the time for maximum titer development may have been significantly extended.

Fluctuations in trypanosome IFAT titers have occurred,[45,56] but sudden decreases to negative or suspicious reactions have not been reported. The sudden decreases in IFAT titers that were observed in T. vivax-infected cattle may have been due to a temporary decrease in circulating antibodies as a result of an antigen/antibody reaction that apparently occurs in some hemoparasite infections.[49] However, no parasitemia preceded the decrease in IFAT titer that occurred in calf 5 (Fig. 5). In addition the decline in IFAT titer observed in calf 9 (Fig. 9) occurred over an 18 day interval with only 2 periods of parasitemias ranging

from 2 to 3 days in duration. Therefore, it was thought that the suspicious and negative titers were more likely due to loss of serologic activity during handling and storage. Some serums from T. vivax- maintenance calves were occasionally subjected to varying temperature due to unavoidable disruptions of electrical power in the laboratory.

Sensitivity was defined as the percentage of positive IFAT reactions detected at a given serum dilution in serums obtained from a given population of T. vivax-infected cattle throughout the course of infection. Using this definition, sensitivity of the IFAT developed in this study was 80% at a serum dilution of 1:100. If the serum dilution were lowered to 1:50, the sensitivity would increase to 97%. However, increasing the sensitivity to the 97% level, increases the possibility of cross reaction with T. evansi-infected cattle as noted below; therefore, the 80% level of sensitivity was adopted to avoid false positive reactions.

The wide ranges encompassed by the 95% confidence limits of the normal approximation to the mean indicate that a larger number of IFAT titer determinations must be made to obtain more definitive results regarding repeatability. With the limited data, it was thought

that the repeatability of the IFAT was approximately 80%. The use of different antigen lots produced from different calves probably does not reduce the repeatability of the test. There has been no statistical analysis of the repeatability of the IFAT for trypanosomes reported from the literature with which to make comparisons.

Results from specificity tests indicate that serological cross reactions between A. marginale, Babesia spp., Eperythrozoon spp. or T. theileri with T. vivax are not likely to occur. These results agree with those of Wilson et al.[57] with regard to T. theileri. There have been no reports pertaining to cross reactivity between A. marginale, Babesia spp. and Eperythrozoon spp. and the IFAT for trypanosomiasis.

Only minimal cross reactivity has been shown to exist between T. vivax antigen and T. brucei positive serum.[44,56,57,61] Because of the close phylogenetic relationship of T. evansi to T. brucei, the 6 of 96 (6.2%) suspicious reactions recorded in Table 10, may be a manifestation of slight cross reactivity. However, it is thought that the distribution of these reactions in Table 10 is not consistent enough to strongly support the above contention.

Even though not a single T. evansi isolate has been recovered from Colombian cattle,[53] its existence in the cattle population is a distinct possibility in view of its presence in Panamanian cattle.[8] Therefore, to minimize the possibility of any cross reaction, a 1:100 serum dilution was selected as the baseline dilution for all surveys conducted in this study.

Persistence of Maternal Antibodies

Due to the lack of serum samples available, the results as indicated in Table 11 can only be regarded as an indication of the duration of maternal antibodies in newborn calves capable of eliciting a positive T. vivax IFAT reaction. Such antibodies may persist up to 24 days. There are no reports in the literature concerning the persistence of trypanosome-specific maternal fluorescent antibodies in newborn calves with which to compare the above observation.

Knowledge regarding the duration of trypanosome-specific maternal IFAT antibody in calves is essential for accurate epidemiological studies. The lack of such knowledge precludes the use of serums obtained from young calves. It would not be possible to differentiate between active or passive immunity in positive IFAT

reactors without subsequent serum titrations.

Use of Blood Saturated Filter Paper Discs as Field Samples in Indirect Fluorescent Antibody Test

Albumin was chosen as the serum component to compare in determining the dilution factor because it migrated slowly and remained distinct from hemoglobin and globulin fractions on cellulose polyacetate support medium during electrophoresis. The resulting dilution factor of 68 ± 11 was considered too low for field surveys if the possibility of T. evansi cross reactions were to be minimized. Consequently, a new dilution factor of 88 ± 14 was selected and attained by increasing the eluent per dried blood sample from 0.100 ml. to 0.125 ml.

