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Annual Research Report

A Research and Training Program in Tropical Veterinary Medicine
(AID-csd-1947, in Colombia)

Program Director: F. D. Maurer, D.V.M., Ph.D.
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NARRATIVE SUMMARY OF ACCOMPLISHMENTS AND UTILIZATION

The hemotropic, arthropod transmitted diseases of cattle have long been recognized as a major impediment in the development of a viable, productive, livestock industry in tropical areas. The USAID project with Texas A&M University was initiated in 1968 for the purpose of generating a better understanding of these diseases and to develop techniques and procedures which would provide a solution to these problems.

During the years, significant progress has been made by the research team located first at Bogota, and more recently at Cali, Colombia, as well as the group studying the same problem at Texas A&M University.

The problem has been well defined as a result of epidemiologic studies of anaplasmosis, babesiosis, and to a lesser extent trypanosomiasis. In conducting these studies, it has been necessary to implement serologic testing procedures for anaplasmosis, and to develop similar tests for the identification of babesiosis and trypanosomiasis. A complement-fixation test has been developed for babesiosis, and a reliable indirect fluorescent antibody technique developed for trypanosomiasis. The areas where anaplasmosis and babesiosis are most common have been identified and similar studies will be done on trypanosomiasis.

A new test for anaplasmosis involving a rapid agglutination reaction (the card test) has been tested in recent months and found to agree very well with the complement-fixation test. A similar test (card test) is being developed for babesiosis and offers promise. Tests of these new techniques are in progress.

Vaccine studies have shown the value of premunition in cattle maintained on the north coast of Colombia.

The drug Imidocarb has been shown to have marked therapeutic effect against anaplasmosis and babesiosis, and to have a prophylactic effect against babesiosis Combinations of drugs, Imidocarb plus Gloxazone, and Gloxazone plus oxytetracycline, have shown even greater promise as sterilants against Anaplasma.

The present approach of hemoparasite disease control on the north coast of Colombia involves a control program aimed at maintenance of infection at sub-clinical levels. This approach is practical and indicated at this time; however, premunition will perpetuate the disease, and it is possible that the initial infection and a continuing chronic infection may cause a loss of productivity. To maintain chronic infection, constant reinfection by natural arthropod vectors is necessary, and this means accepting the detrimental effects of tick infestation, involving damaged hides and a constant drain of animal vitality.

Disease eradication is presumed to be impractical at the present time. An experimental anaplasmosis eradication scheme is currently being tested in a Texas herd numbering 460 head of cattle which showed an infection rate in excess of 80%. This incidence of infection is comparable to many herds in

Colombia. The procedure involving treatment with Imidocarb immediately stopped losses due to anaplasmosis. The herd is being observed for evidence of reinfection.

During the past year, staff changes have been significant. Drs. Todorovic, Adams, Craig, Thompson, and Platt returned to the U.S. from Cali. Dr. Wyss remained in Cali. Dr. Galvin, from Texas A&M, replaced Dr. Adams as project leader in Colombia. Dr. Kuttler remained at Texas A&M.

A total of 10 U.S. graduate students have participated in this program since its beginning, 7 in microbiology, 2 in pathology, and 1 in parasitology. As a result of research in Colombia and academic studies at Texas A&M, 4 have completed the Masters program and 1 the Ph.D. program. Currently 5 students are pursuing the Ph.D. degree and 1 the Masters degree. The results of this program are now becoming evident. In the near future there will be several veterinarians with a Ph.D. degree in a given discipline who will become available for full staff assignment in Tropical Veterinary Medicine.

An added phase of our training program in Colombia has been the supervision of students from South American countries both in Colombia by staff stationed there and in the U.S. by Texas A&M staff. The broad range of participation on the part of students and staff both from the United States and South America has established a rapport which has strengthened the veterinary program in both Colombia and at Texas A&M.

GENERAL BACKGROUND

Disease control is a basic prerequisite for livestock survival and production in any locale, but becomes a major consideration and a decisive factor in the tropics where environmental factors favor the development and transmission of animal disease. Historically, the validity of this statement has been established in nearly every instance where high producing livestock have been introduced into the tropics.

Many tropical and subtropical areas of the world are noted for profuse forage and roughage production, unsuited for human food but capable of utilization by ruminant ungulates which can convert this resource into a high quality protein for human consumption. This production could contribute to the health and economic well-being of millions of people now living on marginal diets under poverty conditions. A major limiting factor, preventing livestock production, in these areas is animal disease. A major group of these diseases comprises the blood parasitic diseases of cattle including anaplasmosis, theileriasis (East Coast Fever), babesiosis (piroplasmosis), and trypanosomiasis. Wilson, et al. (Bulletin World Health Organization, 28, 595-613) estimates that the area in Africa virtually devoid of cattle, as the result of trypanosomiasis alone, exceeds the size of the United States. He estimates that this area could support 125,000,000 head of cattle were it not for this devastating cattle disease. Control of this disease plus the other blood parasitic diseases mentioned above would produce an economic-social impact on affected areas that could hardly be measured. Such a step could rival, if not surpass, the so-called "Green Revolution" that has so significantly contributed to cereal grain production in recent years.

Past research efforts have contributed greatly to our knowledge of these diseases, but more information is needed to provide workable, practical control programs.

It was with this background that our original project was submitted to AID for funding. The research proposal was aimed primarily at the applied, production oriented aspects, but in so doing it was recognized that basic studies would be required to provide the groundwork for applied techniques.

PROJECT OBJECTIVES
(As Submitted January 1968 With Minor Modifications)

1. To conduct research on tropical diseases aimed at developing information and methods for the control of these diseases in order to produce more beef in developing countries, with initial emphasis on arthropod-borne blood diseases (anaplasmosis, babesiosis, theileriasis, and trypanosomiasis).

   A. Anaplasmosis (specific objectives)

      a. To evaluate, under field conditions, vaccines presently available.
      b. To develop a more effective killed vaccine.
      c. To evaluate premunization methods as a means of prophylaxis.
      d. To establish the cattle passages required for reversion of the attenuated Anaplasma to virulence.
      e. To measure possible antigenic variations among Anaplasma organisms.
      f. To investigate the prevalence of disease and natural transmission by arthropod vectors.
      g. To investigate the use of systemic insecticides and other vector control measures as preventive measures.
      h. To evaluate therapeutic compounds for treatment.
      i. To conduct pathogenesis studies.

   B. Babesiosis (specific objectives)

      a. To develop an effective immunological approach.
      b. To determine the prevalence of disease and the potential number of vectors in various geographic areas.

c. To evaluate a tick eradication program as a means of Babesia control.
d. To investigate various ixodicides in achieving tick control.
e. To measure the productivity of infected cattle as compared to non-infected animals.
f. To evaluate therapeutic compounds, their efficacy and toxicity, in relation to treating acute and chronic infections.
g. To develop serologic tests for the detection of latent as well as acute infection.
h. To determine the antigenic variations of Babesia organisms, by serologic and other means.
i. To determine the Babesia incidence in cattle and wild fauna, and the role of wildlife in maintaining infection.
j. To develop non-bovine sources of Babesia organisms from ticks and tissue culture for use in vaccines.
k. To conduct pathogenesis studies.

C. Other related blood diseases, including Trypanosoma and Theileria infections, have been studied.

2. To train graduate students in research methods applicable to tropical diseases and to collect information, specimens, and illustrations for use in this training program.

3. To provide trained faculty and staff to operate veterinary programs in developing countries, and to serve as consultants to related tropical disease problems.

CONTINUED RELEVANCE OF OBJECTIVES

Many of the objectives listed regarding anaplasmosis and babesiosis have been accomplished, with techniques and information evolving which offer hope for significant improvement in present control practices. Even so, losses continue and the disease problem persists. There are many vital questions that have gone unanswered. Continued research is the best way to solve these problems and to reach the ultimate objective of disease control.

As the research on babesiosis and anaplasmosis proceeds to the demonstration of methods which provide effective control, more attention can be

given to trypanosomiasis, theileriasis, or other parasitic diseases for which control techniques are relatively inadequate.

ACCOMPLISHMENTS TO DATE

During the past year, studies have been made which contribute directly to the objectives listed above and are a continuation of results previously reported.

A review paper on East Coast Fever (theileriasis) consisting of 17 pages and 45 references was prepared. A paper entitled "Arthropod Borne Protozoan Infections (Affecting Domesticated, Food-Producing Animals)" including anaplasmosis and babesiosis was prepared which consisted of 45 pages and 88 references. A paper entitled "African Horsesickness" consisting of 17 pages with 25 references has been prepared. These papers, plus an introductory chapter entitled "The Need" emphasizing the justification for research in tropical veterinary medicine, will appear as chapters in the manual Foreign Animal Diseases to be published by the Foreign Animal Disease Committee of the U.S.A.H.A. Abstracts of these papers appear in Appendix I.

Anaplasmosis:

a. To evaluate, under field conditions, vaccines presently available.

The Turipana study reported last year has been completed and results analyzed. The preliminary assessment of this data has been verified and confirmed. It would appear that significant economic advantage can be gained by the control of hemoparasites using a premunition approach. Three additional studies have been instituted at

Turipana to further evaluate premunition and chemoprophylactic measures for control. In addition, the attenuated Anaplasma is being retested as a premunizing organism to replace the virulent A. marginale previously used. The results of these experiments are not yet available, but some death losses have occurred in control cattle in contrast to the apparent protection shown in treated and vaccinated cattle.

b. To develop a more effective killed vaccine.

The key to an improved killed vaccine is the development of high titer antigens free of bovine erythrocytic contaminants. Unsuccessful attempts have been made to produce high Anaplasma parasitemias in sheep for possible use in a killed vaccine.

Antigenic activity has been detected in highly purified bovine antigens. A test to measure the residual red cell antigens of these preparations has not yet been made, even though bovine erythrocyte and Anaplasma antisera have been prepared for this purpose. No killed vaccines have been tested this year.

c. To evaluate premunition as a means of prophylaxis.

Adult cattle premunized over 2 years ago with the attenuated vaccine have withstood a virulent Anaplasma challenge, showing no signs of challenge infection. We have not, however, challenged with the antigenic variant strains that occur in South America. For this reason, the attenuated organism was hand carried to South America this year, and the strain re-established in sheep and cattle for use there. A trial to evaluate possible strain differences based on cross infectivity trials is presently underway.

Premunition experiments in the past have been extensive, and papers reporting these trials are included in Appendix IV. A brief, consolidated statement of general recommendations for premunition is included in Appendix II.

d. To establish the cattle passages required for reversion of the attenuated Anaplasma to virulence.

This work has been previously reported and is essentially completed.

e. To measure possible antigenic variations among Anaplasma organisms.

Discussed under section c.

f. To investigate the prevalence of disease and natural transmission by arthropod vectors.

No extension of serologic surveys in Colombia has been made this year. Several sizeable field trials involving serologic testing and evaluation of epidemiologic factors have been made of herds in Texas.

One herd (the Callaghan - Encinal, Texas) of 469 head of grade Hereford cattle were tested and treated with Imidocarb to eliminate infection. The complement-fixation (CF) test revealed 73.9% positive, 14.5% suspicious, and 11.5% negative. There were obviously acute cases occurring in this herd at the time of testing, since 16% showed 0.5% or greater Anaplasma parasitemias. The test was made in mid-December, well past the normal season for biting flies which are thought to be the principle vector. Extended incubation times could have overlapped the fly season, but it is suspected that a tick vector may have been involved. Both Dermacentor albipictus and D. variabilis were observed when cattle were initially tested. These ticks were the only potential vectors observed at this time. The procedure originally planned was to

treat only the reacting cattle. Because of the high incidence, all cattle were treated. Treatment consisted of 3.5 mg/kg Imidocarb given intramuscularly 2 times at a 14 day interval. Blood samples have been taken periodically since that time to follow the course of infection in the herd and to evaluate the efficacy of treatment.

The high incidence of infection and the rapid spread of infection indicates the presence of a highly efficient vector, not unlike the situation encountered in Colombia. If this anaplasmosis outbreak can be stopped and then eliminated by treatment under these conditions, it might suggest a similar approach in endemic zones of Colombia. This ranch is located about 20 miles from the Mexican border in a subtropical area.

During the bleeding of this herd a card test (CT), developed by the U.S.D.A. for diagnosing anaplasmosis, was performed at the ranch using a car battery to power the portable equipment needed. The test was performed on plasma and the results compared with the CF test conducted on serum at the laboratory. There was an overall agreement of 93%. The CT and CF tests were compared on a second herd of 430 cattle with a low incidence of infection. In this instance, the CT was performed on serum. The overall agreement was 95.8%. The agreement for the 899 samples was 94.3%.

The CT is much more easily run, requiring little more than the simplest equipment. If, as it seems, the test proves adequate it would have wide use in LDC where a rapid diagnostic test is desirable. The antigen and kits will be marketed in the U.S. probably late in 1973.

h. To evaluate therapeutic compounds for treatment.

A complete table of results comprising 42 different treatments on 86 cattle is given in Appendix IV. In summary, results indicate that combination therapy is more effective in eliminating the carrier infection and less toxic. The combination of Gloxazone and Imidocarb is probably most effective given daily for 3 days, or every other day, for a total of 3 treatments. A minimum of 3 mg/kg Gloxazone given intravenously (I.V.) plus 3 mg/kg Imidocarb given intramuscularly (I.M.) was effective. When 5 mg/kg Gloxazone is used, a level of 2 mg/kg Imidocarb is adequate. The schedule of 3 treatments every 24 or 48 hours appears preferable to 2 treatments at 14 day intervals; however, instances of success have been reported for each.

A second combination consisting of Gloxazone and oxytetracycline, given I.V., has also been effective. Good success has been achieved when these drugs were given 3 times at either 24 or 48 hours. This combination was also effective given 2 times at a 14 day interval.

The use of Imidocarb alone was effective in eliminating Anaplasma carrier infections at 3, 4, 5, and 6 mg/kg levels when administered I.M. 3 times at 24 or 48 hour intervals. The drug was also effective when given at the 5 mg/kg level 2 times at 14 day intervals; however, the 3 injection schedule appeared preferable. Imidocarb given I.M. at 2.5 mg/kg 10 times at 2 day intervals was not effective in 1 of 6 calves surviving this treatment schedule. Fatal drug toxicosis occurred in 5 of the 6 calves treated by this method.

The tables of results (see Appendix) list all results. There are instances of failures to clear the infection in most of the treatment schedules. It should be remembered, however, that these trials

for the most part were conducted on splenectomized calves which have little or no natural resistance to the disease. These trials represent a more severe test for the treatment programs than would normally be encountered.

Drug toxicity was encountered only when Imidocarb was used. No toxic response was seen with oxytetracycline when a total dosage used was 66 mg/kg or with Gloxazone when used at levels of 30 mg/kg. Fatal toxicosis was observed on several occasions with Imidocarb when the total dosage rate was 15 mg/kg or over. During the field trials at the Callaghan ranch, a death loss of 5% occurred among 469 cattle following treatment. These cattle received a total of 7 mg/kg, divided into 2 treatments. It is not known if all of these losses were the result of drug toxicity, because several other factors were present which created an artificial stress, but there is a strong possibility that the drug was a contributory factor.

In recognition of this feature, some rather extensive toxicity trials on Imidocarb have begun. The results are not yet complete; however, 15 mg/kg appears to be the LD50. A trial showed the drug was toxic at the 6.75 mg/kg level for Angora goats. Imidocarb apparently acts primarily as a nephrotoxin, causing severe acute tubular necrosis of the proximal convoluted tubules.

Babesiosis:

a. To develop an effective immunological approach.

Immunologic studies on babesiosis have emphasized the principle of premunition. Premunition can be successfully induced by injecting virulent *Babesia spp.* followed by specific therapy at the substerilizing

level. A second approach is to treat with Imidocarb, which is known to have a prophylactic effect, and then place the treated animal in an environment where a continuous Babesia challenge will occur over a period of time, either by natural means or by the regular injection of infected blood under experimental conditions. To date, field trials on the north coast, experimental trials at Nuevo Laredo, and needle challenge experiments at Texas A&M suggest that premunition by this method is feasible. The theory is that as the drug effect gradually wears off exposure will finally produce a parasitemia, but because of drug residues, this parasitemia never becomes acute to the point of producing clinical illness. The animal's immune response then has time to control the infection, establishing premunition and, hence, resistance to future Babesia infection.

An interesting aspect of these studies is the observation of an extremely mild response in adult cattle to Babesia bigemina when the infection is induced by the inoculation of infected blood from a carrier calf. We have also found that Babesia in these mild reactions is difficult to demonstrate and sometimes complicates the data in that the presence or absence of infection cannot be unequivocally established.

b. To determine the prevalence of disease and the potential number of vectors in various geographic areas.

c. To evaluate a tick eradication program as a means of Babesia control.

d. To investigate various ixodicides in achieving tick control.

During the past year, a unique situation developed in Texas in that the tropical fever tick, Boophilus microplus, was observed in a large area of south Texas, north of any previously infected areas. The Institute staff has become involved in investigative procedures and have

participated as consultants to groups considering the eradication of this tick. Working with Texas wildlife personnel, the U.S.D.A., and the Texas Animal Health Commission, we have kept abreast of developments in this area which are directly applicable to similar problems in Colombia and elsewhere. The tick, B microplus, is well adapted to sustain itself on the Texas white-tailed deer. In the present tick quarantine area, it is estimated that there are more deer than cattle. The question as to whether the ticks can be eradicated by a cattle dipping program is presently unanswered. If this approach is successful, then a similar approach in South America should work There are no areas in Colombia where wildlife approaches the concentrations seen in Texas. The versatility of the B. microplus tick with regards to the variety of hosts it can attach on is one reason that eradication has seemed impractical in South America. The success or failure of this program could well influence our future research efforts in South America.

Presently the dipping program is using Co-Ral (0 2 - 0.25%) as the acaracide, with a dipping interval of 18 days with a 3 day grace period, the maximum dipping interval being 21 days

In Colombia, blood samples are being taken at approximately 3 month intervals from 9 herds involving 325 head of cattle located in the Llanos Orientales. Frequent applications of insecticides have practically eliminated ticks from these cattle, resulting in little or no transmission of hemoparasites. Complement-fixation titers have reverted to low or negative status indicating that the cattle are becoming free of infection, but simultaneously may soon be susceptible to reinfection.

Colonies of Boophilus microplus, B. annulatus, Amblyomma cajennense,

A. auricularium, Dermacentor nitens, and D. albipictus are being maintained for experimental and training purposes. Transmission studies are currently being conducted with B. microplus and D. albipictus. Plans have been made to obtain Ixodes scapularis and A. americanum for study.

f. To evaluate therapeutic compounds for treatment and prevention of Babesia infections.

Imidocarb has been and is continuing to be tested for its therapeutic efficacy against Babesia. A single injection of 0.5 mg/kg has been shown effective in eliminating B. bigemina infection. A single injection of 2 mg/kg has been effective in eliminating B. argentina infection. When compared to Anaplasma, Imidocarb is at least 30 times more effective against B. bigemina and 7.5 times more effective against B. argentina. At these low levels it seems probable that toxicity problems will not become apparent. Lower levels will be tried so the final potential of this drug has not yet been described.

The therapeutic and prophylactic effect of Imidocarb has been measured in calves being exposed periodically to known Babesia infected B. microplus ticks. The diproprionate salt at the rate of 5 mg/kg was injected into calves 14 days after tick exposure (when a Babesia parasitemia was evident) 14, 28, and 42 days before tick exposure. The drug was responsible for an immediate remission of the Babesia parasitemia when given 14 days after tick exposure. The ticks recovered from this calf failed to transmit Babesia when tested on a splenectomized calf. The drug given 14 days before tick infestation prevented infection in the calf and the ticks. The drug given 28 days before tick infestation prevented evidence of infection in the calf, but the ticks either became

infected or retained their Babesia infection on this calf. The drug given 42 days before tick infestation prevented clinical evidence of Babesia infection but did not prevent the development of a Babesia parasitemia and did not prevent the infection of ticks. Control calves confirmed the presence of Babesia infection and substantiates the above observations. Details of this experiment are given in Appendix IV.

g. To develop serologic tests for the detection of babesiosis.

During the past year, Babesia CF antigens for both B. bigemina and B. argentina have been prepared at Texas A&M and Colombia. Using techniques similar to that used in anaplasmosis, a card test antigen for Babesia has been prepared. Preliminary results with the babesiosis card test are encouraging. It was possible to detect specific B. bigemina antibodies in the sera of infected cattle 6 to 8 days post infection. The agglutination reaction was persistent up to 6 weeks post infection, at the time of writing this report. The significance of the babesiosis card test was demonstrated by the fact that when Giemsa stained blood films of infected cattle were negative, the babesiosis card test was positive. Experiments are in progress to determine specificity and sensitivity of the agglutination reaction observed in the babesiosis card test.

h. To determine the antigenic variations of Babesia organisms.

Studies of possible antigenic differences in B. bigemina isolates from 2 different localities of Colombia have been concluded. These studies involved heterologous and homologous challenge of previously premunized cattle. A B. bigemina isolate from the Llanos and another

from the north coast of Colombia were used for these comparisons. No antigenic differences were detected by these procedures.

On the basis of data obtained from experiments designed to study antigenic variation of B. bigemina isolated from acute and chronic blood-borne and tick-borne infections, it can be concluded that variation exists and may play a role in developing resistance to babesiosis.

j. To develop non-bovine sources of Babesia organisms from ticks or tissue culture.

Attempts to grow B. bigemina in tissue culture systems, in an effort to provide a more economical source of antigenic material for immunization of cattle and serological tests, were continued. Although the study is not yet completed, efforts have been unsuccessful thus far.

Attempts to infect cattle with macerated tick tissues obtained from cattle known to be infected with B. argentina have been unsuccessful. It was hoped that this approach would provide a non-bovine source of infection but unfortunately we were unable to transmit infection by these measures.

k. To conduct pathogenesis studies.

A study has been made of the clinical, serological, and pathological manifestations of concurrent anaplasmosis and babesiosis in experimentally infected calves. The biological relationship of A. marginale and B. bigemina during the concurrent infection appeared to be one of independency. Neither an inhibitory nor a synergistic relationship was apparent. The clinical and pathological manifestations of concurrent

infection were more severe than those observed during infection with either of the hemoparasites alone and were attributed to the concurrent infection being additive in nature.

Trypanosomiasis:

Trypanosomiasis, caused by Trypanosoma vivax, is known to occur in Colombia, but the extent of infection, vectors, and the economic significance is not fully understood. In the absence of a specific diagnostic test information on these subjects has been difficult to obtain. A specific diagnostic test was developed for the diagnosis of T. vivax infection using an indirect fluorescent antibody test. This test has been used to screen over 3,000 serum samples, but to date the data has not been compiled in final form.

The principle of this test involves blood smears containing T. vivax, which serves as the antigen. The unknown bovine serum samples are placed on the slide If antibodies are present, they will attach to the antigen (T. vivax) on the slide. The slide is then counter stained with a fluorescein tagged anti-bovine globulin rabbit serum. If bovine globulin in the form of T. vivax antibody has attached to the T. vivax antigen, then the bovine globulin antisera will attach to the bovine globulin on the parasite and fluoresce, giving a positive diagnosis.

Theileriasis:

A Theileria resembling T. mutans has been isolated in Texas and passed one time in a splenectomized calf. No pathogenic effects of this organism have been detected at this time.

SUPPORTING EVIDENCE

Attached to this report is Appendix IV which provides greater detail of the experimental work.

RESEARCH DESIGN

The major emphasis in Colombia is on babesiosis and trypanosomiasis. In Texas, the major emphasis is on anaplasmosis and babesiosis.

It is intended that there be 2 senior staff plus 2 or 3 junior staff positions in Colombia, with similar equivalents at Texas A&M.

The work in Colombia and Texas compliments one another, and contributes a greater understanding and appreciation of the problem by providing an up-to-date exchange of information between all professional staff, which facilitates implementation of research findings and allows rapid confirmation, or second party examination, of research findings. Our present approach has worked well; however, there is room for improvement. An opportunity for the research staff to assemble more frequently than the present annual review would facilitate the exchange of information. Greater participation by research staff in field trials both in Colombia and the U.S. might increase the yield of information and accelerate progress.

The research approach is basically sound and workable for the type work in which we are engaged. We are basically following the procedures and plans as outlined in our previous proposals.

DISSEMINATION AND UTILIZATION OF RESEARCH RESULTS

A list of titles and abstracts of presentations made to scientific meetings and publications are listed in Appendix I. The relationship of

disease to productivity as applied primarily to this project plus an outline of anaplasmosis premunition techniques are presented in Appendix II.

The research capabilities established first at ICA, and later CIAT, have been used not only by our staff but also by Colombian veterinarians and students working on similar problems, and often in cooperation with our team. This project has markedly stimulated local interest in these problems and has contributed to a better appreciation for what can and should be done. The Colombian government (ICA) has constructed a modern laboratory at Turipana for the study of tropical hemotropic diseases.

Research reports concerning the efficacy of 4A65 have stimulated company officials (Burroughs Wellcome) to seek clearance from FDA for further experimental field testing in the U.S. Field trials based on premunition and treatment are underway in Colombia, utilizing past research experience.

The program has been fortunate in working with Colombian veterinarians with common interests. There have been a large number of these people who have worked with Texas A&M staff as students and associates, both in Colombia and Texas, who continue to cooperate and assist in our program.

During the past year we have had several visitors from overseas who have come for varying periods of time to become familiar with our techniques and research approach primarily in the area of anaplasmosis. Dr. Yusuf Aliu, a Nigerian veterinarian pursuing a M.S. in pharmacology and physiology, is working about 20 hours a week with us and being trained in techniques routinely used in our research effort. Miss Juliet Jarganauth, a laboratory technician from Guyana, spent about 2 months with us. Her major interest was to become thoroughly familiar with the complement-fixation test and related laboratory procedures. Dr. R. Rogers, a visiting

scientist from Australia, came in April and will spend 4-6 months working with us principally in the area of improved Anaplasma diagnostic antigens.

STATEMENT OF EXPENDITURES AND OBLIGATIONS AND CONTRACTOR RESOURCES

Funds were spent in the U.S. and in Colombia for budgeted items including manpower, equipment, travel, etc In Colombia local expenditures for wages, travel, etc., are made by CIAT on our behalf, and CIAT is periodically reimbursed from the AID account maintained at Texas A&M by the Office of International Programs. All other purchases and staff salaries (in Texas and Colombia) are handled through this office. At the present time, Dr. Galvin is paid half-time by CIAT, Dr. Adams half-time by the Department of Veterinary Pathology, and Dr. Todorovic half-time by the Department of Veterinary Microbiology. Most items of equipment and supplies for use in Colombia are first delivered to Texas A&M for transshipment to Colombia.

This has been a year of transition for staff both in Texas and Colombia, with a major relocation occurring for most. Drs. Adams and Todorovic moved from Colombia to Texas. Dr. Galvin moved from Texas to Colombia. Among the research assistants, Drs. Thompson, Craig, and Platt returned to the U.S. Dr. Wyss came to the U.S. for a few weeks but returned to Colombia. In January 1973, Mr. Michael Clark was assigned on temporary duty to the veterinary station at Turipana to assist this project.

In the coming year it is planned that Drs. Hopps, Thompson, and Corrier will be stationed in Cali to assist Dr. Galvin. Dr. Wyss will return to the U.S. Drs. Adams and Todorovic will remain at Texas A&M on a half-time status. Dr. Kuttler will remain on full-time status at Texas A&M.

State and industry support of activities at the Institute of Tropical

Veterinary Medicine has shown a significant increase during the past year, amounting to about $54,000.00. We have or will soon receive funds totaling $16,500.00 from Burroughs Wellcome Laboratories for continued research on Imidocarb. We have received about $17,500.00 from the state by way of salary and operation support funds. Closely related to our effort has been a $20,000 state grant to the Department of Parasitology to study the Boophilus tick problem and means of acaracide application for tick eradication.

In part because of the renewed local interest in anaplasmosis and babesiosis, we have recently been asked to prepare proposals dealing with these diseases for consideration of added financial support. There is an increasing awareness of the need for Boophilus tick research and the role this vector plays in the transmission of disease. The Chairman of the Texas Animal Health Commission has suggested the establishment of a laboratory in the U.S. where these problems could be studied. Such a facility plus the accompanying financial support could well contribute significantly to our primary aims to control hemotropic diseases of the tropics.

A budget statement for 1972-73 outlining expenditures and obligations for each of the major inputs and the major targets is given (Appendix III).

WORK PLAN AND BUDGET FORECAST FOR THE COMING YEAR

In general the programs at Texas A&M and Colombia will be oriented toward the completion of present projects and utilization of past research results to evolve systems and techniques for the more efficient and economic control of hemotropic diseases of food-producing ungulates.

Work in Colombia will include studies on all 3 major hemotropic diseases: anaplasmosis, babesiosis, and trypanosomiasis, but with an emphasis on the last two.

Research on hemotropic diseases at Texas A&M in the past has been productive, even though handicapped to some extent in that field studies on all agents are impossible. Even though the pathogenic cattle Babesias and T. vivax are not thought to be endemic in Texas, studies with Babesia, Trypanosoma, and Anaplasma are in progress. During the past year a pathogenic Babesia was isolated in Texas and the presence of B. microplus ticks confirmed. Greater emphasis on Babesia and the Boophilus ticks is expected in the coming year in Texas. Research emphasis will continue on anaplasmosis, largely because it is a local problem, field studies are possible, and the agent is readily available.

It is anticipated that data will be completed on the field trials at Turipana in which tests are being made of immunization and therapeutic programs for control of the hemotropic diseases. The study of tissue culture growth of Babesia will be completed. Using data from the trypanosome indirect fluorescent antibody test the incidence of T. vivax infection will be more geographically plotted.

Additional pathologic studies will be made, particularly of the toxic effects of Imidocarb and other possible chemotherapeutic agents. Work is continuing on tissue culture growth of Anaplasma, and while as yet unsuccessful it is hoped that future work may be more encouraging. Work will proceed using new therapeutic agents and combinations for treatment of anaplasmosis and babesiosis. Increased emphasis will be placed on the production of a more purified killed Anaplasma vaccine. Future field

trials will be conducted with the attenuated *Anaplasma* vaccine.

A budget statement for 1973-74 showing planned expenditures for each of the major inputs and the major work targets is given (Appendix III).

APPENDIX I

Institute Staff Publications
1968 - 1973

APPENDIX I

KUTTLER, K. L., ZARAZA, H. and ROBERTS, E. D.: Hematologic and Clinical Response to Anaplasmosis Vaccines and the Comparative Efficacy of These Vaccines, As Measured by Field and Experimental Challenge. Proceedings of the 5th National Anaplasmosis Conference, February 28-29, 1968, Stillwater, Oklahoma.

Twenty, 3-month-old calves were divided in 4 equal groups. Group 1 was inoculated with an attenuated Anaplasma marginale, group 2 received an A. marginale adjuvant vaccine, group 3 was infected with virulent A. marginale followed by treatment, and group 4 remained as unvaccinated controls. All animals were moved into an Anaplasma endemic zone 3 months later and allowed to undergo natural field challenge. Evidence of acute anaplasmosis was observed in all calves, except those premunized by virulent A. marginale. No significant evidence of protection was produced by either the attenuated A. marginale or the adjuvant vaccine when compared to the unvaccinated controls. The group premunized with virulent A. marginale failed to respond to natural exposure.

Hematologic response to virulent, attenuated, and killed A. marginale vaccines was measured in 18 mature cattle divided into 3 groups. The group receiving virulent A. marginale was treated 25 days after infection (Burroughs Wellcome Compound 356C61). No death losses occurred in this group, but moderate infections were observed to result in a significant reduction of PCV. The attenuated A. marginale vaccine produced a low level parasitemia, a marked serological response as measured by the complement-fixation test (CF), and a very slight drop in PCV, which was not significantly different from values observed in an unvaccinated, non-infected, control group. The group receiving adjuvant vaccine showed only a low level, transient, CF serological response.

An experimental challenge was administered 8 weeks after vaccination to cattle receiving the attenuated and adjuvant vaccines along with a group of 5 unvaccinated controls. All controls reacted to challenge with severe acute signs of anaplasmosis. One animal was allowed to die, a second would probably have died had it not been treated. Cattle receiving the attenuated vaccine showed no signs of active infection resulting from challenge. Cattle receiving the adjuvant vaccine reacted to challenge, but less severely than did the controls.

ZARAZA, H. and KUTTLER, K. L.: Respuesta Hematologica y Clinica a Diferentes Vacunas de Anaplasmosis y la Eficacia Comparative de estas, Evaluadas por la Inoculacion Experimental. Revista ICA, (Diciembre de 1968), 3, (4): 323-331. (Spanish translation of above.)

BISHOP, J. P. and KUTTLER, K. L.: Infectivity and Immunogenicity of Irradiated Babesia rodhaini. Journal of Protozoology (submitted).

Babesia rodhaini parasitized mouse blood exposed to varied doses of gamma radiation up to 30,000 r was inoculated into mice. Mice inoculated with non-irradiated B. rodhaini developed progressive infections and died 7 to 11 days after inoculation. Mice infected with B. rodhaini parasitized blood exposed to doses up to and including 22,000 r developed progressive parasitemias which were delayed in comparison to mice inoculated with nonirradiated B. rodhaini. Some mice receiving parasitized blood irradiated at 26,000 r did not develop progressive parasitemias. Progressive infections were prevented by exposure to irradiation at 30,000 r.

The results of two separate experiments revealed that one inoculation of parasitized blood exposed to 30,000 r or higher apparently stimulated a resistance to a challenge infection with nonirradiated parasitized blood. While 20 out of 20 control mice died as a result of challenging infections, 9 out of 28 mice previously exposed to irradiated parasitized blood survived.

The injection of irradiated nonparasitized blood did not produce a discernable acquired resistance to B. rodhaini. Presumably the irradiated parasitized blood was responsible for the development of acquired resistance to B. rodhaini.

CARSON, C. A.: An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of Anaplasma marginale. A Thesis submitted to the Graduate College of Texas A&M University in partial fulfillment of the requirement for the degree of Master of Science, August 1969.

An antigenic and serologic study was conducted using virulent strains of Anaplasma marginale from Texas and Colombia and an attenuated strain of Anaplasma marginale. Soluble antigens of the three A. marginale strains were compared by agar gel diffusion and immunoelectrophoresis. Serum proteins from calves infected with each of the three A. marginale strains were separated electrophoretically and reacted with rabbit anti-bovine serum in immunoelectrophoresis systems.

No differences between the soluble antigens of the three A. marginale isolates were detectable by agar gel diffusion. All three antigens moved to the same mobility zone in agar gel electrophoresis systems and each antigen formed an arc of precipitation when reacted with serum from calves infected with homologous or heterologous strains of A. marginale.

A beta and a gamma serum protein component, not exhibited in normal bovine serum, were present in the serums of animals infected with either of the virulent A. marginale strains or the attenuated strain.

CORRY, G. L. and KUTTLER, K. L.: Serological Activity of a Soluble Antigen of *Theileria cervi*. Experimental Parasitology (submitted).

Two basic cellular erythrocytic antigens were prepared from erythrocytes obtained from a white-tailed deer (*Odocoileus virginianus*) infected with *Theileria cervi*. The first antigen was prepared from erythrocytes lysed by freezing, the second from erythrocytes lysed with distilled water. The serologic activity as determined by the complement-fixation (CF) test was greater in the antigen lysed by freezing. Both antigens when solubilized at pH 7.2 using ultrasonic disintegration increased markedly in titer.

The two antigens were pooled and disrupted by ultrasonic disintegration in buffered mediums, ranging from pH 5 to pH 11. Optimal solubilization and serologic activity as measured with the CF test was obtained at pH 11.

The antigen solubilized at pH 11 was used to determine antibody in sera from infected deer by (CF) and by passive hemagglutination (PHA) tests. Both tests resulted in similar but not identical antibody titers.

A gel diffusion test and a ring (interfacial) test gave no valid results.

INDEX DESCRIPTORS: *Theileria cervi* effect of soluble antigen on complement-fixation and passive hemagglutination.

Theileria cervi is a hemoparasite of the white-tailed deer (*Odocoileus virginianus*), first described by Schaeffler (1961). It was thought by Marburger and Thomas (1965) and Robinson *et al*. (1967) to be a contributing factor of death losses among deer in Texas.

Other organisms of the same genus occur in different parts of the world where they cause diseases of varying severity in domestic and wild animals.

Laboratory diagnosis of these diseases depends primarily on microscopic detection of the parasites in stained smears. Serologic tests have been described by Schaeffler (1963), Kuttler and Robinson (1967), Kuttler *et al*. (1967), and Gadir *et al*. (1970). The antigens used in these tests were, for the most part, particulate. In this state, the cell membrane is most responsible for antigenic and serologic activity with the internal structure of the parasite cell not so greatly involved. It is not unreasonable to assume that the cytoplasm of the parasite cells contains a mosaic of antigens which when dispersed in molecular phase, i.e., when in solution, are capable of more specific or sensitive reactions. Such antigens could find wide use in serologic tests, and possibly prove of greater value in elucidating the antigenic relationship of similar intraerythrocytic parasites.

The purpose of this work, therefore, was to investigate the possibility of establishing a method of obtaining a soluble antigen from the erythrocytic stage of *T. cervi* that would react with the homologous antibody in an *in vitro* system.

KUTTLER, K. L.: Serial Passage of an Attenuated Anaplasma marginale in Splenectomized Calves. Proceedings of the 73rd Annual Meeting of the USAHA, October 12-17, 1969, Milwaukee, Wisconsin, 131-135.

Twelve serial passages of an attenuated Anaplasma marginale were made in splenectomized calves by blood inoculation. The severity of infection produced at the twelfth passage level in 4 splenectomized calves was compared to the infection occurring in 4 similar calves at a second passage level. Significantly higher parasitemias and lower packed cell volumes occurred in the twelfth passage group, suggesting an increased virulence. No deaths occurred among animals of the second passage group; whereas, 1 of 4 died in the twelfth passage group.

KUTTLER, K. L., ADAMS, L. G. and ZARAZA, H.: An Epidemiologic and Geographic Survey of Anaplasma marginale and Trypanosoma theileri in Colombia. 106th Annual AVMA Convention, July 1969, Minneapolis, Minnesota. Journal of the American Veterinary Medical Association, (June 1, 1969), 154: 1398, (abstract).

Anaplasmosis complement-fixation tests, packed cell volumes, and stained blood smears were made on 603 cattle located at 5 experiment station farms in Colombia. These farms were situated in differing climatic zones varying from 2,600 meters to 13 meters in altitude and from 13°C to 28°C in mean temperature. Specific reference was made to breed susceptibility, the influence of age, and climatic condition on the incidence and severity of infection.

A direct correlation was noted between mean temperature and incidence of anaplasmosis. At 13°C the incidence was nil; whereas, at 28°C over 90% infection was noted. The mean temperature is directly associated with altitude.

Incidence of infection in enzootic areas was generally greater in older animals, but the effect of infection as characterized by anemia was more noticeable in young animals. The incidence of anaplasmosis in European breeds did not appear greatly different when compared to native and Zebu cattle, but in some instances PCVs were significantly lower in European breeds. This was most marked at the lower elevations.

Blood cultures for T. theileri from 71 cattle at 2 experiment stations resulted in a pattern of infection similar to anaplasmosis. A high incidence of infection was noted at the lower elevation with a high mean temperature and no evidence of infection at 2,600 meters with a low mean temperature.

KUTTLER, K. L. and ZARAZA, H.: Premunization With An Attenuated Anaplasma marginale. Proceedings of the 73rd Annual Meeting of the USAHA, October 12-17, 1969, Milwaukee, Wisconsin, 104-112.

An attenuated Anaplasma marginale infection has been established in 21 calves and 12 mature cattle. The resulting infections were found to be significantly less severe than virulent A. marginale in 12 calves and 5 mature cattle. A slightly milder response to the attenuated A. marginale occurred in calves at Bogota with a mean temperature of 14°C when compared to calves similarly infected at Palmira with a mean temperature of 24°C.