The method used by the author for the preparation of filter paper blood samples was considered to be superior to the procedure employed by Bailey et al.[6] Construction of wells directly on antigen slides was tedious and required more time than preparing slides for the standard IFAT procedure. In contrast, the wells of microtiter trays could be filled rapidly with eluent and dried blood samples. The resulting eluates were then removed with modified Pasteur pipettes and placed directly on slides as in the standard IFAT procedure

described above. An added advantage was that once filled with eluent and sealed, microtiter trays could be held overnight at 4 C, thus providing a convenient stopping point.

The efficacy of the IFAT over the TBST in detecting exposure of cattle to T. vivax was adequately demonstrated in surveys I, II and III (Table 13) and supports the findings of other workers.[36,51,55,61]

Applying the classification system of Wilson and Cunningham,[56] it appears that the high rates of efficacy of the IFAT over the TBST observed in surveys I and III probably indicate a status of latent herd infection because the percentage of IFAT reactors in the herd did not change significantly over a 6 month period. Conversely, the lower rate of IFAT efficacy seen in survey II probably indicates a more recent infection.

The above conclusions were verified by the history of the herds surveyed. Surveys I and III were made in the same herd 6 months apart. No problem of clinical trypanosomiasis was reported in this herd for over one year prior to or during the interval between surveys. Survey II was made on a herd at the onset of an acute trypanosomiasis outbreak.

The high degree of correlation between conventional

and eluate sample IFAT results in surveys I and II (Table 14), coincide with the findings of Cunningham *et al.*[12] A similar comparison of IFAT results of survey III was not made because the dried blood samples were stored at a higher temperature for a longer period of time than the samples of surveys I and II. It was thought that the prolonged storage resulted in a loss of serological activity, accounting for the 4 fold difference between IFAT results presented in survey III (Table 13).[6] The disagreements in survey I (Table 14) were attributed to technical errors in preparing samples for the IFAT.

Positive/suspicious (P/S) combinations between conventional serum and eluted filter paper blood samples from the same source were considered agreements for the following reasons. Normal fluctuations of plus or minus 1 IFAT titer as seen in Table 3 can account for a P/S combination. In addition, the total number of P/S combinations of conventional and eluted samples respectively were nearly equal to the number of P/S combinations of eluted and conventional samples respectively.

Although initial results indicate that filter paper blood samples can be used successfully in field

surveys, additional correlation studies are required. The main thrust of these studies should be directed at determining the rate at which serological activity is lost in filter paper blood samples collected and handled under various field conditions. High temperature and humidity of the tropics may result in sufficient loss of activity so that serums collected from cattle during the initial stages of *T. vivax* infection when IFAT titers are characteristically low,[56] may be found to give false negative results.

Trypanosoma vivax Survey of Colombian Cattle Using Indirect Fluorescent Antibody Test

The results of chi square statistical analysis presented in Table 16 were derived from data collected from sites in which there was sufficient categorization of data to permit analysis. Each site was analyzed separately. The results pertaining to age distribution were not considered to be additive because the samples were not thought to be homogeneous.

The finding that there was no overall significant difference in the incidence between sexes of *T. vivax*-IFAT-positive cattle agrees with the observations of Wain *et al.*[51] The significant differences between

incidence of IFAT-positive cattle among age categories at sites 2, 3, 45(10/68) and 45 (4/72) may be an indication that younger cattle are less susceptible to T. vivax infection than older cattle (Table 16). However, this hypothesis is considered unlikely in view of the observations of others[51,53] as well as the ease with which it was possible to infect calves under 6 months of age with T. vivax in this study.

It is more likely that the difference in incidence of IFAT-positive cattle within age groups reflects the rate of infection that exists in a given area. In areas with low infection rates, a higher percentage of IFAT-positive older cattle would exist because of greater exposure time to infection, whereas in areas with high infection rates, the differences between age classes would be expected to be less pronounced.

Consequently, the differences observed between age groups at sites 2 and 3 were thought to be due to sporadic outbreaks. The absence of infected calves may be an indication that an outbreak occurred at least 6 months prior to the time serum samples were collected. The outbreak at site 3 probably occured even earlier. Both sites 2 and 3 are located in Los Llanos where alternating dry and wet seasons occur. Consequently,

the abundance of potential arthropod vectors could be affected, resulting in outbreaks of a sporadic nature.

The development of significant differences in the incidence of IFAT-positive cattle between age categories from October 1968 to April 1972 at site 45, was thought to be an indication of declining infection rate. It can be argued that the differences were due to a seasonal decline because samples were collected at different times of the year. If such were the case, the percentage of IFAT-positive cattle older than 18 months would not be expected to differ significantly between years. However, when the incidence of IFAT-positive cattle older than 18 months on both sample dates was compared, a significant difference ($P < .1$) was observed.

Additional evidence indicating a declining infection rate at site 45 was the decline in total incidence of IFAT-positive cattle within the herd from 18.8% in October 1968 to 10.8 and 11.7% in April and November of 1972. The differences between the incidence of the latter 2 dates and the incidence in October 1968 were significant at $P < .05$ and $P < .1$ respectively. Furthermore, it was noted that Wells _et al._[53] in 1967 found via the TBST that 23 of 196 (11.7%) cattle were

infected with T. vivax. Using the same technique, the author found 1 of 184 (<0.5%) cattle infected in April 1972 and none of 204 cattle in November 1972. Clearly the incidence of T. vivax infection had decreased and with it the exposure rate.

Serological identification of cattle exposed to T. vivax in the departments of Boyacá, Córdoba, Meta and Valle compliments earlier findings in which T. vivax was detected in the preceding departments via the TBST.[53] In addition, T. vivax has also been identified in cattle via the TBST in the departments of Atlántico, Bolívar, Caldas, Cundinamarca, Guajira, Huila, Magdalena, Santander, Santander del Norte and Tolima. The serological identification of T. vivax-exposed cattle in Sucre reveals for the first time the presence of T. vivax in this department.

The major areas from which no clinical cases of T. vivax have been reported and for which no serological data is available include the eastern section of Los Llanos Orientales, the Pacific coast and the southern boundary departments of Nariño, Putamayo and Amazonas. It is impossible to know if T. vivax is present in these areas without conducting further clinical and serological surveys.

Despite extensive sampling in Caquetá where environmental conditions are ideal for the arthropod transmission of T. vivax, not a single serum sample was positive. This observation was considered unusual in view of the fact that no physical barriers separate Caquetá from Meta where T. vivax is established. It is possible that this trypanosome has not yet reached Caquetá. The lack of extensive cattle movement into and within this department would hinder the spread of T. vivax. The cattle industry of the Pacific coast and southern boundary departments, with the exception of the high altitude country around Pasto, is similar to that of Caquetá and, therefore, it is not likely that T. vivax exists in these areas.

An increase in the prevalence of T. vivax can be anticipated throughout los Llanos and elsewhere as the cattle industry of Colombia expands. No area will be exempt except possibly the higher altitudes. In both the author's serological survey and the results of other studies,[53] T. vivax was not found in natural infections at altitudes greater than approximately 1500 meters.

Photography

Photographs taken automatically were consistently more accurate in reproducing the degree of fluorescence as seen in the microscopic field than photographs taken at pre-determined times. Figures 14, 15, 16 and 17 are accurate representations of trypanosomal fluorescence; however, the background in these figures is darker than that which is normally observed.

SUMMARY

Trypanosoma vivax with a mean length of 19.8 ± 1.5 u was isolated from a cow in the Department of Córdoba, Colombia. The isolate of *T. vivax* was pleomorphic in blood varying from predominately slender forms with tapering posterior ends during ascending parasitemias, to broader forms with large rounded posterior ends which were present during the latter phases of longer standing parasitemias. The trypanosome was purified by passing it through a sheep which was simultaneously treated with Cloxazone and oxytetracycline. Subsequently, the isolate was maintained only in calves because low temperature storage of the organisms was unsuccessful.