Calves and mature cattle previously premunized with the attenuated organism appeared to be immune to virulent challenge using a Texas isolate of A. marginale. Experimental and natural challenge with a Colombian isolate resulted in evidence of acute anaplasmosis in both vaccinated and non-vaccinated animals.

TODOROVIC, R. A., ADAMS, L. G. and ROBERTS, D. E.: A Study of Bovine Babesiosis in Colombia, South America. Proceedings of the 106th Annual AVMA Convention, July 1969, Minneapolis, Minnesota. Journal of the American Veterinary Medical Association, (June 1, 1969), 154: 1399, (abstract).

Our research program on bovine babesiosis is a part of the Institute of Tropical Veterinary Medicine, College of Veterinary Medicine, Texas A&M University, with the research program being sponsored by the Rockefeller Foundation and conducted at the Laboratorio de Investigaciones Medicas Veterinarias laboratories, Bogota, Colombia, in cooperation with the Instituto Colombiano Agropecuario. This research effort is directed mainly toward the study and control of bovine babesiosis and the training of Colombian veterinarians and graduate students involved in these research projects.

Although bovine babesiosis is eradicated in the United States, the disease still occurs in most of the world and is of great importance as a threat to livestock industry, especially in the tropical areas of Latin American countries. In Colombia, babesiosis was first described by Lleras (1908) and later recognized as a widely distributed disease, causing great losses in purebred dairy cattle imported into enzootic areas. At the present time the incidence of babesiosis in Colombia is difficult to estimate. The disease exists as a mixed infection of Babesia bigemina, Babesia argentina, and Babesia major, and the incidence of infection appears to be related to the occurrence and activity of the tick vectors at the various altitudes.

The experiments were carried out to identify the existing Babesia species occurring in Colombia by morphologic, immunoserologic, pathologic, and chemotherapeutic methods. The immunoserologic relationship of Babesia spp. and strains were studied by gel-double diffusion precipitation, immunoelectrophoresis, and fluorescent antibody techniques. Attempts were made to develop a sensitive and practical serologic test for the diagnosis of the latent Babesia infection. Several groups of intact and splenectomized calves were inoculated with various antigens isolated from the blood of cattle with acute babesiosis and the blood from patent carriers, respectively. Response to vaccination, premunition, and challenge by tick-borne Babesia was recorded. The results of these experiments were discussed.

ZARAZA, H., KUTTLER, K. L. and ROBERTS, E. D.: Efectos de la Descarga Natural de Anaplasma marginale en Terneros Vacunados y no Vacunados. Revista ICA, (September 1969), 4, (3). (Spanish translation of: Kuttler, K. L., Zaraza, H. and Roberts, E. D.: Hematologic and Clinical Response to Anaplasmosis Vaccines and the Comparative Efficacy of These Vaccines, As Measured by Field and Experimental Challenge.)

ADAMS, L. G., HIPOLITO, O., MORALES, H., GONGORA, S. and JONES, L. P.: Dermatofilosis Bovina (Estreptotricosis cutanea) en Colombia. Revista ICA, (March 1970), 5, (1): 3-16.

Four cases of bovine dermatophilosis were diagnosed in Cordoba, Colombia and confirmed by bacteriological culture methods. Macroscopic and microscopic descriptions were made of the lesions caused by Dermatophilus congolensis.

ADAMS, L. G. and KUTTLER, K. L.: Toxicity of Alpha-Ethoxyethylglyoxal Dithiosemicarbazone in Cattle. American Journal of Veterinary Research, (August 1970), 31: 1493-1495.

Alpha-ethoxyethylglyoxal dithiosemicarbazone, administered 10 consecutive days at the dose rate of 5 mg/kg/day, caused axonal and myelin degeneration of the vagus nerve in 2 of 7 calves. Of the 7 experimental calves, 6 died of tympanites.

ADAMS, L. G. and TODOROVIC, R. A.: A Study of the Pathogenesis of Anaplasmosis in Intact Calves: Including Clinical, Clinical Pathological, Serological, and Immunofluorescent Techniques. Proceedings of the VI Congreso Panamericano de Medicina Veterinaria y Zootecnia, September 28 - October 3, 1970, Santiago de Chile, 37.

Twelve, 4-month-old, male, hemotropic disease-free, Holstein calves were inoculated subcutaneously with blood containing a Colombian isolate of Anaplasma marginale. Previous to inoculation 3 control samples were taken for bone marrow and blood determination.

Thereafter, samples were collected every 2 days and one calf was euthanatized every 2 days to collect a complete set of tissues for gross and microscopic pathological lesions as well as for the immunofluorescent study using the indirect technique. Results obtained are discussed, except those related to immunofluorescent study.

CARSON, C. A., ADAMS, L. G. and TODOROVIC, R. A.: An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of Anaplasma marginale. American Journal of Veterinary Research, (June 1970), 31, (6): 1071-1078.

Soluble antigens of 3 Anaplasma marginale strains were compared by agar gel diffusion and immunoelectrophoretic techniques. Serum proteins from calves infected with each of the 3 A. marginale strains were separated electrophoretically and tested with rabbit anti-bovine serum in immunoelectrophoretic systems. There was no detectable difference between the soluble antigens or the 3 A. marginale strains. A beta globulin arc, which was not detectable in normal bovine serum, was present in serum of acutely affected calves, and a gamma globulin arc was lengthened in the latter serum as compared with that in serum of normal calves.

KUTTLER, K. L. and ADAMS, L. G.: Comparative Efficacy of Oxytetracycline and a Dithiosemicarbazone in Eliminating Anaplasma marginale Infection in Splenectomized Calves. Research in Veterinary Science, (July 4, 1970), 2: 339-342.

Comparisons between oxytetracycline and a dithiosemicarbazone (356C61) were made in 11 splenectomized, Anaplasma marginale infected calves. Oxytetracycline was administered at the rate of 11 mg/kg intravenously (i.v.) for 5 and 10 consecutive days. Compound 356C61 was administered at the rate of 5 mg/kg i.v. for 5 and 10 consecutive days.

Compound 356C61 appeared to be relatively more effective in the treatment of anaplasmosis, as indicated by the relative increase in packed cell volume (PCV) following treatment, and by the apparent elimination of the carrier status in animals receiving the 10 daily treatments. Compound 356C61 administered daily for 10 consecutive days resulted in rumen atony, tympanites, and death.

KUTTLER, K. L., ADAMS, L. G. and ZARAZA, H.: Estudio Epizootiologico del Anaplasma marginale y et Trypanosoma theileri en Colombia. Revista ICA, (June 1970), 5, (2). (Spanish translation of: Kuttler, K. L., Adams, L. G. and Zaraza, H.: An Epidemiologic and Geographic Survey of Anaplasma marginale and Trypanosoma theileri in Colombia.)

KUTTLER, K. L. and ZARAZA, H.: A Preliminary Evaluation of a Dithiosemicarbazone for the Treatment of Anaplasmosis. Research in Veterinary Science, (July 4, 1970), 2: 334-338.

Trials were conducted on 3 splenectomized calves treated with a single intravenous (i.v.) inoculation of a dithiosemicarbazone (356C61) using 5 mg/kg, at different stages of induced anaplasmosis infection. When compared to an untreated control this compound was effective in reducing the severity of the infection. Hematological response was least severe in the animal receiving treatment before signs of parasitemia or a decrease in packed cell volume had occurred.

Treatment with compound 356C61 (5 mg/kg i.v.) of 5 splenectomized calves and 6 intact adult cattle early in the course of an artificially induced Anaplasma marginale infection prevented death loss and reduced the severity of the subsequent reaction when compared with non-treated controls.

TODOROVIC, R. A.: Babesiellosis Bovina en Australia. Revista de la Facultad de Medicina Veterinaria y de Zootecnia, (1970), 32, (1 & 2): 45-59.

Bovine babesiosis is still of great importance as a threat to the livestock industry in Australia. Due to the complexity of the epidemiology of this disease and other factors, the eradication of this hemoprotozoan malady is not possible at the present time.

The Commonwealth Scientific and Industrial Research Organization (CSIRO) is actively engaged in control and research on Babesia. Other research and teaching institutions involved in the same problem include: the University of Queensland; New South Wales, Department of Agriculture, Cattle Tick Research Station; Queensland State Department and Animal Health Station. All of these research projects on Babesia are sponsored mainly from the Government of Australia.

The Australian research workers have contributed more than a hundred scientific publications on the various areas of Babesia research; they are foremost in this field and the best trained in the world. The research laboratories are equipped with modern scientific tools, and staffed with well-trained technicians who successfully operate these instruments The facilities are excellent and designed particularly for Babesia research. (Slides of these facilities are available for those who are interested.)

The experience from this visit and knowledge obtained through discussion with Australian scientists working on different research projects will be invaluable for organizing a similar research program on Babesia in Colombia, South America. Furthermore, the Australian scientists with whom I visited all realized the importance of our mission in South America and expressed their willingness to cooperate with us in any manner in the future. They will be able to come to Colombia and spend time on short or long-term assignments if funds are available.

TODOROVIC, R. A., ADAMS, L. G., VIZCAINO, O. and GONZALEZ, E.: Research and Control of Bovine Babesiosis in Colombia. Proceedings of the VI Congreso Panamericano de Medicina Veterinaria y Zootecnia, September 19 - October 3, 1970, Santiago de Chile, 36

Research was carried out to develop an effective program for the control of bovine babesiosis in Colombia.

Experiments were carried out at the Palmira Instituto Colombiano Agropecuario (ICA) experimental station in Valle del Cauca (altitude 1,000 meters) to produce co-infectious and sterile immunity against bovine babesiosis. Calves randomly selected were divided into four groups according to the experimental design used to evaluate the immunoserological responses to vaccination against babesiosis and tick-borne challenge The degree of this immunity was determined by tick- and blood-borne challenge. The percentage of parasitemia (P), body temperature (T), and percentage of mortality (M) were used as the basis for comparing the reaction produced after vaccination and challenge. Experiments were conducted to evaluate the prophylaxis, therapy, effects, dosage, route of infection, toxicity, and response of the animals injected with a new Burroughs Wellcome babesiacidal drug No 4A65

On the basis of the observations made from these experiments, conclusions can be drawn that some degree of sterile immunity exists, besides the well known co-infectious (premunition) immunity in Babesia infections. To understand the exact mechanism of this type of immunity, more work needs to be done. The degree of resistance and the duration of immunity in relationship to different environmental conditions, strain differences, and the pathogenicity of the Babesia spp., and the quality of tick-borne challenge need to be determined.

TODOROVIC, R. A., GONZALEZ, E. F. and ADAMS, L. G.: Immune Response of Cattle Vaccinated Against Babesiosis in Colombia, South America. Proceedings of the American Veterinary Medical Association and the American Association of Veterinary Parasitologists, June 22, 1970, Las Vegas, Nevada.

Attempts to produce co-infectious and sterile immunity in cattle against Babesia infections have been carried out by vaccinating animals with live or killed Babesia vaccines at Palmira, Valle del Cauca, Colombia (altitude 1,000 meters) Immune responses of the vaccinated animals were evaluated by several immunoserologic methods The degree of resistance to tick-borne challenge (Boophilus microplus naturally infected with Babesia spp.) was determined by the percentage of recovery to normal parameters used in this study.

According to the experimental design used, a total of 110 animals were divided in 5 experimental groups to ascertain the immunologic responses. The first group consisted of 20 male, 85 kg, Holstein, 3-month-old calves which were premunized with Babesia bigemina, Babesia argentina, and 4 weeks later were exposed to tick-borne (Boophilus microplus) challenge. The second group consisted of 20 male, 95 kg, Holstein, 4-month-old calves subdivided into 4 groups and vaccinated with a killed Babesia vaccine derived from the erythrocytes and plasma, respectively, of animals acutely infected with Babesia bigemina and Babesia argentina The animals were inoculated with vaccine with or without Bacto-Adjuvant Complete H 37 Ra. The third group of 40 male, 80 kg, Holstein, 3-month-old calves was divided into sub-groups. The first sub-group consisted of 20 animals which were premunized with Babesia bigemina and Babesia argentina and 8 days later were treated with a new experimental babesiacidal drug The second sub-group which consisted of 20 animals was simultaneously premunized with Babesia spp. and Anaplasma marginale and later treated with their respective specific drugs The fourth group consisted of 20 female, 75 kg, Holstein, 3-month-old calves prophylactically treated with drug No. 4A65 and 3 weeks later exposed to Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina. The fifth group consisted of 10 animals used as controls. Responses to vaccination and tick-borne challenge were evaluated by packed cell volumes, percentage of parasitemia, body temperatures, body weight, complement fixing antibody titers, general physical conditions, and percent recoveries after tick-borne challenge. Results in general indicate that resistance to babesiosis can be produced by co-infectious or sterile immunity Experiments in prophylaxis, based on residual action of the babesiacidal drug, have given consistent and satisfactory results. In the future, it may be possible to develop control programs against bovine babesiosis based on these observations. The present status of these studies was described

TODOROVIC, R. A., GONZALEZ, E., MATEUS, G. and ADAMS, L. G.: Simultaneous Control of Helminths, Anaplasmosis and Babesiosis in Cattle. Revista de la Facultad de Medicina Veterinaria y Zootecnia, Bogota (accepted for publication).

A group of 50 male, Holstein-Friesian calves, 3 to 4 months old, were used to evaluate a control program for gastrointestinal and hemotropic parasites. The experiment was conducted at the ICA experimental station in Palmira, Valle del Cauca, at an elevation of 1,000 meters. The animals were divided into 3 groups.

Twenty animals were premunized against anaplasmosis and babesiosis simultaneously; 8 days later they were treated against babesiosis using the compound 4A65 at a dosage of 1 mg/kg of body weight, and 21 and 56 days after premunition they were treated intravenously with the compound 356C61 (5 mg/kg IV) against anaplasmosis.

Twenty animals were premunized against anaplasmosis as it was done with the animals in Group I. Animals in this group were vaccinated with AGS plus adjuvant vaccine against babesiosis. The vaccine was repeated 14 days later. Animals in Groups I and II were treated twice during the experiment with Ripercol (Tetramisol) against gastrointestinal parasites.

Ten animals were not treated and were used as controls.

All three groups of calves were kept under the same environmental conditions and the same management. The experiment was carried out during a period of 8 months. Blood samples were collected to evaluate anemia and parasitemia. The antibody titer was determined by the complement-fixation test. The body weights were measured and the fecal samples were examined for the presence of gastrointestinal parasites. Animals in Groups I and II had a high degree of resistance to babesiosis and anaplasmosis infections as a result of effective premunition and vaccination techniques. However, the animals in the control group had clinical babesiosis and anaplasmosis and high infestation with gastrointestinal parasites.

The importance of simultaneous control of gastrointestinal and hemotropic parasites is pointed out and methods to control these parasites are given.

ADAMS, L. G. and FERREIRA, W. L.: Necrobacilosis Neo-Natal en Ovinos. Revista ICA, (September 1971), 6, (3).

Five cases of ovine neo-natal necrobacilosis, in the Sabana of Bogota, were diagnosed in lambs less than 2 weeks of age. Macroscopic and microscopic lesions were described and the diagnosis was confirmed by bacteriological cultural techniques. This report constitutes the first known notice of the disease in neo-natal lambs in Colombia.

BISHOP, J. P.: Immune Response of Cattle Inoculated With Irradiated Babesia bigemina. A Dissertation submitted to the Graduate College of Texas A&M University in partial fulfillment of the requirement for the degree of Doctor of Philosophy, December 1971.

Babesia bigemina parasitized blood exposed to varied doses of gamma radiation up to 60 kRad was inoculated into calves. Calves infected with 1×10^{10} B. bigemina parasitized erythrocytes exposed to doses up to and including 30 kRad developed progressive parasitemias. Some calves receiving 1×10^{10} parasitized erythrocytes irradiated at levels of 36 and 42 kRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 kRad or higher. Subinoculations into susceptible splenectomized calves from parasites thus treated failed to produce active infections.

A degree of acquired resistance to infection with B. bigemina developed in calves after 1 inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The resistance was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction and a smaller increase in rectal temperatures following challenge. Presumably, the irradiated parasites were responsible for the development of resistance since irradiated nonparasitized blood did not produce a discernable acquired resistance.

The acquired resistance to infection with B. bigemina developed in calves inoculated with 1×10^{10} B. bigemina irradiated at 48 and 60 kRad was similar to the acquired resistance developed in calves inoculated with 1×10^{10} nonirradiated B. bigemina. It seems likely that the protective immunity produced with irradiated B. bigemina may be similar to that produced with living pathogenic B. bigemina in non-fatal infections. The acquired resistance to infection with B. bigemina developed in calves inoculated with 1×10^{10} B. bigemina irradiated at 48 and 60 kRad was much greater than the acquired resistance to infection developed in calves inoculated with 1×10^{10} heat killed B. bigemina. Thus, it seems likely that immunization with irradiated Babesia may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite. The Babesia parasites could be irradiated and frozen without apparent loss of immunizing properties.

DALEY, C. A.: A Sequential Study of the Pathogenesis of Disease Caused by Trypanosoma vivax in Experimentally Infected Calves, Utilizing Clinical, Pathological, Histopathological and Immunofluorescent Techniques. A Thesis submitted to the Graduate College of Texas A&M University in partial fulfillment of the requirement for the degree of Master of Science, May 1971.

Trypanosoma vivax obtained from a clinically sick cow near Neiva, Colombia, was passed in a sheep and a calf and inoculated into the jugular vein of 14 Holstein-Friesian calves. Fever occurred by 24 hours, and recurring parasitemia commenced after 72 hours. Associated with the first and subsequent parasitemias were decreases in hemoglobin, PCV, M:E ratio, serum albumin, A:G ratio and neutropenia.

All calves exhibited gradual weight loss by 2 weeks and later submandibular edema usually became evident. Consistent post mortem lesions seen after 4 weeks were conspicuously hypertrophied, edematous lymph nodes, hypertrophied hemal lymph nodes, emaciation, rounded right heart, palpably firm liver, atrophied thymus and hypertrophied femoral bone marrow.

Associated with T. vivax of the infecting inoculum and succeeding parasitemias were generalized endothelial hypertrophy and mononuclear cell infiltration along blood and lymph vessels with proteinuria and bone marrow hyperplasia. At 3 weeks there were aggregations of macrophages containing engulfed material distributed along capillaries in pulmonary interalveolar tissue, and this lesion in combination with the anemia and apparent cardiac insufficiency were thought important in the development of anoxia, and probably contributed to the single fatality observed.

GONZALEZ, E. F., TODOROVIC, R. A. and ADAMS, L. G.: Ultraestructura de la Babesia bigemina. Revista ICA, (March 1971), 6, (1): 89-112.

The morphology and some aspects related to the reproductive and feeding mechanism of Babesia bigemina have been studied by means of electron microscopy.

Although there are reports in the literature of the fine structure of Babesia canis which affects dogs, Babesia caballi which affects horses, and Babesia rodhaini which affects rodents, there is no report on the ultrastructure of B. bigemina which infects cattle.

B. bigemina was isolated from naturally infected cattle in the Valle del Cauca, Colombia, and maintained in splenectomized calves in the Laboratorio de Investigaciones Medicas Veterinarias in Bogota. Blood samples were collected from the splenectomized animals at a time when the percentage of parasitized erythrocytes was 25%, and these samples were used for electron microscopic studies.

By means of the electron microscope, different stages of B. bigemina were revealed such as oval, conoid and most commonly, pear shaped. The sizes of these forms were 2.5 to 6.5 microns in length by 2.3 microns in width. The young forms of the parasite were 1.5 by 2.5 microns. All these forms of parasites are surrounded by a dense cytoplasmic membrane which contained endoplasmic reticulum in the form of vesicles; these vesicles are composed of granules of different density. The endoplasmic reticulum appears as a homogenous mass with transparent vacuolar structures which are oval and spherical in shape. In addition to the endoplasmic reticulum, well defined dense polar bodies were found which appeared as oval shaped organelles, which communicated with the conoid part of the parasite by canals. The nucleus is the largest internal structure of the parasite and occupies one-fourth to one-third of its body. The nucleus is surrounded by a single membrane. Nucleoli were not revealed by electron microscopy.

Reproduction of B. bigemina appears to be carried out in two ways - by budding and binary fission. On the basis of these observations it is not clear which means of reproduction is more predominant. It is possible that both forms take place at the same time.

The feeding mechanism is not apparent. It appears that polar bodies play some role in this mechanism. These polar bodies could assume the function of food reservoirs of the parasite. It was also revealed that food vacuoles are similar to those in malarial parasites. The formation of food vacuoles probably results from an end process of pinocytosis as was described for Plasmodium species. We believe that both processes are involved in the feeding mechanism of Babesia parasites. Results of this study confirm the previously reported observation that there is no formation of pigment granules in Babesia; this implies that digestion of the host hemoglobin is complete; in contrast, malarial parasites form hemozoin, a blood pigment, as an end product of metabolism.

KUTTLER, K. L.: Efficacy of Oxytetracycline and a Dithiosemicarbazone in the Treatment of Anaplasmosis. American Journal of Veterinary Research, (September 1971), 32: 1349-1352.

The combination of a dithiosemicarbazone (356C61) and oxytetracycline proved more efficacious in the treatment of anaplasmosis than did either drug administered alone. The Anaplasma marginale carrier state in splenectomized calves was suppressed for as long as 120 days and was possibly eliminated by 3 injections of 356C61 (5 mg/kg) and oxytetracycline (11 mg/kg) given simultaneously at 48-hour intervals.

KUTTLER, K. L.: Promising Therapeutic Agents for the Elimination of Anaplasma marginale In the Carrier Animal. Proceedings of the 75th Annual Meeting of the USAHA, October 27, 1971, Oklahoma City, Oklahoma, 92-98 (published 1972).

Two new drugs, a dithiosemicarbazone (356C61) and 3,3'-bis(2-imidazolin-2-yl)-carbanilide dihydrochloride (4A65) have been successfully used to treat splenectomized calves with anaplasmosis. Carrier infections were eliminated with 5 or 10 mg/kg 356C61 and 11 mg/kg oxytetracycline when given 3 times at either a 24- or 48-hour interval. In addition, 5 mg/kg 356C61 plus 2 mg/kg 4A65 given 3 times at 24-hour intervals was effective in eliminating A. marginale infections. Levels of 4 and 6 mg/kg of 4A65 given 3 times at 24-hour intervals has proven successful in eliminating A. marginale infection.

KUTTLER, K. L., GRAHAM, O. H. and JOHNSON, S. R.: Apparent Failure of Boophilus annulatus to Transmit Anaplasmosis to White-Tailed Deer (Odocoileus virginianus). Journal of Parasitology, (June 1971), 57: 657.

Transovarial transmission of anaplasmosis occurred when two splenectomized calves were infested with unfed larvae of Boophilus annulatus, but no evidence of infection was detected in 2 intact white-tailed deer after they were infested with other larvae of common origin. All attempts to isolate Anaplasma marginale from the 2 deer by transfer of blood into splenectomized calves were unsuccessful.

MULLENAUX, C. H. and ADAMS, L. G.: La Oncocercosis Equina Asociada con el Mal de la Cruz en Colombia: Descripcion de dos casos. Revista ICA, (September 1971), 6, (3).

Two cases of equine fistulous withers were diagnosed in which Onchocerca spp. was found to be present in the affected tissues. One of the horses had a brucellosis antibody titer of 1:50 using the rapid plate agglutination method and, in the same animal, Brucella spp. was cultured from the suppurative materials of the nuchal bursitis of the withers. Macroscopic and microscopic pathological lesions caused by the nematode Onchocerca spp. were described.

TODOROVIC, R. A. and ADAMS, L. G.: Serologic Diagnosis of Babesiosis. Proceedings of the XIX World Veterinary Congress, August 15-22, 1971, Mexico City, 1114-1116.

The detection of the carrier state of bovine babesiosis has presented a particularly difficult problem because the blood from a high percentage of carrier animals does not contain sufficient Babesia parasites on which to base the diagnosis. Therefore, a great deal of past interest was concerned with the development of serologic techniques which would aid in diagnosing babesiosis.

In this review an attempt has been made to summarize and discuss the recent advances on sero-diagnosis of babesiosis in infected cattle with special attention to the serologic procedures used in the Laboratorio de Investigaciones Medicas Veterinarias located in Bogota, Colombia. In the last two decades fundamental knowledge concerning the immuno-serology of several Babesia spp. has led to the development of sero-diagnostic procedures for detection of Babesia antibodies. The antigens used in these techniques originated from parasitized erythrocytes and serum or plasma of animals with acute babesiosis, and they were applied in several serologic tests. The complement-fixation reaction constituted one of the earliest tests for the diagnosis of babesiosis. In recent years considerable progress was made to improve the complement-fixation test for the diagnosis of babesiosis. In addition, gel precipitation, fluorescent antibody, and agglutination techniques were applied for the detection of specific Babesia antibodies utilizing antigens from the parasitized erythrocytes as well as acute serum.

The investigations described in this report were conducted to develop the new techniques and to evaluate existing techniques for diagnosing bovine babesiosis. Research was executed in collaboration with the Instituto Colombiano Agropecuario in the Laboratorio de Investigaciones Medicas Veterinarias in Bogota, Colombia. Antigens of Babesia spp. were isolated by means of two techniques and used in the complement-fixation test for the detection of Babesia antibodies in cattle experimentally and naturally infected. By means of the complement-fixation test it was possible to detect specific antibodies in the serum of cattle 8 days after blood-borne infection. A total of 5,420 serum samples of cattle infected with babesiosis were tested. The cattle were from several Colombian experimental herds with known histories of babesiosis located in Valle del Cauca, Rio Magdalena, Llanos and Monteria and from cattle artificially infected in the Laboratorio de Investigaciones Medicas Veterinarias in Bogota. Approximately 95% of these samples were positive; whereas, about 5% gave discordant reactions. In addition to the complement-fixation test used in our laboratory, attempts were made to apply the double-gel diffusion for characterization of Babesia spp. antigen-antibody reactions. A cross reaction was noted between Babesia bigemina and Babesia argentina in this system. The application of latex-agglutination and hemo-agglutination tests for the detection of the Babesia antibodies are still under investigation in our laboratory. As a result of these investigations and observations, it is apparent that more investigation is needed for the development of a practical serologic technique for the diagnosis of babesiosis and to help solve this complex biological disease problem in tropical and sub-tropical areas of the world.

TODOROVIC, R. A., LUQUE, G. F. and ADAMS, L. G.: Contribution to the Study of the Tick Distribution in Colombia, South America. Revista de la Facultad de Medicina Veterinaria y Zootecnia (accepted for publication).

The purpose of this work was to collect and identify tick species involved in the epizootiology of bovine babesiosis in Colombia. Bovine babesiosis was reported in Colombia in 1888 but there is not any published evidence about tick species involved in the transmission of the disease. Although Boophilus microplus is the predominant tick in medium and hot climates, the exact distribution of the tick in the different regions of Colombia is not known. To develop an effective control program, the distribution of tick species needs to be determined. This is the first attempt to obtain this information about tick distribution in Colombia.

Animals naturally infected with Babesia bigemina and Babesia argentina were used as a tick collection source. The infectivity of these animals was determined by blood smears and complement-fixation techniques. The animals were located on farms in Palmira (Valle del Cauca) and Turipana (north coast), Magdalena River and Sumapaz River. Ticks were collected from different breeds: Holstein-Freisian, Zebu, Blanco Orejinegro, and Costeno con Cuernos, in animals of different ages. The ticks were collected from different parts of the animal bodies and preserved in Ethanol 95%. Adults, nymphs, and larvae were collected from both sexes for identification purposes during a 12-month period (January - December).

Dermacentor nitens was found in the animals infected with babesiosis in the Valle del Cauca, north coast, Sumapaz River; Amblyomma cayennense was found in animals infected with babesiosis and anaplasmosis in the Magdalena River and the north coast; Boophilus microplus was found in the same animals infected with babesiosis and anaplasmosis used in this experiment. Until the present time the significance of the findings of Amblyomma and Dermacentor ticks in epidemiology of babesiosis is not clear.

Experiments are in progress to determine the population and distribution of the tick species in other parts of Colombia for the purpose of investigating the exact role of Dermacentor nitens and Amblyomma cayennense in the transmission of bovine babesiosis.

ZARAZA, H. and KUTTLER, K. L.: Comparative Efficacy of Different Immunization Systems Against Anaplasmosis. Tropical Animal Health & Production, (1971), 3: 77-82.

Animal response to anaplasmosis vaccination was measured using an attenuated organism, a killed adjuvant vaccine, and a virulent Anaplasma marginale. A total of 7 calves (2-4 months of age) and 5 heifers (18 months of age) received the attenuated organism; 8 calves were given the adjuvant vaccine; 7 calves were premunized with virulent A. marginale; and 7 calves remained as non-vaccinated controls. The animals were vaccinated at Tibaitata on the Bogota Savannah and later moved to the north coast of Colombia, an anaplasmosis enzootic area.

All vaccination methods produced positive CF results. The live agents resulted in low parasitemias in most instances, although the attenuated organism was particularly mild in the younger animals.

Protection from field challenge was observed in all calves premunized with virulent organism, and in 2 of 5 heifers premunized with the attenuated organism. All other vaccinated animals developed anaplasmosis which was equally as severe as seen in the non-vaccinated controls.

ADAMS, L. G.: Epizootia Espontanea de Hepatitis Toxica en Porcinos Atribuida a Aflatoxicosis. Revista ICA (accepted for publication).

Nine of the 56, 4- to 6-month-old Duroc male and female pigs died 2 months after consuming a ration consisting of 8.75% moldy peanut meal. The pigs exhibited weight loss, roughened hair coats, anorexia, lethargy, icterus, melena, increased followed by decreased rectal temperature and death. The livers of the remaining 45 pigs were condemned due to cirrhosis. Serum sorbitol dehydrogenase activities, glutamic-oxaloacetic transmission activities, bilirubin concentrations, serum beta globulin levels, serum gamma globulin levels, and total serum protein concentrations were increased as serum albumin/globulin rations, albumin levels, packed cell volume and hemoglobin contents were decreased. No changes were observed in total leukocyte counts or serum alpha globulin levels.

The principal macroscopic lesions consisted of generalized icterus, petechial and ecchymotic hemorrhages with yellow transudates occurring in the body activities. Subendocardial as well as subserosal ecchymotic hemorrhage were commonly observed. Ulceration of the gastric fundus occurred which filled the stomach, duodenum, jejunum, ileum, and colon with free digested and undigested blood. The liver was pale yellowish-brown, firm (increased cutting resistance), and cirrhotic with very accentuated hepatic lobules outlined by translucent bands. Hundreds of irregular round yellow to brown foci of hepatic nodular regeneration were interspersed throughout the hepatic parenchyma. The gall bladder was moderately edematous and contained a small amount of light green bile. The principal microscopic lesions of the liver were disorganization of the hepatic architecture, acinus formation, severe sinusoidal fibrosis, mild biliary hyperplasia, advanced hepatic nodular regeneration, extensive hepatocellular megalocytosis, hepatocellular anisocytosis, mild hepatocellular necrosis, fatty metamorphosis, and moderate cholangiolar bile plug formation. The diagnosis and etiology of these 4 cases of porcine chronic toxic hepatitis was attributed to aflatoxicosis apparently produced by Aspergillus flavus growing on peanut meal. The present article is the first report of aflatoxicosis in Colombia.

ADAMS, L. G., CRAIG, T. M., PLATT, K. B. and WYSS, J. H.: Bovine Eperythrozoonosis in Colombia. Revista ICA (accepted for publication).

Eperythrozoon wenyoni, E. teganodes and E. tuomii were diagnosed in 14 of 37 splenectomized, Holstein-Freisian, 4- to 11-month-old calves that originated from the Sabana de Bogota. Eleven calves had pure infections of E. wenyoni, 2 calves had dual infections of E. wenyoni and E. teganodes, and 1 calf had a pure infection of E. tuomii. The diagnosis was determined on Giemsa-stained blood smears, and morphological descriptions of the Eperythrozoon spp. were given. Six splenectomized calves exhibited depression and anorexia, but all 14 calves had elevated rectal temperatures. Two calves had serous conjunctivitis with excessive lacrimation. The increase in rectal temperature coincided with the onset of parasitemia while the packed cell volume decreased after the onset of parasitemia. The average incubation period and standard deviation was 14.9 ± 3.5 days post-splenectomy. Treatment with 2-di-(Beta, gamma-dioxipropil)-(aminofenol)-(4 arseno 5)-Beta-(benzaxozalil)-(2)-mercaptol-proprionato de sodio at 29 mg/kg intramuscularly caused the parasitemia to become undemonstrable within 24 hours with further recrudescence occurring within 6 weeks. This is the first report of bovine eperythrozoonosis due to Eperythrozoon wenyoni, E. teganodes and E. tuomii in Colombia.

ADAMS, L. G. and TODOROVIC, R. A.: The Chemotherapeutic Efficacy of Imidocarb Dinydrochloride in the Elimination of Concurrent Bovine Anaplasmosis and Babesiosis. Tropical Animal Health & Production (accepted for publication).

Intact Anaplasma marginale, Babesia bigemina and Babesia argentina carrier calves treated intramuscularly 5 or 10 times with 2.5 mg/kg of imidocarb dihydrochloride at 48-hour intervals eliminated the Babesia infections, but not Anaplasma infections. The parasitemias became microscopically undemonstrable within 4 days following the first treatment, and the packed cell volumes increased significantly within 18 days. Intoxications resulting in fatalities occurred in 5 of 6 calves given 10 intramuscular treatments of 2.5 mg/kg of imidocarb dihydrochloride at 48-hour intervals.

ADAMS, L. G. and TODOROVIC, R. A.: Chemotherapy of Experimental Concurrent Bovine Anaplasmosis and Babesiosis With Imidocarb Dihydrochloride. Tropical Animal Health & Production (accepted for publication).

The chemotherapeutic efficacy of imidocarb dihydrochloride (3,3'-bis(2-imidazolin-2-yl)-carbanilde dihydrochloride) administered as single intramuscular doses of 1.0, 2.0 and 2.5 mg/kg, against concurrent bovine anaplasmosis and babesiosis, is reported. Dosages of 2.0 and 2.5 mg/kg of imidocarb dihydrochloride rapidly inhibited acute ascending concurrent parasitemias of Anaplasma marginale, Babesia bigemina and Babesia argentina; however, 1.0 mg/kg had a minimal effect on A. marginale, but was very effective against B. bigemina and B. argentina. Imidocarb dihydrochloride at 1.0, 2.0 and 2.5 mg/kg inhibited the development of immunity of the acute Babesia spp. infections, making the calves more susceptible to babesiosis upon challenge. The inhibition of A. marginale parasitemias was directly related to increasing doses of imidocarb dihydrochloride; however, recrudescing and persisting post-treatment parasitemias also occurred more frequently at higher doses.

ARMSTRONG, J. M. and TODOROVIC, R. A.: Anaplasmosis of Cattle. Texas Agricultural Extension Service, A Fact Sheet, 1972, (abstract).

A brief description of anaplasmosis, with special emphasis on recent achievements in the field of diagnosis and control was discussed and summarized for Texas A&M University Extension Service publication. This fact sheet was written principally for livestockmen to make them aware of recent developments in the field of anaplasmosis control and action that can be taken for prevention and treatment of this hemotropic disease.

BISHOP, J. P. and ADAMS, L. G.: Babesia bigemina: Immune Response of Cattle Inoculated With Irradiated Parasites. Experimental Parasitology (accepted for publication).

Investigations were undertaken to study the effects of various radiation dosages on the infectivity and immunogenicity of Babesia bigemina. Calves infected with 1×10^{10} B. bigemina parasitized erythrocytes exposed to 24 kRad developed progressive parasitemias. Some calves receiving 1×10^{10} parasitized erythrocytes irradiated at 36 kRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 and 60 kRad. A degree of acquired resistance to infection with B. bigemina developed in calves after inoculation with parasites irradiated at 48 and 60 kRad. The resistance developed was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections due to challenge with nonirradiated parasites. Irradiated parasites were frozen without apparent loss of immunizing properties.

BISHOP, J. P. and ADAMS, L. G.: Combination Thin and Thick Blood Films for the Detection of Babesia Parasitemia. American Journal of Veterinary Research (accepted for publication).

A method for preparing and examining combination thin and thick blood films for the detection of Babesia spp. parasitemias was developed. A technique for staining the combination thin and thick films, using a phosphate-buffered Giemsa stain solution containing alkyl phenoxy polyethoxy ethanol was also described.

BISHOP, J. P., ADAMS, L. G., THOMPSON, K. C. and CORRIER, D. E.: The Isolation, Separation and Preservation of Babesia bigemina. Tropical Animal Health & Production (accepted for publication).

Experiments were performed to separate Babesia bigemina from contaminating organisms. Babesia bigemina was passaged serially through 5 splenectomized calves. The first calf was inoculated with blood carrying several different organisms, and subsequent subinoculations were done soon after blood smears from each calf were found to be positive for B. bigemina. Five blood passages were carried out in 6.5 days. Babesia argentina, B. major and A. marginale were eliminated as contaminants of the B. bigemina isolated after four passages. A frozen stabilate of the isolated B. bigemina was established.

HERNANDEZ, J. D., ROBERTS, E. D., ADAMS, L. G. and VERA, T.: Pathogenesis of Hepatic Granulomas in Turkeys Infected with Streptococcus faecalis var. liquefaciens. Avian Diseases, (January - March 1972), 16, (2): 201-216.

The pathogenesis of hepatic granulomas in turkeys has been studied by reproducing the lesions experimentally with Streptococcus faecalis var. liquefaciens isolated during a field outbreak of turkey hepatic granulomas in Colombia. The 170 turkey poults (Bronze) used were 4 weeks old. Groups of poults were inoculated intravenously or orally with 0.1 ml of a 24-hour culture of Streptococcus faecalis var. liquefaciens at a dilution of 3 x 10 on the MacFarland Nephelometer Standard 10. The oral route of inoculation reproduced a disease most similar to the naturally occurring disease.

Clinically, the acute phase of infection was characterized by a high mortality rate in the first to seventh days but only sporadically thereafter. The septicemic phase produced the formation of septic thrombi which localized in various organs, producing infarction with heterophilic infiltration. Once the septicemic phase of the problem passed, the disease was manifested primarily by a focal hepatitis initiated primarily as a focal necrotic cholangial lesion. The biliary epithelium had hyperplastic to degenerative processes which participated in the formation of biliary thrombi. Granulomas were characterized by focal areas of necrosis surrounded by Langhans-type giant cells and macrophages.

KUTTLER, K. L.: Combined Treatment With A Dithiosemicarbazone and Oxytetracycline to Eliminate Anaplasma marginale Infections in Splenectomized Calves. Research in Veterinary Science, (November 1972), 13, (6): 536-539.

A total of 12 treatment schedules combining oxytetracycline and an alpha-dithiosemicarbazone (356C61) were tested on 36 splenectomized calves carrying Anaplasma marginale infections. Anaplasma infection was eliminated following the administration of 5 or 10 mg/kg 356C61 combined with 11 mg/kg oxytetracycline, and given 3 times at 24 or 48 hour intervals. Treatments employing lower levels, fewer injections, or at greater time intervals failed to eliminate infection.

Treated, splenectomized calves failing to show evidence of an A. marginale relapsing infection within 62 days were found to be free of infection on the basis of infectivity trials conducted an average of 87 days after treatment, and by re-inoculation with A. marginale an average of 164 days after treatment.

KUTTLER, K L.: Comparative Response to Premunization Using Attenuated Anaplasma marginale, Virulent A. marginale and A. centrale in Different Age Groups. Tropical Animal Health & Production, (1972), 4: 197-203.

Premunizing infections using virulent Anaplasma marginale (VAM), attenuated A. marginale (AAM) and A centrale (AC) have been induced in 46 mature cattle, 33 intact calves, and 38 splenectomized calves, for the purpose of comparing the relative response to these infections.

The VAM produced significantly more severe reactions in adult cattle and splenectomized calves, and a slightly more severe response in intact calves; however, these animals were relatively more resistant to all three infections. There was no detectable difference between the reactions caused by AAM and AC when measured in adult cattle and intact calves. Among splenectomized calves, however, the AAM infections resulted in a milder response as measured by the relative drop in packed cell volume and percent parasitemia. The CF response was significantly lower in the AC infection.