The health of *T. vivax*-maintenance calves was monitored via packed cell volume, rectal temperature and the extent and frequency of parasitemia. Packed cell volumes declined to their lowest value within 31 days post inoculation (PI), after which a slow gradual incline was observed. No direct relationship between degree of parasitemia and rectal temperature was observed. The frequency and intensity of parasitemias decreased sharply 60 days post parasitemia (PP).

Circulating trypanosomes were seen as long as 180 days PP.

An isolate of T. evansi originally derived from a vampire bat was maintained by storing stabilates at -70 C. Concurrently, the isolate was maintained in calves up to 143 days PI. Thick blood smear examination was more efficient than wet mount examination for detecting parasitemias during the initial period of infection. Trypanosoma theileri was isolated from the CIAT herd and maintained through 6 passages on modified blood agar.

Trypanosoma vivax organisms for IFAT antigen preparation were obtained by infecting hemoparasite-free calves. High levels of parasitemia were easily attained in splenectomized steriod-treated calves. Adequate levels of parasitemia for antigen production occurred when 12 to 15 trypanosomes were observed per x 1000 field of a stained thin blood smear. Trypanosoma vivax-infected sheep failed to develop adequate levels of parasitemia. While it was possible to serially pass T. vivax organisms from an infected calf through 5 rat passages, it was not possible to consistently obtain suitable levels of parasitemia.

Trypanosoma evansi organisms for IFAT antigens were obtained by infecting rats with stabilates. Culture forms of *T. theileri* were used for IFAT antigen.

Indirect fluorescent antibody test antigen war prepared by evenly distributing *T. vivax*-infected citrated blood over the entire surface of glass microscope slides. The smears were air dried and fixed in 60:40::acetone:methanol at -20 C for 30 minutes. Dried, fixed antigen preparations were stored dry at -35 C or -70 C and used successfully in the IFAT for periods up to 144 and 116 days respectively. *Trypanosoma evansi* and *T. theileri* IFAT antigen was similarly prepared.

Indirect fluorescent antibody test serum titers of 1:100 or greater were observed at a mean time of 15.9 days PP with a range of 6 to 32 days PP. Maximum titers of 1:400 developed in 14 to 33 days PP in 8 calves. In 1 calf, 109 days were required before the maximum titer was attained. Serum titers persisted in calves up to 278 days PP. Maternal antibodies capable of causing a positive fluorescent reaction at a 1:100 serum dilution, persisted for 24 days after birth in 1 calf.

At serum dilutions of 1:100 the IFAT detected positive reactions in 80.4% of 133 serum samples obtained from 9 calves at various times during the course of T. vivax infections. At serum dilutions of 1:50, the percentage of positive reactions increased to 97%.

No cross reactivity was observed between T. vivax and Anaplasma marginale, Babesia argentina, Babesia bigemina, Eperythrozoon spp. or Trypanosoma theileri at 1:50 serum dilutions. Suspicious reactions were occasionally observed when Trypanosoma evansi-positive serum diluted 1:50 and 1:100 was used in the IFAT for T. vivax. No false positive reactions were observed when serums from 36 hemoparasite-free calves were tested.

Results from the IFAT were able to be repeated within plus or minus 1 dilution approximately 80% of the time using the same or different antigen lots.

Samples obtained for the IFAT by eluting dried serum from blood-impregnated filter paper discs produced results nearly equal to those obtained by using conventional serum samples when dried samples were stored at 0 C for 10 days or less prior to use after delivery from the field.

In field surveys the IFAT was up to 21 times more effective in detecting T. vivax-positive cattle on the basis of antibody presence than the thick blood smear technique.

Results from a survey involving 2,053 cattle from 11 departments and territories in Colombia revealed the presence of T. vivax antibodies in cattle from the departments of Boyacá, Córdoba, Meta, Sucre and Valle. No T. vivax IFAT antibodies were detected in serums obtained from cattle in the departments of Antioquia, Cauca, Caquetá, Cundinamarca, Tolima and Vichada.

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The typist for this thesis was Jean E. Platt.