KUTTLER, K. L., GRAHAM, O. H., JOHNSON, S. R. and TREVINO, J. L.: Unsuccessful Attempts to Establish Cattle Babesia Infections in White-Tailed Deer (Odocoileus virginianus). Journal of Wildlife Diseases, (January 1972), 8: 63-66.

Attempts to induce a demonstrable cattle Babesia infection by feeding known infected ticks on two white-tailed (Odocoileus virginianus) deer were unsuccessful. The injection of known Babesia carrier blood into an intact and a splenectomized deer failed to result in evidence of infection.

All deer were checked for possible sub-patent infections by inoculating their blood into splenectomized calves at weekly intervals for 5 weeks following exposure, but no infections were produced in the calves.

Babesia infected ticks having undergone one generation on deer were unable to transmit infection to splenectomized calves on the succeeding generation.

CORRIER, D. E. and ADAMS, L. G.: Observations During Concurrent Anaplasmosis and Babesiosis in Experimentally Infected Calves. Proceedings of the 6th National Anaplasmosis Conference, March 1973, Las Vegas, Nevada.

The clinical, serological and pathological manifestations of disease in intact calves concurrently infected with Anaplasma marginale and Babesia bigemina were investigated. Clinical signs were more severe in the concurrently infected calves than in singularly infected controls. Decreases in packed cell volume, albumin:globulin ratio, myeloid:erythroid ratio and increases in the number of reticulocytes, total serum proteins and serum gamma globulins were more pronounced in the concurrently infected calves. The concurrent infections had no apparent effect on the production of complement fixing antibodies. Gross lesions observed in the concurrently infected calves included: pleural and peritoneal transudates; splenomegaly; hepatomegaly; and moderate lymph node enlargement. Histological lesions included: moderate hepatocellular degeneration; lymphoid hyperplasia in the spleen and lymph nodes; and hemosiderosis of the spleen, lymph nodes, liver and kidneys. The relationship of A. marginale and B. bigemina during the concurrent infections appeared to be one of independency. The increased severity of the clinical and pathological signs of disease in the concurrently infected calves was attributed to the concurrent infections being additive.

KUTTLER, K. L.: Current Status of the Tropical Cattle Fever Tick Boophilus microplus and Texas Wildlife. Presented to the Wildlife Society, Texas Chapter, Annual Meeting - April 5, 1973, Barnet, Texas, (abstract).

The increasing presence of both Boophilus annulatus and Boophilus microplus have created considerable concern among both the Texas livestock industry and those interested in the preservation and maintenance of wildlife. Even though both ticks can complete their life cycles on deer, B. microplus appears better adapted to deer. This tick is very versatile and is capable of maintaining itself on several wildlife species. Neither B. annulatus nor B. microplus are capable of transmitting any known diseases from deer to cattle or from cattle to deer.

Eradication plans have been successful in the past, particularly where B. annulatus was present. The feasibility of B. microplus eradication by similar means was discussed.

KUTTLER, K. L.: East Coast Fever (Theileriasis, Theileriosis, Rhodesian Tick Fever, Rhodesian Red Water). Submitted for publication in the 1973 revision of the "Foreign Animal Diseases" manual, U. S. Animal Health Association, Committee on Foreign Animal Diseases (In Press)

A review of the literature with a comprehensive discussion of East Coast Fever is given. This hemoprotozoal agent (Theileria parva) is considered by many to be the single most serious tick-borne disease occurring in East Africa. The 3 host tick, Rhipicephalus appendiculatus, is difficult to control and most ranches where it occurs will dip their cattle every week. The disease organism is readily transmitted by this and other tick vectors and produces a disease which may produce mortality of over 90%. This disease is limited to the African continent; however, similar infections caused by T. annulata and T. mutans have a much broader distribution. Theileria mutans is generally considered non-pathogenic, but T. annulata is a major disease producing hemoparasite, but generally of less virulence than T. parva

KUTTLER, K. L.: Review: Current Status of Control and Treatment With Drugs. Proceedings of the 6th National Anaplasmosis Conference, March 1973, Las Vegas, Nevada, (abstract).

A review of the literature was given, emphasizing those treatment techniques and reports since the initial use of the tetracyclines for anaplasmosis in 1951. Two new drugs, Imidocarb and Gloxazone, were discussed. These drugs, while more effective than the tetracyclines, have not been cleared by the FDA and remain experimental. At the present time, the tetracyclines are the only effective therapeutic compounds available commercially for the treatment of anaplasmosis. Current recommendations for the elimination of carrier infections are to feed an oral tetracycline for 60 days at the rate of 5 mg/pound of body weight.

KUTTLER, K. L. and TODOROVIC, R. A.: Arthropod Borne Protozoan Infections (Affecting Domesticated, Food-Producing Animals). Submitted for publication in the 1973 revision of the "Foreign Animal Diseases" manual, U. S. Animal Health Association, Committee on Foreign Animal Diseases. (In Press)

A review of the literature with a comprehensive discussion of anaplasmosis and babesiosis is given. In addition, tables of different Babesia spp., Theileria spp., Trypanosoma spp., Anaplasma spp., and Leucocytozoon spp. are given with reference to animals affected, morphology of the organism, and potential vectors. Brief descriptions of besnoitiosis and leucocytozoonosis are also given.

Despite progress in developing more effective acaracides and more efficient therapeutic agents, the arthropod borne hemoparasites remain a major disease problem in the tropics and the subject of intense research.

KUTTLER, K. L. and TODOROVIC, R. A.: Techniques of Premunization for the Control of Anaplasmosis. Proceedings of the 6th National Anaplasmosis Conference, March 1973, Las Vegas, Nevada.

Attempts at Anaplasma premunization in varying age groups have been reported using a virulent A. marginale of Texas origin, a virulent A. marginale of Colombian origin, an attenuated A. marginale, and A. centrale. Results of premunization response and the response to field and artificial challenge have been reported.

Premunization is a practical approach for the prevention of clinical losses associated with anaplasmosis, but a series of variables must be considered if success is to be achieved. Some of these factors are: (1) age of the animals being premunized, (2) virulence, potency, and size of the premunizing inoculum, and (3) the strain, or size, of the expected challenge exposure. In some instances, the use of a highly virulent A. marginale in adult cattle resulted in overly severe reactions even with treatment. Gloxazone (356C61) and Imidocarb (4A65) were superior to oxytetracycline in moderating the premunizing infection. Attenuated strains of A. marginale when used in young intact calves failed to produce the desired premunizing effect; however, attenuated strains were very effective in adult cattle. Premunization is dependent on establishing an active infection, and in the absence of such infection, susceptibility to field or experimental challenge occurs.

Successful premunization resulted in almost complete protection following challenge with antigenically similar A. marginale isolates. Protection was only partial, however, in instances where heterologous challenge was encountered.

MAURER, F. D.: The Need. Introductory Chapter for the 1973 revision of the "Foreign Animal Diseases" manual, U. S. Animal Health Association, Committee on Foreign Animal Diseases. (In Press)

As the introduction to a text on the diagnosis, prevention and control of diseases foreign to the United States, it is a chapter of justification for a national interest in exotic animal diseases. Justification is based upon the need to protect U.S. livestock industries; hence, U.S. food supply. Further justification is based upon world food deficits in relation to population growth and the need for ruminant food animals to utilize the world's vast forage and range resources that could not otherwise be converted to human food.

MAURER, F. D. and McCULLY, R. M.: African Horsesickness. Submitted for publication in the 1973 revision of the "Foreign Animal Diseases" manual, U. S. Animal Health Association, Committee on Foreign Animal Diseases. (In Press)

The chapter describes the history, geographic distribution, etiology, clinical character, pathology, diagnosis, preventive immunity, epidemiology and control of this most destructive disease of equine animals. As a major threat to the world's equines, African Horsesickness not only threatens the 13 billion dollar U.S. horse industry but threatens the food supply and economies of the many countries which still rely heavily on horses for agricultural power and transportation.

TODOROVIC, R. A.: Bovine Babesiosis: Its Diagnosis and Control. Proceedings of the 7th Panamerican Veterinary Congress, July 1973, Bogota, Colombia.

The investigation was conducted to develop new systems and to evaluate existing ones for the diagnosis and control of bovine babesiosis in Colombia, South America. Antigens of Babesia bigemina and Babesia argentina were isolated and used in the complement fixation and rapid agglutination tests for the diagnosis of babesiosis in cattle. Three systems were evaluated for the control of bovine babesiosis: (1) vaccination of susceptible cattle with killed Babesia spp. vaccine to produce resistance based on sterile immunity; (2) premunition of cattle with virulent Babesia spp., followed by chemotherapy to produce resistance based on co-infectious immunity; and (3) chemoprophylaxis based on the activity of babesiacidal compounds with prolonged residual action. All these systems were found effective in controlling bovine babesiosis under the conditions of these experiments. The epizootiological conditions of babesiosis enzootic areas will indicate which system is applicable. In zones with a high incidence of Boophilus microplus, the premunition is indicated; in areas where the tick population is controlled, or in areas at constant risk of tick exposure, the system of inducing resistance with killed Babesia spp. or chemoprophylaxis is indicated.

TODOROVIC, R. A. and KUTTLER, K. L.: Development of Rapid Agglutination Test for Diagnosis of Bovine Babesiosis. (manuscript prepared 1973)

Splenectomized calves were treated with an immunosuppressive drug (Azium) before they were infected with Babesia bigemina or Babesia argentina. These calves were used as a source of infective blood for isolation of B. bigemina or B. argentina agglutinating antigens for the diagnosis of bovine babesiosis. By means of a rapid agglutination technique it was possible to detect specific Babesia spp. antibodies in serum of cattle experimentally infected with B. bigemina or B. argentina when the antigen was used within 20 days after preparation and stored at 3-5°C. However, non-specific agglutination was observed with antigen stored more than 20 days at refrigeration temperature. Experiments are in progress to evaluate different systems of antigen preservation and application of the rapid agglutination test for diagnosis of cattle naturally infected with babesiosis.

TODOROVIC, R. A., GONZALEZ, E. F. and ADAMS, L. G.: Bovine Babesiosis: Co-Infectious Immunity to Babesia bigemina and Babesia argentina. Revista ICA (accepted for publication).

A total of 48 intact and 8 splenectomized calves were used to evaluate different systems of immunization against Babesia bigemina and Babesia argentina infections. These systems were based on premunition or co-infectious immunity in which the continued presence of a relatively small number of Babesia parasites protects the bovine host against otherwise lethal infections. Babesia spp. parasites were isolated from naturally infected cattle in the Cauca Valley and Monteria and pure isolates of B. bigemina and B. argentina were maintained in calves at the laboratory. The infectivity and pathogenicity of B. bigemina and B. argentina were established in splenectomized calves.

Co-infectious immunity was produced by two methods: (1) acutely infected blood with B. bigemina and B. argentina was used as the source of inoculum and the post injection reactions were controlled with Imidocarb or Ganaseg; and (2) by artificially inducing babesiosis and anaplasmosis with the blood of patent carriers of B. bigemina and B. argentina without chemotherapy. Since babesiosis and anaplasmosis occur as concurrent infections under tropical conditions, attempts were made to produce co-infectious immunity against both diseases.

Immune responses of calves to premunization and challenge were assessed by hematocrit values (H), parasitemia (P), temperature (T), body weight (BW), complement fixing antibody titers (AT), and mortality. The degree of resistance was determined by blood-borne and tick-borne challenges. Ticks were collected from all animals and identified as Boophilus microplus and Dermacentor nitens. Premunized cattle demonstrated a high degree of resistance to babesiosis; however, non-premunized suffered severe attacks of babesiosis and were treated with specific therapy to prevent death losses.

TODOROVIC, R. A., GONZALEZ, E. F. and ADAMS, L. G.: Bovine Babesiosis: Sterile Immunity to Babesia bigemina and Babesia argentina Infections. Revista ICA (accepted for publication).

Killed Babesia bigemina and Babesia argentina vaccine was prepared from the infected erythrocytes (AG-E) and from the infected plasma (AG-S) collected from acutely infected calves with Babesia bigemina and Babesia argentina. The vaccine was tested in Colombian cattle under field conditions in the Cauca Valley. A total of 40 calves two and one-half months of age received killed-Babesia vaccine, and 5 calves were not vaccinated; they served as controls. Vaccinated and non-vaccinated control calves were exposed to field-borne challenge with Boophilus microplus infected ticks. On the basis of the data obtained in these experiments, it was found that a high degree of sterile immunity to Babesia bigemina and Babesia argentina can be produced in calves injected with killed-Babesia vaccine. It appears that sterile immunity plays an important role in the mechanism of acquired immunity to babesiosis other than well known co-infectious immunity known as premunition.

TODOROVIC, R. A., VIZCAINO, O. G., GONZALEZ, E. F. and ADAMS, L. G.: Chemoprophylaxis (Imidocarb) Against Babesia bigemina and Babesia argentina Infections. American Journal of Veterinary Research (accepted for publication).

Twenty-nine calves were used for the evaluation of chemoprophylactic effects of Imidocarb (3,3'-bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride) for bovine babesiosis. The compound was found to have prophylactic and therapeutic properties in calves artificially or naturally infected with Babesia bigemina and Babesia argentina of Colombian (South American) origin. Administered intramuscularly at a level of 2 mg/kg, Imidocarb suppressed the development of acute babesiosis in calves treated 46 days previously and later exposed to a lethal dose of Babesia spp. infected blood. Imidocarb failed to protect against Anaplasma marginale infection. Calves treated intravenously with 2 mg/kg Imidocarb and challenged 20 days later with a lethal challenge of Babesia spp. infected blood were protected. For 90 days after challenge, all calves were found negative for Babesia spp. parasitemia and also by blood-subinoculation into fully susceptible splenectomized calves. Calves intravenously treated 21 days previously with 3 mg/kg Imidocarb resisted tick-borne challenge of Boophilus microplus. This resistance was evidenced for 15 weeks of field exposure by negative blood examinations and the mortality of non-treated calves from acute babesiosis. All calves treated with Imidocarb and subsequently exposed to blood- or tick-borne Babesia spp. responded with a rise of complement fixing antibodies. Imidocarb readily controlled very severe acute infections with B. bigemina and B. argentina at dose rates of 1 mg/kg by both intramuscular or subcutaneous routes. Acute signs of toxicity were observed following intravenous injections of 3 mg/kg of Imidocarb. Three calves died following this injection with symptoms of embarrassed respiration, oral respiration, excessive salivation, muscular fasciculations, urination, defecation, incoordination, and prostration. Toxic signs were milder for intramuscular or subcutaneous injections of Imidocarb.

APPENDIX II

1.) The Relationship of Disease to Productivity

2.) Anaplasmosis Premunition, General Recommendations

APPENDIX II-1

THE RELATIONSHIP OF DISEASE TO PRODUCTIVITY

From an animal health viewpoint, hemotropic diseases represent the greatest single deterrent to meat and milk production in the tropics. These diseases are transmitted by arthropods such as tsetse fly, ticks, and mosquitoes. They are usually responsible for severe to moderate anemias, which are associated with weight losses, drops in milk production, and, in cases of acute infection, death. Local indigenous cattle are thought to be resistant, in that deaths are not a common occurrence. Field and laboratory investigations in recent years confirm the relatively low mortality among indigenous cattle, but show an extremely high incidence of infection, accompanied by anemia, lack of growth, and low milk production. Frequently under these conditions the sick animals are not individually identified, since nearly all are infected. The resulting poor growth rate and low milk output is then tolerated as being normal for the area. To a degree this may be true, but a cursory review of history shows that animal production can be greatly increased by the elimination of hemotropic infections. Prior to 1920 the Texas cattle industry was severely hampered by *Babesia bigemina*, which prevented the successful introduction of clean, genetically improved breeds into the area and reduced the growth rates and production of indigenous cattle. These conditions were similar to those currently existing in Colombia. The elimination of this disease was accompanied by a rapid increase in beef and milk production.

The solution of the problem of babesiosis in Texas was facilitated when research showed that a single one host tick, *Boophilus annulatus*, was responsible for its spread. The problem is compounded in many tropical

countries, Colombia being a good example, by multiple tick vectors, and combined infections caused by Anaplasma, B. bigemina, B. argentina, B. major, and Trypanosoma. Notwithstanding these problems, recent research has revealed several approaches which offer the hope for more effective control and even possible eradication.

The economics of these diseases, or accurate calculations as to the dollar value of losses from these infections, is difficult but estimates are possible.

In 1962, Dr. W. T. Oglesby (Louisiana State University) estimated annual U.S. losses from anaplasmosis to be at least $35,000,000.00. A national survey of anaplasmosis is currently being conducted by the U.S.D.A. under the supervision of B. R. McCallon. On the basis of preliminary data, Dr. McCallon has estimated that current annual losses approach $100,000,000.

There are approximately 31,500,000 cattle in those states where anaplasmosis is most common. On the basis of a 16,232,697 cattle population in Colombia (Departmento Administrativo de Estadistica) it may be expected that an approximate $50,000,000.00 annual loss would be incurred if cattle values were equivalent. Assuming cattle values to be 66% of those in the U.S., then a loss of 34-35 million dollars might be expected annually in Colombia. This figure is really only a part of the annual losses due to hemotropic diseases, when we consider babesiosis and trypanosomiasis. Based on experimental results obtained at Turipana (1971-1972) losses from babesiosis are probably equal to those from anaplasmosis. These diseases are endemic throughout Latin America, therefore, this figure should be multiplied many times to acquire a true picture of the actual losses of this area.

In addition to the economic loss associated with blood parasites, a significant toll can be expected from the ticks which spread these diseases. Scientists in Australia have shown that an average daily infestation of 50 or more engorging adult Boophilus ticks caused an annual loss in growth rate of 1.67 pounds per tick, or meanly, 4 tons of beef in a herd of 100 animals. Similar loss of productivity can be expected in dairy cattle and would be reflected most often as a drop in milk production.

Perhaps of greater concern than the actual monetary losses which these diseases cause is the lack of productivity, the failure to achieve the full potential for which the land is capable, resulting in an absence of food and animal protein which are irreplaceable and can only aggravate the already serious shortages which exist in the world. Control of the hemotropic diseases and the arthropods responsible for transmission is an essential prerequisite to achieve the full measure of livestock productivity in tropical areas of the world.

Contributions by Colombia to This Program:

The seriousness of this problem is generally recognized throughout Latin America, and particularly so in Colombia. Colombian government agencies have contributed generously to the support of our program since its beginning in 1967. Until 1971 our staff and graduate students were housed at the LIMV laboratories of the Instituto Colombiano Agropecuario (ICA) in Bogota. During this period we received assistance in the acquisition and maintenance of laboratory animals, and also the use of laboratory space and facilities, at no cost to our project. In early 1971 a new veterinary laboratory at Turipana, on the north coast near Monteria, was completed, primarily for the study of hemotropic diseases. This laboratory

is headed by a former graduate student at Texas A&M. Field trials in cooperation with the ICA-Turipana station are presently underway in attempts to control hemoparasitic diseases.

The facilities available to us at Turipana are ideal for field testing new methods of control. The cooperation and assistance of local authorities has been excellent and shows every indication of continuing.

In 1971 our staff moved to the new Centro Internacional de Agricultura Tropical (CIAT) laboratories at Cali. The Colombian government made land available for CIAT and has a close relationship with this organization which cooperates with ICA.

Summary of Research Accomplishments:

(1) Premunization with virulent *Anaplasma* and *Babesia* in indigenous cattle maintained under lowland, tropical conditions has proven effective in significantly reducing mortality and production losses. The initial animal response to these virulent organisms was modified by specific chemotherapy.

(2) A new vaccine for anaplasmosis has been tested in Colombia and offers some promise despite initial setbacks. This vaccine premunizes cattle with practically no adverse reaction. Premunition of young calves with virulent organism can produce a persistent anemia for up to 50 days; whereas, the vaccine produces a milder response and an anemia that persists less than 8 days.

(3) A therapeutic approach has been developed which will completely eliminate infection with anaplasmosis with 2 or 3 injections which is more effective than previous methods.

(4) Since the inception of the Institute of Tropical Veterinary

Medicine, over 58 scientific papers have been presented for publication. This plus unpublished research data is forming the basis of current and future control programs for the containment of these costly diseases.

(5) Like the U.S., some areas of Colombia are free of these diseases. A survey has been completed which identifies problem areas and establishes very definite epidemiologic patterns of infection.

(6) Progress has been accelerated by the discovery of 2 new drugs, a dithiosemicarbazone (356C61) and Imidocarb (4A65), which show much greater activity as therapeutic agents, particularly when given together or in combination with other drugs.

(7) Imidocarb has been demonstrated to have prolonged prophylactic value against Babesia, and to prevent or eliminate infection in the tick vector.

(8) A card test is being tested as a babesiosis diagnostic procedure and shows considerable promise.

(9) Immunological studies have shown that a sterile immunity in Babesia can be induced in susceptible cattle, which is contrary to previously held theories that carrier status or co-infectious immunity was essential.

(10) Babesia argentina and B. bigemina have been shown to be immunologically different. A B. argentina challenge of a B. bigemina carrier produces a more severe reaction than the opposite challenge.

(11) Purified isolates have been made of A. marginale, B. bigemina, B. argentina, T. vivax, and T. evansi from cattle in Colombia.

(12) Non-infected Boophilus microplus ticks have been selectively infected with pure isolates of B. argentina and B. bigemina.

Training or Teaching Accomplishments:

(1) Four U.S. graduate students have completed their work for the M.S. degree and 1 has completed and received his Ph.D.

(2) We now have 1 candidate for the M.S. degree and 5 candidates for the Ph.D. degree currently pursuing their work.

(3) The research programs plus the training of Colombian students has focused attention on these hemotropic diseases to the extent that more work is being done to control these diseases by Colombians in their own country.

(4) In June 1971, Dr. R. A. Todorovic went to Peru at the request of the USAID mission there to aid in projecting an evaluation program for control of hemotropic diseases in cattle. This activity further emphasizes the point that much of the work done in Colombia will be applicable throughout Latin America.

Application of Research Findings:

A field program has been underway for over 2 years at Turipana putting into practice the scientific principles developed in the laboratory. Direct data from these experiments is not yet available, but it is known that when anaplasmosis and babesiosis are absent or controlled that productivity as measured by growth rates will increase.

The effects of moving clean, adult, dairy type stock from a hemotropic disease-free area, such as the Bogota Savanah, to the north coast without adequate vaccination or treatment is invariably followed by drastic weight loss, an almost complete drop in milk production, and in many instances death within 60 days. Treatment can in most instances prevent death, but the persistent re-exposure pattern prevents economic milk production. Weight gains of 4-6 month old calves moved to Turipana

showed the effects of hemotropic disease. The untreated controls showed an average gain of 36 pounds in the first 60 days, and for the next 60 days an average loss of 4 pounds for a net gain of 32 pounds. Among calves premunized with the attenuated vaccine an average gain of 52 pounds was recorded for a net increase of 20 pounds or 62% per calf. Among calves premunized with virulent Anaplasma an average weight gain of 56 pounds was recorded for a net increase of 30 pounds or 94% per calf.

If these figures are representative and we reflect this increased weight gain to a ranch weaning 1,000 calves we could expect 20-30,000 pounds more in live weight or 10-15,000 pounds more from essentially the same feed input.

The potential for increase in beef and milk production through the control of these hemotropic diseases is one which is well worth pursuing.

An increasing number of veterinarians and livestock producers are utilizing the therapeutic and immunologic approaches which have been demonstrated effective in preventing losses. Most of the research papers resulting from this work are published in spanish for greater distribution in Colombia and South America.

A recent field trial at Turipana (Colombia's north coast) has recently been completed. This trial demonstrated significant advantages to the use of vaccination and treatment procedures in indigenous calves to prevent hemoparasite losses. Twenty calves in which disease control was achieved showed an 89% increase in weight gains during a 257 day observation period, when compared to untreated controls.

ANAPLASMOSIS PREMUNITION - GENERAL RECOMMENDATIONS

Premunization involves the establishment of a chronic or carrier infection, preferably without inducing an acute phase. The resulting immunity is probably the most secure of any, and in the case of anaplasmosis the only practical immunization presently available for use in the tropics. There is a killed vaccine (Anaplaz) currently being marketed in the U.S. which produces a partial immunity and generally prevents death losses. This vaccine, while successfully used in the U.S., has been inadequate in Colombia.

There are several variables involved in premunization which must be considered if acute and possibly fatal initial reactions are to be prevented, and if adequate infection is induced to produce solid premunization. Some of these variables are:

(1) Age of the animal. (Calves are most resistant with susceptibility generally increasing with age)

(2) The strain or isolate of Anaplasma used for premunization.

(3) The size and virulence of the infecting inoculum.

(4) The drug used to moderate infection.

(5) Timing and dosage rates of the therapeutic agent.

Complete immunity can be expected only against antigenically homologous organisms. Even in these instances, if challenge is sufficient a transitory response of varying intensity is possible. It is believed that premunition, if properly done, will generally provide sufficient cross protection to prevent severe challenge response even with heterologous strains.

Infecting Organisms:

Premunization data ia available on 4 *Anaplasma* isolates:

(1) Colombian virulent (Monteria), CAM.

(2) Texas virulent (Texas A&M laboratory strain), TAM.

(3) Illinois attenuated (Univ. of Illinois - developed by and obtained from M. Ristic).

(4) *Anaplasma centrale* (Veterinary Laboratory - Kabete, Kenya).

Drugs Used to Moderate Infection:

(1) Oxytetracycline (Chas. Pfizer & Co.) or Chlortetracycline (Lederle, American Cyanimid Co.).

(2) Gloxazone (356C61) - (Burroughs Wellcome Co.).

(3) Imidocarb (4A65) - (Burroughs Wellcome Co.).

Infecting Inoculum:

(1) 1-5 ml blood injected S/C, freshly collected from a carrier calf in sodium citrate, heparin, or EDTA. Blood should not be stored longer than 1 hour at room temperature or 24 hours at 5°C. Extreme heat or cold should be avoided. Freezing at -20°C destroys infectivity.

(2) Blood from an acutely infected animal may be mixed with a cryoprotectant (Glycerol or dimethylsulfoxide, DMSO) and frozen for periods of up to 2 years at temperatures of -60°C or below.

Method 1: Glycerol is added, to give 8.2% V/V, to whole blood collected in heparin.

Method 2: Red cells are washed 1 time in PSS and added in equal volumes to 4M DMSO in PSS.

These frozen stabilates should be checked for their infectivity after freezing. The incubation time for the infecting inoculum should preferably be between 15 and 30 days.

Recommendations for Premunization

I. Calves less than 8 months of age.

A. Colombian virulent: treatment is recommended.
Tetracycline at the rate of 12 mg/kg should be given when parasitemia is apparent but does not exceed 3%. One to 3 injections should be given I/V, I/M, or I/P at 1 week intervals, or as needed.

B. Texas virulent: no treatment is recommended.

C. Illinois attenuated: no treatment is recommended.
Because of the mildness of this strain it is not recommended for premunition in this age group when the infecting inoculum has been previously frozen.

D. *Anaplasma centrale*: no treatment is needed.

II. Cattle 8 months of age and older.

A. Colombian virulent: Treatment consisting of 5 mg/kg 356C61 plus 12 mg/kg tetracycline given I/V is recommended in the early stage of infection, preferably when parasitemia is 1-3%. Treatment given 1 time is usually adequate, but a second treatment 1 to 2 weeks later may be indicated. One injection of Imidocarb at the rate of 5 mg/kg given S/C or I/M may be used in the early stages of infection. Clinical relapse has

more frequently followed the use of this drug. In the absence of 356C61 or 4A65, multiple injections of 12 mg/kg oxytetracycline given early in the course of infection is effective.

B. Texas virulent: Treatment is recommended in the early stage of infection.

(1) Tetracyclines at the rate of 6.6 mg/kg I/M or I/V given 2 or 3 times at 3 day intervals, to coincide with onset of parasitemia.

(2) 5 mg/kg 356C61 plus 11 mg/kg oxytetracycline given I/V one time, plus a second injection 1 week later if indicated.

(3) Younger animals of this age group may not require treatment.

C. Illinois attenuated: With the exception of lactating dairy animals, no treatment is recommended. In lactating dairy cattle, 1 to 2 treatments of 6 to 8 mg/kg I/V of tetracycline given 3 to 4 days apart may be desirable. Generally treatment is not recommended.

D. Anaplasma centrale: This organism has essentially the same virulence as the Illinois attenuated organism, hence, essentially the same treatment recommendations.

Host stress may influence the premunizing infection. When virulent organisms are used, mild to cool temperatures are recommended. This is not so much a factor with the attenuated organisms. Cattle movements should be delayed until recovery from the premunizing infection is complete. Frozen

inocula of the attenuated strain are often of marginal infectivity; for this reason, fresh bovine blood (no more than 2 passages away from sheep) or sheep blood is recommended for this procedure.

APPENDIX III

Budget Statement for Past Year (1972-73)

Statement of Expenditures According to Major Work Goals

	Anaplasmosis 30%	Babesiosis 48%	Trypanosomiasis 20%	Theileriasis 2%	Total (100%)
Personnel Salary & Allowances	55,286.00	88,458.00	36,857.00	3,686.00	184,287.00
Travel and Transportation	8,159.00	13,055.00	5,439.00	544.00	27,197.00
Equipment	1,326.00	2,122.00	884.00	88.00	4,420.00
Supplies and Operation	21,177.00	33,884.00	14,118.00	1,412.00	70,591.00
Overhead	2,546.00	4,075.00	1,698.00	170.00	8,489.00
Total	$88,494.00	$141,594.00	$58,996.00	$5,900.00	*$294,984.00

* $282,086.00 actually spent this year. The larger amount ($294,984.00) reflects committments and obligations incurred during this 12 month period.

Appendix 3

Budget Statement for the Coming Year (1973-74)

Statement of Expenditures According to Major Work Goals

	Anaplasmosis 34%	Babesiosis 45%	Trypanosomiasis 20%	Theileriasis 1%	Total (100%)
Personnel Salary & Allowances	68,320.00	90,424.00	40,188.00	2,009.00	200,941.00
Travel and Transportation	8,466.00	11,205.00	4,980.00	249.00	24,900.00
Equipment	2,720.00	3,600.00	1,600.00	80.00	8,000.00
Supplies and Operation	23,227.00	30,742.00	13,663.00	683.00	68,315.00
Overhead	19,667.00	26,030.00	11,569.00	578.00	57,844.00
Total	$122,400.00	$162,001.00	$72,000.00	$3,599.00	$360,000.00

Annual Research Report

A Research and Training Program in Tropical Veterinary Medicine
(AID-csd-1947, in Colombia)

Program Director: F. D. Maurer, D.V.M., Ph.D.
Associate Dean
College of Veterinary Medicine
Texas A&M University
College Station, Texas 77843

Period Covered by Report: April 1, 1972 to March 31, 1973

Appendix IV

Studies on Hemotropic Diseases

Papers and Manuscripts Prepared for the Period
April 1, 1972 to March 31, 1973

F. D. Maurer, D.V.M., Ph.D.
Director
Institute of Tropical Veterinary Medicine

Professional Staff:

L. G. Adams, D.V.M., Ph.D. - Texas A&M University - College Station

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BOVINE EPERYTHROZOONOSIS IN COLOMBIA *

L. GARRY ADAMS, THOMAS M. CRAIG,

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INTRODUCTION

Bovine eperythrozoonosis in Palestine was first described in 1934 by Adler and Ellenbogen (1). Since then, it has been diagnosed by several investigators (2, 4, 5, 6, 7, 8, 9, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, 25, 28, 29, 32, 32). Bovine eperythrozoonosis was reviewed by Weinman (30), Seamer (23) and Kreier (18).

Eperythrozoon wenyoni (Erythrocytozoon bovis) (18), stained by the Giemsa method, was described as reddish-purple discoid, thread-shaped, coccoid, rod-shaped or ring-shaped organisms (0.3 - 1.5 microns in diameter) found on the surface of erythrocytes and platelets or free in the plasma. The pleomorphic organisms may completely cover the periphery of the erythrocytes (2, 6, 14, 15, 17, 18, 21, 25, 28). Fluorescent antibody-stained E. wenyoni are similar to Giemsa-stained organisms (17), but acridine orange-stained E wenyoni appear as coccoids or chains of coccoids, 0.5 microns in diameter (18). Eperythrozoon wenyoni observed with phase contrast microscope appear as spheres (17). Ultrastructural studies of E. wenyoni revealed it to be a short rod or disk with granular masses in the cytoplasm, and it more closely resembled rickettsiae or bacteria than protozoa (17).

Eperythrozoon wenyoni is species specific (16, 17, 18, 22, 30) and possibly may be transmitted by nymphs of Ixodes ricinus (30), by Dermacentor marginatus and Rhipicephalus bursa (12) or by Hyalomma sp. (8). Eperythrozoon wenyoni exists in Japan (15), Madagascar (28), Iran (5), East Africa (2), Algeria (8), Australia (14), South Africa (21), Iran, Jerusalem, France (30), United States (4, 7, 15, 18, 20), Russia (12), Holland (31), Finland (25),

and possibly Venezuela (11). Concurrent infections of E. wenyoni with Anaplasma marginale (10) or Babesia bigemina (13) were shown to interfere with one another in the development of parasitemia. Furthermore, cross-reactions between E. wenyoni and A. marginale were demonstrated by the complement fixation and direct fluorescent antibody tests (17), but not by the passive hemagglutination test (9).

Eperythrozoon wenyoni exhibits a variable parasitemia which causes a mild anemia with little or no clinical manifestations in intact cattle, however, Crocker and Sutter (4) reported a case that apparently was related to E. wenyoni, causing clinical manifestations and mortality of Hereford cattle in the United States. Increased body temperature (1, 2, 10, 14, 15, 16, 25, 30), anorexia (25), moderate anemia (10, 14, 15, 16, 17, 25, 30), icterus (21, 25, 31), debilitation (25, 31), lacrimation and hyperemia of the conjuntivae (15) were observed in splenectomized cattle, Clinical pathological findings in splenectomized cattle were described as: decreased packed cell volumes (10, 15, 17, 25), hemoglobin concentrations (15, 17, 25), erythrocyte counts (17, 25) and serum globulin concentrations (7); increased leukocyte counts and sedimentation rates (15); unchanged mean corpuscular volumes and mean corpuscular hemoglobin contents (17). The mild anemia caused by E. wenyoni was described as normochromic-and normocytic, which suggests an anemia due to erythrocytic destruction (17).

On a morphological basis only, Eperythrozoon teganodes (14, 18, 19) and E. tuomii (3, 24, 26, 27, 28, 29, 32) were also described in cattle. Giemsa stained Eperythrozoon teganodes were described as occurring free in the plasma (i.e. not attached to erythrocytes) as reddish-purple rings (0.4 - 1.2 microns in diameter), rods or threads, and rings with rods (0.2 - 0.3 microns X 0.3 -

3.5 microns) attached (14, 28). The pathogenecity of E. teganodes induced infections in splenectomized calves was not different from E. wenyoni infections (14, 28, 29). Giemsa stained Eperythrozoon tuomii were described as reddish-purple small rings or ovals (0.3 - 1.0 microns) adhered to the periphery of thrombocytes with occasional free rings forms (24, 26, 27, 28, 29, 32). Eperythrozoon tuomii completely encircled thrombocytes, forming a dark purple corona around them as E. wenyoni does erythrocytes (29). Eperythrozoon tuomii caused only moderate body temperature elevations and was incapable of producing anemia (29, 32).

The following describes the first cases of bovine eperythrozoonosis reported in Colombia.

HISTORY

During bovine hemoparasitic disease investigation, 37 male Holstein-Freisian, 4 to 11-month-old cattle, were splenectomized within 3 weeks to 3 months after their arrival at the research facilities. Three groups of calves came from one farm in the northern part of the Sabana de Bogotá and one group of calves came from another farm in the Central Sabana de Bogotá. The two farms were tick free; however, Linognathus vitali (the long nosed cattle louse) occurred frequently on both farms. Twenty calves were splenectomized and housed in an open barn at Bogotá, 18 calves were splenectomized and housed in a tick moated, screened barn at Palmira, Valle.

Each of the 4 groups of splenectomized calves was housed in separate pens and, in an adjacent pen, 6 other splenectomized calves were housed for a period of 3 weeks prior and 4 months after splenectomy. Biting flies were occasionally

observed in small numbers, but ticks were never seen in either of the barns. The calves were each fed 1.5 kg of the concentrate ration (Table I) plus 1.0 kg of ground corn cobs per day.. Blood samples for packed cell volumes and Giemsa stained blood smears were collected weekly before and twice after splenectomy. Rectal temperatures were taken every second day. Body weights were determined monthly.

CLINICAL SIGNS

Eight calves had sub-clinical infections, and 6 other infected calves exhibited depression and anorexia. All of the calves had elevated rectal temperature (Fig. I). Two calves had serous conjunctivitis with excessive lacrimation. Of the six calves showing clinical signs, 2 had infectious pododermatitis. The 14 affected splenectomized calves gained 177 g/day while the 6 unaffected splenectomized calves gained 277 g/day for the 31 days.

MICROSCOPIC AND CLINICAL PATHOLOGICAL FINDINGS

Microscopic examination of Giemsa-stained, Ph 7.05 blood smears from 14 (37.8%) of the 37 splenectomized calves revealed variable parasitemias of Eperythrozoon sp. Predominantly curved filamentous rods (0.3 X 1.5 microns), coccoids (0.2 - 0.4 microns in diameter) and, less commonly, rings (0.4 - 1.2 microns in diameter) were found to be located upon the surface of erythrocytes, and occasionally free in the plasma (Figs. 2 & 3) of 11 of the 14 affected calves. The pleomorphic organisms stained pinkish-purple and were determined to be E. wenyoni morphologically.

TABLE I

MEZCLA DE CONCENTRADOS

Maíz desgranado	430 kg
Torta de Algodón o Soya	300 kg
Salvado de Trigo	230 kg
Sal Yodada	10 kg
Harina de Hueso	20 kg
*Premezcla de Vitaminas	10 kg
**Premezcla de Minerales	10 kg
	1.000 kg

*PREMEZCLA DE VITAMINAS

Salvado de Trigo	10 kg
Vit A 750,000 i.u./gm y Vit. D 150,000 i.u./gm	8 kg
	10 kg

** PREMEZCLA DE MINERALES

Sulfato de Cobre	1.95 kg
Sulfato de Hierro	7.47 kg
Oxido de Zinc	1.24 kg
Sulfato de Magnesio	3.09 kg
Sulfato de Cobalto	0.20 kg
Yoduro de Potasio	0.07 kg
Salvado de Trigo	85.98 kg
	100.00 kg

Blood smears of 2 of the 14 affected calves revealed large numbers reddish-purple filamentous rods of threads (0.3 X 3.0 microns) with much fewer numbers of rings and rings with rods attached (0.4 - 1.3 microns in diameter) occurring free in the plasma, which were considered morphologically to be E. teganodes (Fig. 4). Also observed in the blood of these two calves was a low level of E. wenyoni affected erythrocytes.

In one of the 14 affected calves, blood smears demonstrated very small reddish-purple rings or coccoids (0.3 - 0.8 microns) which adhered to and completely encircled the thrombocytes (Fig. 5). In addition, small numbers of rings (0.4 - 1.0 microns) were free in the plasma. Based upon the morphology of the organism, this calf was determined to have a E. tuomii infection. Therefore, 11 calves had E. wenyoni infections only, 2 had a dual infection of E. wenyoni and E. teganodes, and one calf had an E. tuomii infection only. The parasitemias varied from 2 or 3% to 80 or 90% infected erythrocytes. Prior and during the onset of parasitemia, thrombocytes noticeable increased in Giemsa-stained blood smears although no specific counts were made. The results of the packed cell volumes determination are given in Fig. 1. The distribution of the first onset of parasitemias after splenectomy are given in Fig. 6. The average incubation period and standard deviation was 14.9 ± 3.5 days post-splenectomy with a range of 9 to 22 days.

CONTROL

Twelve of 14 affected splenectomized calves were treated once intramuscularly with 29 mg/kg of 2-di-(Beta, gamma-dioxipropil)-aminofenol-(4 arseno 5)-(benzoxazolil)-mercaptol-proprionato de sodio*, an average and standard deviation

* Spirotrypan-Fuerte, Farbwerke Hoechst AG, Frankfurt, Germany

of 1.2 ± 1 day following the onset of parasitemia. One day following treatment, no parasitemias were demonstrable.

DISCUSSION

The 14 affected calves were most likely infected prior to arrival at the research facilities, because the calves exhibited parasitemia within 22 days following splenectomy; 29 other splenectomized calves in contact with them did not have demonstrable parasitemias. Because the cattle were not exposed to ticks, but were exposed to lice and biting flies prior to leaving the farms, it is suggested that lice and/or biting flies may have been the vectors in this case, in addition to possible mechanical means of transmission.

The clinical signs and microscopic findings of the affected calves correspond to those described by other investigators (1, 2, 4, 6, 8, 14, 15, 16, 17, 18, 19, 21, 25, 28, 29, 30, 31, 32). The morphological characteristics of the organisms found in the blood of the 14 affected calves were strikingly similar to the description of E. wenyoni (1, 4, 6, 14, 15, 17, 21, 25, 28,30), E. tegenodes (14, 18, 19) and E. tuomii (24, 26, 29, 32). The average incubation period and standard deviation of 14.9 ± 3.5 days and the distribution of the onset of parasitemia post-splenectomy (Fig. 6) is generally in agreement with that reported by other investigators (1, 4, 6, 14, 15, 17, 20, 25, 28, 29, 31, 32). The clinical manifestations were mild, but 6 unaffected splenectomized infected calves gained an average of 100 g/day more than the calves with eperythrozoonosis. As indicated in Fig. 1, the packed cell volumes increased following splenectomy and decreased following the onset of parasitemia. The temperature increased sharply with the onset of parasitemia and decreased

as the parasitemia became undemonstrable. Following the intramuscular injection of 2-di-(Beta, gamma-dioxipropiol)-aminofenol-(4 arseno 3)-Beta-(benzotiazolil-(2)-mercaptol)-proprionato de sodio at 29 mg/kg, the parasitemia became undemonstrable within 24 hours with further recrudescence occurring in 2 of 12 calves 35-40 days following treatment. The 2 untreated infected calves both had low porcentages of infected erythrocytes and one calf had a slightly decreased packed cell volume which was followed by a recrudescense of parasitemia in 14 days.

RESUMEN

Eperythrozoon wenyoni, E. teganodes y E. tuomii fueron diagnosticados en 14 de 37 terneros de raza Holstein-Freisian de 4 a 11 meses de edad, traídos de la Sabana de Bogotá. Once terneros mostraron infecciones puras de E. wenyoni, dos terneros mostraron infecciones simultáneas de E. wenyoni y E. teganodes y un ternero mostró una infección pura de E. tuomii. El diagnóstico fué determinado por medio de muestras de sangre con tinción por el método Giemsa; tambien, se hizo una descripción morfológica de Eperythrozoon sp. Seis terneros esplenectomizados mostraron depresión y anorexia, sin embargo, todos los 14 terneros tenían elevada temperatura rectal. Dos terneros mostraron conjuntivitis serosa con lacrimación excesiva. El aumento de la temperatura coincidió con el primer ataque de parasitemia mientras que el hematocrito disminuyó después del ataque de parasitemia. El promedio y desviación estandard del periodo de incubación fué de 14.9 ± 3. 5 días después de la esplenectomía. El tratamiento con 2-

di-(Beta, gama-dioxipropil)-aminofenol-(4 arseno 5)-Beta-(benzaxozalil-(2)-mercaptol)-proprionato de sodio, a razón de 29 mg/kg intramuscular, causó la parasitemia la cual se hizo indemostrable en 24 horas con mas recrudecimientos dentro de seis semanas. Este es el primer informe sobre eperythrozoonosis en Colombia debido a Eperythrozoon wenyoni, E. teganodes y E. tuomii.

SUMMARY

Eperythrozoon wenyoni, E. teganodes and E. tuomii were diagnosed in 14 of 37 splenectomized, Holstein-Freisian, 4 to 11-month-old calves that originated from the Sabana de Bogotá. Eleven calves had pure infections of E. wenyoni, 2 calves had dual infections of E. wenyoni and E. teganodes, and one calf had a pure infection of E) tuomii. The diagnosis was determined on Giemsa-stained blood smears, and morphological descriptions of the Eperythrozoon sp. were given. Six splenectomized calves exhibited depression and anorexia, but all 14 calves had elevated rectal temperatures. Two calves had serous conjunctivitis with excessive lacrimation. The increase in rectal temperature coincided with the onset of parasitemia while the packed cell volume decreased after the onset of parasitemia. The average incubation period and standard deviation was 14.9 ± 3.5 days post-splenectomy. Treatment with 2-di-(Beta, gamma-dioxipropil)-aminofenol)-(4 arseno 5)-Beta-(benzaxozalil-(2)-mercaptol-proprionato de sodio at 29 mg/kg intramuscularly caused the parasitemia to became undemonstrable within 24 hours with further recrudescence

occurring within 6 weeks. This is the first report of bovine eperythrozoonosis due to *Eperythrozoon wenyoni*, *E. teganodes* and *E. tuomii* in Colombia.

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THE CHEMOTHERAPEUTIC EFFICACY OF IMIDOCARB DIHYDROCHLORIDE IN THE ELIMINATION OF CONCURRENT BOVINE ANAPLASMOSIS AND BABESIOSIS

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SUMMARY

Intact Anaplasma marginale, Babesia bigemina and Babesia argentina carrier calves treated intramuscularly 5 or 10 times with 2.5 mg/kg of imidocarb dihydrochloride at 48-hour intervals eliminated the Babesia infections, but not Anaplasma infections. The parasitaemias became microscopically undemonstrable within 4 days following the first treatment, and the packed cell volumes increased significantly within 18 days. Intoxications resulting in fatalities occurred in 5 of 6 calves given 10 intramuscular treatments of 2.5 mg/kg of imidocarb dihydrochloride at 48-hour intervals.

INTRODUCTION

Imidocarb dihydrochloride* (3,3'-bis(2-imidazolin-2yl) carbanilide dihydrochloride) (IMDC) was shown to be highly effective in the treatment and control of bovine babesiosis by Callow and McGregor (1970), Brown and Berger (1970), Roy-Smith (1971) and Hart, Roy-Smith, Berger, Simpson and McHardy (1971). Callow and McGregor (1970), Berger quoted in Brown and Berger (1970) and Hart, et al. (1971) showed that single doses of IMDC down to 0.6 mg/kg sterilized Babesia bigemina infections, and Callow and McGregor (1970) further demonstrated that single doses down to 2 mg/kg sterilized Babesia argentina infections of cattle.

Hart, et al. (1971) and Roby (1972) demonstrated the high degree of chemotherapeutic efficacy of IMDC in the treatment of bovine anaplasmosis. Kuttler (1971) reported that 3 intramuscular treatments at 24-hour intervals of IMDC at base weight 4, 5, or 6 mg/kg sterilized Anaplasma marginale infections in splenectomized carrier calves. Roby and Mazzola (1972) showed that 2 subcutaneous or intramuscular injections at 14-day intervals of IMDC

*Imidocarb, Burroughs-Wellcome & Co., Ltd., London, England.

or imidocarb diproprionate at salt weight 5.0 mg/kg eliminated the A. marginale infection in intact adult carrier cattle.

This paper presents the results of an attempt to sterilize concurrent A. marginale, B. bigemina and B. argentina infections of intact carrier cattle with imidocarb dihydrochloride.

MATERIALS AND METHODS

Twelve A. marginale, B. bigemina and B. argentina carrier, 330± 18 days old, male, intact Holstein-Friesian calves were used in the experiment which was executed in tick-free quarters at the Instituto Colombiano Agropecuario, Laboratorio de Investigaciones Medicas Veterinarias located in Bogotá, Colombia (altitude 2,600 m). Each of the 12 calves was previously inoculated subcutaneously on day -41 with 10 ml of blood containing 3×10^9 A. marginale bodies and day -38 with 10 ml of blood containing a mixture of 9×10^7 B. bigemina and B. argentina. The parasites were identified by the examination of Giemsa stained thin blood films. The number of parasites was estimated by determining in quadruplicate the percentage of organisms in Giemsa stained thin blood films, and multiplying the average percentage of parasitaemia by the average total erythrocyte count per cubic centimeter also determined in quadruplicate. The isolates of hemoparasites were obtained from a splenectomized calf exposed to a natural environment where Boophilus microplus were abundant in a pasture located at the Turipaná Instituto Colombiano Agropecuario experimental farm near Montería, Córdoba, Colombia (altitude 24 m). The isolates were further twice passaged by inoculating 100 ml of blood into intact calves to help eliminate the possibility of concurrent infectiousdiseases. The calves were demonstrated to be carriers by the presence of positive parasitaemias, positive titers of complement fixing (CF)

antibodies for A. marginale, B. bigemina and B. argentina and decreasing packed cell volumes (PCV). The carrier-calves were in poor physical condition as evidenced by weight loss, roughened hair coats and generalized debilitation.

Imidocarb dihydrochloride salt was dissolved in sterilized, distilled water to make a 5% w/v solution. The drug was injected 5 times intramuscularly on days 0, 2, 4, 6 and 8 at 2.5 mg/kg into the 6 calves of Group I. The 6 calves of Group II were injected 10 times intramuscularly on days 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 with 2.5 mg/kg of IMDC.

Fifty ml aliquots of blood collected on day 81 from each of the 6 calves of Group I were pooled and inoculated intravenously into a splenectomized calf. A 100 ml aliquot of blood collected on day 81 from the one surviving calf of Group II was inoculated intravenously into another splenectomized calf. Blood samples from each of the 2 splenectomized calves were collected daily for 2 weeks, and thereafter, twice weekly for 60 days for the detection of parasitaemias.

The interval of collection of blood and serum samples of Groups I and II was as depicted in Figures 1 and 2. Blood samples were collected from the jugular vein using 1.2 g/l of disodium ethylenediamine tetraacetate as anticoagulant. The packed cell volumes (PCV) were determined by the microhematocrit method after spinning for 5 minutes at 11,500 rpm in a microhematocrit centrifuge. Combination thin and thick Giemsa stained blood films were processed according to a modified method of Mahoney and Saal (1961). Parasitaemias for A. marginale and Babesia spp. were ascertained by examining a transect (75 fields using a X 100 oil immersion objective with X 10 oculars) approximately 3 to 6 mm from the tail of the thin blood films.

All complement-fixation (CF) serum antibody titers were performed by the microtiter method described by Hidalgo and Dimopoullos (1967). Anaplasma marginale CF antibody titers were determined by a microprocedural adaptation of a standard method (Anonymous, 1958), using the standard United States Department of Agriculture, Agriculture Research Service antigen. Babesia bigemina and B. argentina CF antibody titers were ascertained by a microprocedural adaptation of the method described by Mahoney (1962), using distilled water suspensions and extracts of parasitized bovine erythrocytes as antigens according to the method of Mahoney (1967).

RESULTS

The results of the 5 and 10 treatments of 2.5 mg/kg of IMDC of Groups I and II respectively are given in Figures 1 and 2. Each point on the curves of the graphs represents the mean and the bar through each point indicates one standard deviation for a given day. From day 0 to day 18, the PCV of Groups I and II increased significantly ($P < 0.01$) from 23.5± 2.9% to 34.8± 1.5% and from 23.0± 4.0% to 33.0± 2.0% respectively. Following day 18, the PCV of both groups remained at normal levels. By day 4, the A. marginale, B. bigemina and B. argentina parasitaemias became microscopically undemonstrable and remained so throughout the rest of the experiment.

Calves treated 10 times with IMDC developed negative CF titers for A. marginale, B. bigemina and B. argentina 10 to 20 days before calves treated 5 times, and all cattle had negative titers by day 60. The Babesia CF titers usually became negative before the Anaplasma titers.

From day 0 to 10, Group I had an average daily weight loss of 36± 22 g/day, and from day 0 to 18, Group II had an average daily weight loss of

112± 56 g/day. From day 10 to 81, Group I had an average daily gain of 358± 137 g/day, and from day 18 to 81, the surviving calf of Group II had an average daily gain of 196 g/day. The physical status of the calves of Group I improved markedly, but the physical status of the calves of Group II remained static.

No mortalities occurred in Group I, however, 5 of 6 calves of Group II died on days 16, 18, 19, 36 and 63 with the following macroscopic lesions: petechiation of serosal surfaces, hydrothorax, hydroperitoneum and enlarged, pale, swollen kidneys. Excessive salivation, lachrymation and mild dyspnoea persisted for 15-30 minutes following each inoculation, and repetitive treatments did not increase the severity or duration of the signs. Minimal local inflammatory responses occurred at the sites of injection, but usually disappeared within 24 hours.

Neither of the splenectomized calves inoculated with pooled blood from Groups I and II demonstrated any evidence of B. bigemina or B. argentina. The splenectomized calf subinoculated with pooled blood from Group I developed an A. marginale parasitaemia on day 22 post-inoculation and died on day 34 with a PCV of 8.0% and a parasitaemia of 16.0%. The splenectomized calf inoculated with the blood of the surviving calf of Group II developed an A. marginale parasitaemia on day 32 post-inoculation which increased to a maximum parasitaemia of 12% on day 40 and a minimum PCV of 14% on day 43.

DISCUSSION

Imidocarb dihydrochloride rapidly inhibited the parasitaemias of A. marginale, B. bigemina and B. argentina, making them microscopically undemonstrable within 4 days following the first treatment of 2.5 mg/kg of IMDC. The inhibitory effect of IMDC on the parasitaemias was paralleled

by rapidly increasing PCV and an improvement in the physical status of the calves following treatment.

The babesiosis carrier status of intact calves was eliminated by 5 and 10 intramuscular treatments of 2.5 mg/kg of IMDC at 48-hour intervals which agrees with the results of Callow and McGregor (1970), Berger quoted in Brown and Berger (1970) and Hart, et al. (1971) who demonstrated lower single doses sterilized Babesia infections.

The anaplasmosis carrier status of intact calves was not eliminated by either 5 or 10 intramuscular treatments of 2.5 mg/kg of IMDC at 48-hour intervals, yet Kuttler (1971) sterilized A. marginale infections in splenectomized calves with 3 intramuscular treatments at 24-hour intervals of IMDC at base weights of 4, 5 or 6 mg/kg, and Roby and Mazzola (1972) eliminated A. marginale infections in intact adult cattle with 2 intramuscular treatments of salt weight 5.0 mg/kg of IMDC or imidocarb diproprionate. Apparently, a minimal chemotherapeutic blood or tissue level of IMDC must be reached and maintained for the elimination of A. marginale carrier infections which is more easily attained with higher dosages at preferably shorter intervals, however, the increased possibility of drug toxicity must be considered with this regimen. Ten intramuscular applications of IMDC at 2.5 mg/kg at 48-hour intervals was fatal in 5 of 6 calves. The calves had slight daily weight losses during the period of chemotherapy, but made rapid weight gains following treatment, indicating that repetitive chemotherapy with IMDC may reduce metabolic efficiency and result in decreased weight gains of calves during the treatment period.

Roby and Mazzola (1972) indicated that elimination of the carrier state of anaplasmosis in adult cows should be accompanied by loss of CF

antibody titers. Kuttler (1971) found that IMDC treated splenectomized calves which failed to relapse within 62 days following treatment were consistently free of anaplasmosis as determined by infectivity trials. Although intact calves are not directly comparable with splenectomized calves or adult cows, the intact calves in our experiment treated 5 or 10 times with IMDC at 2.5 mg/kg did not develop relapsing A. marginale parasitaemias within 81 days post-treatment and had negative CF A. marginale antibody titers by 60 days post-treatment, yet remained patent carriers of anaplasmosis upon subinoculation into splenectomized calves. These results suggest that a negative anaplasmosis CF antibody titer and the lack of a relapsing parasitaemia are good indicators, but not sufficient to verify elimination of the anaplasmosis carrier state, making subinoculation into splenectomized calves necessary for confirmation.

ACKNOWLEDGEMENTS

This study was financially assisted by the United States Agency for International Development, the Rockefeller Foundation and the Instituto Colombiano Agropecuario. The authors acknowledge the assistance provided by Dr. C. H. Tellez, Dr. L. A. Serrano, Dr. G. Mateus and Mr. L. E. Ramirez.

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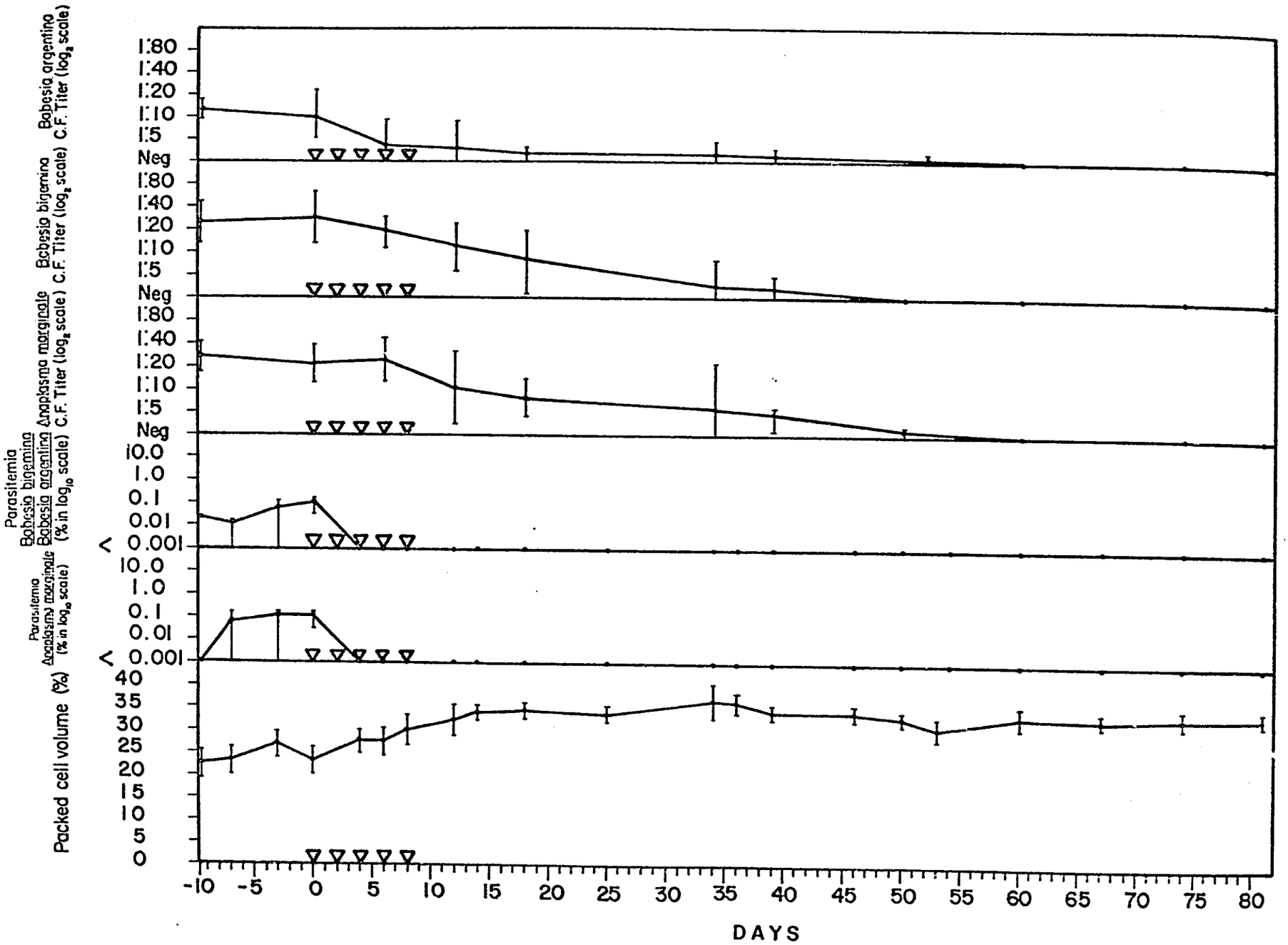
Figure 1. Hematologic and serologic results of 6 concurrent A. marginale, B. bigemina and B. argentina carrier calves (Group I) given 5 intramuscular treatments of 2.5 mg/kg of imidocarb dihydrochloride. The mean and one standard deviation are represented for each given day.

▽ = day of treatment.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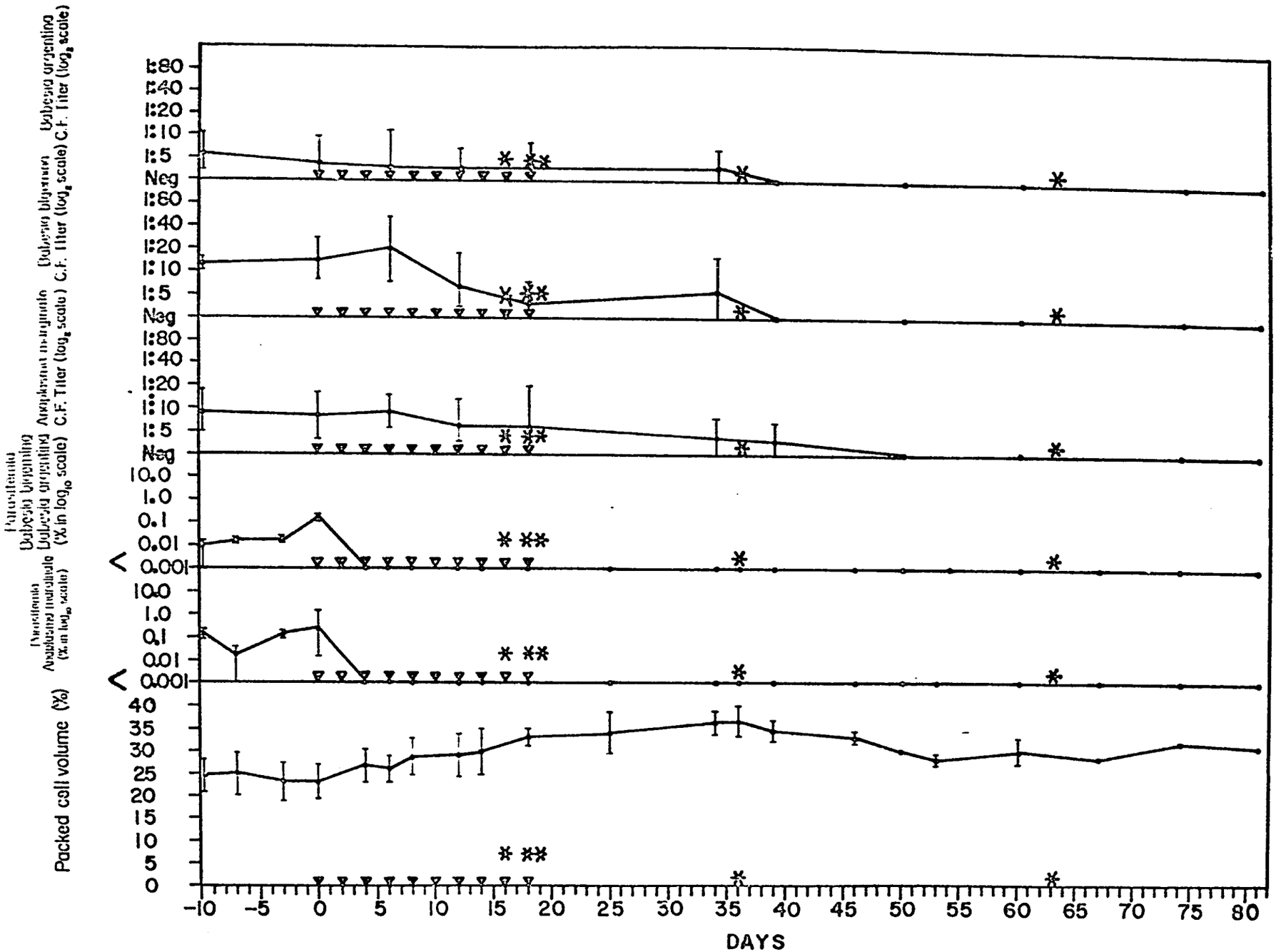
1:40
1:20
1:10
1:5
Neg
Babesia bigemina C.F. Titer (log2 scale)
1:80
1:40
1:20
1:10
1:5
Neg
Anaplasma marginale C.F. Titer (log2 scale)
1:80
1:40
1:20
1:10
1:5
Neg
Parasitemia Babesia bigemina Babesia argentina (% in log10 scale)
10.0
1.0
0.1
0.01
< 0.001
Parasitemia Anaplasma marginale (% in log10 scale)
10.0
1.0
0.1
0.01
< 0.001
Packed cell volume (%)
40
35
30
25
20
15
10
5
0
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
DAYS

Figure 2. Hematologic and serologic results of 6 concurrent A. marginale, B. bigemina and B. argentina carrier calves (Group II) given 10 intramuscular treatments of 2.5 mg/kg of imidocarb dihydrochloride. The mean and one standard deviation are represented for each given day. ▽ = day of treatment and * = death of one calf.


Dabepsia argentina C.F. Titer (log_2 scale)
Dabepsia blyanum C.F. Titer (log_2 scale)
Anaplasma marginale C.F. Titer (log_2 scale)
Dabepsia argentina (% in log_{10} scale)
Anaplasma marginale (% in log_{10} scale)
Packed cell volume (%)
1:80
1:40
1:20
1:10
1:5
Neg
10.0
1.0
0.1
0.01
< 0.001
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65
70
75
80
DAYS

CHEMOTHERAPY OF EXPERIMENTAL CONCURRENT BOVINE ANAPLASMOSIS AND BABESIOSIS WITH IMIDOCARB DIHYDROCHLORIDE

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SUMMARY

The chemotherapeutic efficacy of imidocarb dihydrochloride (3, 3' - bis(2-imidazolin-2 yl) carbanilide dihydrochloride) administered as single intramuscular doses of 1.0, 2.0 and 2.5 mg/kg, against concurrent bovine anaplasmosis and babesiosis, is reported. Dosages of 2.0 and 2.5 mg/kg of imidocarb dihydrochloride rapidly inhibited acute ascending concurrent parasitaemias of Anaplasma marginale, Babesia bigemina and Babesia argentina; however, 1.0 mg/kg had a minimal effect on A. marginale, but was very effective against B. bigemina and B. argentina. Imidocarb dihydrochloride at 1.0, 2.0 and 2.5 mg/kg inhibited the development of immunity of the acute Babesia spp. infections, making the calves more susceptible to babesiosis upon challenge. The inhibition of A. marginale parasitaemias was directly related to increasing doses of imidocarb dihydrochloride; however, recrudescing and persisting post-treatment parasitaemias also occurred more frequently at higher doses.

INTRODUCTION

Schmidt, Hirt and Fischer (1969) described the babesicidal effects of the basically substituted carbanilides on Babesia rodhaini in mice, of which 3, 3' -bis(2-imidazolin-2 yl) carbanilide was the most effective. Beveridge (1969) compared 3, 3' -bis(2-imidazolin-2 yl) carbanilide dihydrochloride* (IMDC) with 3 other babesicides against B. rodhaini infections in rats and mice and demonstrated that IMDC had the greatest babesicidal effect. Callow and McGregor (1970) demonstrated the sterilizing, chemotherapeutic and chemoprophylactic effects of IMDC against Babesia bigemina and Babesia argentina infections of cattle. Brown and Berger (1970) further described the high degree of chemotherapeutic efficacy of IMDC for B. bigemina infections of cattle. Wood (1971) reviewed and

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* Imidocarb, Burroughs-Wellcome & Co., Ltd., London, England.

summarized the activity of IMDC against Babesia infections of cattle. Hart, Roy-Smith, Berger, Simpson and McHardy (1971) demonstrated the efficacy of IMDC against Anaplasma marginale in addition to B. bigemina and B. argentina infections of cattle. Kuttler (1971) and Roby (1972) subsequently showed that IMDC and imidocarb diproprionate eliminated A. marginale from carrier cattle. Roby (1972) further substantiated the inhibitory effect of IMDC on acute anaplasmosis in splenectomized calves.

This paper describes the results of chemotherapeutic experiments with IMDC against blood-borne concurrent B. bigemina, B. argentina and A. marginale infections of intact cattle.

MATERIALS AND METHODS

Male, Holstein-Friesian calves, 300± 18 days old, born and raised in a Boophilus microplus and babesiosis free Bogotá savanna (altitude 2,600 m), were used in the chemotherapeutic trials. The investigation was performed in tick-free quarters at the Instituto Colombiano Agropecuario, Laboratorio de Investigaciones Medicas Veterinarias located in Bogotá, Colombia. Fifty ml aliquots of blood from each of 18 calves were injected intravenously into a splenectomized calf to give an indication of the absence of latent haemoparasitic infections. In addition, Giemsa stained combination thin and thick blood films, determinations of complement fixing antibodies for B. bigemina, B. argentina and A. marginale, and wet mounts of blood were performed 4 times before inoculation to further indicate that each of the 18 calves were free of most haemoparasites, particularly anaplasmosis and babesiosis.

Eighteen calves were inoculated subcutaneously with 5 ml. of blood containing 2×10^9 A. marginale marginal bodies per cm^3 on day -16, and on day -5, the calves were again inoculated subcutaneously with 5 ml of blood

containing a mixture of 2 x 10^9 B. bigemina and B. argentina. The parasites were identified by examination of Giemsa stained thin blood films. The number of parasites was estimated by determining in quadruplicate the percentage of organisms in Giemsa stained thin blood films, and multiplying the average percentage of parasitaemia by the average total erythrocyte count per cubic centimeter also determined in quadruplicate. The haemoparasites were isolated from a splenectomized calf which was exposed to a natural environment where Boophilus microplus ticks predominated in a pasture of the Turipana Instituto Colombiana Agropecuario experimental farm located at Montería, Córdoba, Colombia (altitude 24 m). The isolates were twice passaged by intravenous inoculation of 100 ml of blood into intact calves to check the purity of the isolates prior to inoculation.

Imidocarb dihydrochloride was dissolved in sterilized, distilled water to make a 5% W/V solution. The drug was injected once intramuscularly on day 0 into the 6 calves of Group I at 1 mg/kg, the 5 calves of Group II at 2 mg/kg and the 5 calves of Group III at 2.5 mg/kg. The 2 calves of Group IV remained as untreated controls.

The 14 surviving calves were challenged on day 81 post-treatment (PT) by injecting subcutaneously 10 ml. of blood containing 3 x 10^9 homologous A. marginale bodies, and on day 84 PT, by injecting subcutaneously 10 ml of blood containing a mixture of 9 x 10^7 homologous B. bigemina and B. argentina.

The interval of collection of blood and serum samples was as depicted in Figures 1, 2, 3 and 4. Blood samples were collected from the jugular vein using 1.2 g/l of disodium ethylenediamine tetraacetate as anticoagulant. The packed cell volumes (PCV) were determined by the microhaematocrit method

after spinning for 5 minutes at 11,500 rpm in a microhaematocrit centrifuge. Combination thin and thick Giemsa stained blood films were processed according to a modified method of Mahoney and Saal (1961). Parasitaemias for A. marginale and Babesia spp. were ascertained by examining the thick blood films, and estimates of parasitaemias were made by examining a transect (75 fields using a X 100 oil immersion objective with X 10 oculars) approximately 3 to 6 mm from tail of the thin blood films.

All complement-fixation (CF) serum antibody titres were performed by the microtitre method described by Hidalgo and Dimopoullos (1967). Anaplasma marginale CF antibody titres were determined by a microprocedural adaptation of a standard method (Anonymous, 1958), using the standard United States Department of Agriculture, Agriculture Research Service antigen. Babesia bigemina and B. argentina CF antibody titres were ascertained by a microprocedural adaptation of the method described by Mahoney (1962), using distilled water suspensions and extracts of parasitized bovine erythrocytes as antigens according to the method of Mahoney (1967).

RESULTS

Figures 1, 2, 3 and 4 show the results of the subcutaneous inoculation with A. marginale, B. bigemina and B. argentina in 18 susceptible calves; the intramuscular treatment with 1.0, 2.0, 2.5 and 0.0 mg/kg of IMDC; and subsequent homologous subcutaneous challenge, as assessed by packed cell volumes, parasitaemic levels and CF antibody titres. Each point on the curves of the graphs represents the mean, and the bar through each point indicates one standard deviation for a given day.

Parasitaemias of A. marginale and Babesia spp. preceded the lowest PCV values of the 4 groups by 1-3 days. These lowest values occurred on

days 3 and 4 PT. The PCV of the treated and untreated groups never returned to preinoculation levels during the period of 81 days PT. The slow rate of recovery of the PCV of all groups was accompanied by a persistent variable A. marginale parasitaemia. The PCV of the treated groups of calves decreased markedly following challenge on days 81 and 84 PT and was concurrent with a Babesia spp. parasitaemia. The surviving untreated control calf had no decrease in PCV.

Imidocarb dihydrochloride at 1.0, 2.0 and 2.5 mg/kg reduced the A. marginale parasitaemia to 1.0% or less within an average of 2.3 days PT while untreated controls had a persistent parasitaemia of more than 1% for at least 7 days PT. Dosages of 2 and 2.5 mg/kg reduced the A. marginale parasitaemia to less than 0.1% within 3 and 4 days PT respectively, but 1 mg/kg failed to do so. A slight increase in A. marginale parasitaemia occurred following challenge.

The Babesia spp. parasitaemias were not demonstrable after day 1 PT in any of the treated calves, but the untreated control calves had a parasitaemia that persisted 11 days. Babesia spp. parasitaemias developed in treated and untreated calves following homologous challenge on day 84 PT; however, the treated calves had higher and more persistent parasitaemias with one mortality occurring in the group treated with 2.5 mg/kg.

All calves had positive A. marginale CF antibody titres in accordance with the recrudescing and persisting parasitaemias. A slight increase in A. marginale CF antibody titres occurred following homologous challenge on day 81 PT.

All calves developed positive B. bigemina and B. argentina CF titres by day 0. The untreated calves had persistently positive CF titres for

B. bigemina and B. argentina, but all of the treated calves developed negative titres for B. argentina and B. bigemina within 22 and 35 days PT respectively. Calves treated with 2.0 and 2.5 mg/kg had negative B. bigemina CF titres before calves treated with 1.0 mg/kg. Babesia bigemina and B. argentina CF titres of all surviving treated calves became positive within 10 to 17 days after homologous challenge on day 84 PT. The surviving untreated control calf had a persistent positive CF antibody titer for B. bigemina and B. argentina with a minimal response after challenge.

The 5 mortalities that occurred during the experiment are described as follows: Group I - 1 calf died of anaplasmosis on day 10 PT with a 10% PCV, and 1.3% A. marginale and negative Babesia spp. parasitaemias; Group II - 1 calf died of chronic relapsing anaplasmosis on day 21 PT with a 12% PCV, and 0.05% A. marginale and negative Babesia spp. parasitaemias, another calf died of chronic relapsing anaplasmosis on day 56 PT with a 10% PCV, and 2.3% A. marginale and negative Babesia spp. parasitaemias; Group III - 1 calf, challenged on day 84 PT, died of acute babesiosis on day 105 PT with a 12% PCV, and 5% Babesia spp. and negative A. marginale parasitaemias; Group IV - 1 control calf died of concurrent anaplasmosis and babesiosis on day 4 with a 10% PCV and 4% A. marginale and 3% Babesia spp. parasitaemias.

A very slight local inflammatory response occurred and disappeared within 3 days at the site of inoculation of the dosages used. Mild transitory dyspnoea, excessive salivation and lachrymation occurred and subsided within 30 minutes post-treatment. The average daily weight gains of surviving calves in Groups I, II, III and IV were 271±70, 142±41, 254±49 and 199 gm/day respectively from day 0 to day 119 PT.

DISCUSSION

Imidocarb dihydrochloride at 1.0, 2.0 and 2.5 mg/kg rapidly reduced

the Babesia spp. parasitaemias indicating a high degree of chemotherapeutic effectiveness. These findings closely parallel those of Callow & McGregor (1970) and Brown & Berger (1970). The absence of Babesia spp. parasitaemias, and the development of negative CF titers for B. bigemina and B. argentina following treatment are highly suggestive that the Babesia spp. of the treated calves were eliminated. The death of one treated calf due to acute babesiosis following challenge on day 84 PT and the positive challenge response of the other treated calves to homologous Babesia spp. further indicate that IMDC apparently sterilized the Babesia infections. Callow and McGregor (1970) demonstrated that 0.6 mg/kg of IMDC sterilized B. bigemina infections and 2.0 mg/kg of IMDC sterilized B. argentina infections. Hart et al. (1971) and Berger quoted in Brown & Berger (1970) showed that 5.0 mg/kg and 0.75 mg/kg of IMDC respectively sterilized B. bigemina infections. Our results indicate that 1, 2 and 2.5 mg/kg of IMDC apparently sterilized acute ascending parasitaemias of B. bigemina and B. argentina, making the calves more susceptible to challenge 84 days following treatment. Callow and McGregor (1970) suggested that cattle sterilized of Babesia spp. infections by excessive IMDC treatment may suffer a clinical attack upon further exposure to babesiosis. These findings disagree with those of Hart et al. (1971) and Wood (1971) whose results indicate that IMDC did not interfere with the development of immunity to bovine babesiosis. Because the calves in our experiment had demonstrable Babesia spp. parasitaemias for only 3 days before treatment, the duration of immunity may be related to the longevity of initial infection prior to chemotherapy.

The ascending A. marginale parasitaemias of acute infections were rapidly decreased by 2.0 and 2.5 mg/kg of IMDC, but not by 1.0 mg/kg. The

reduction in A. marginale parasitaemias was directly related to increasing dosages of IMDC. These findings closely agree with the observations of Hart et al. (1971) and Roby (1972). The occurrence of recrudescing and/or persisting A. marginale parasitaemias and the associated reduction in PCV was relatively augmented as the dosage of IMDC increased which agrees rather well with the results of Hart et al. (1971). Sterilization of anaplasmosis did not occur following treatment, because the A. marginale CF antibody titres and parasitaemias were persistently present, and only a mild response resulted following homologous challenge. Kuttler (1972) demonstrated that 3 intramuscular injections of 24-hour intervals of 4.0, 5.0 or 6.0 mg/kg of IMDC eliminated the A. marginale carrier status of splenectomized calves. Roby and Mazzola (1972) showed that 2 intramuscular or subcutaneous injections at 14-day intervals of 5.0 mg/kg of IMDC or imidocarb dipropionate eliminated the A. marginale carrier status in intact adult cattle. Therefore, it is apparent that increased amounts of IMDC are required for complete elimination of bovine anaplasmosis.

One calf treated with 1.0 mg/kg of IMDC and 2 calves treated with 2.0 mg/kg of IMDC died of either subacute or chronic recrudescing anaplasmosis on days 10, 21 and 56 PT indicating that these dosages were probably not adequate to control the relapsing infection. All of the treated groups of calves had higher average daily weight gains than the control calves except Group II which had lower weight gains, apparently related to the continuous recurrence of anaplasmosis.

The dual chemotherapeutic efficacy of IMDC against bovine anaplasmosis and babesiosis is very beneficial; however, smaller doses, which are adequately effective against babesiosis, appear to have a minimal effect on

anaplasmosis. Conversely, larger doses which are adequately effective against anaplasmosis may be too effective against babesiosis, and sterilize the infection and possibly make the cattle resusceptible to babesiosis upon the next exposure. The diversity of the chemotherapeutic efficacy of a single dose of IMDC against concurrent bovine anaplasmosis and babesiosis in relation to the sterilization and re-exposure to babesiosis warrants further evaluation. In addition, the relationship between the dosage of IMDC and the occurrence and severity of recrudescing anaplasmosis requires further elucidation.

ACKNOWLEDGEMENTS

This study was financially assisted by the United States Agency for International Development, the Rockefeller Foundation and the Instituto Colombiano Agropecuario. The authors acknowledge the assistance provided by Dr. Elmer Tellez, Dr. Luis Alberto Serrano, Dr. Guillermo Mateus and Mr. Luis Eduardo Ramirez.

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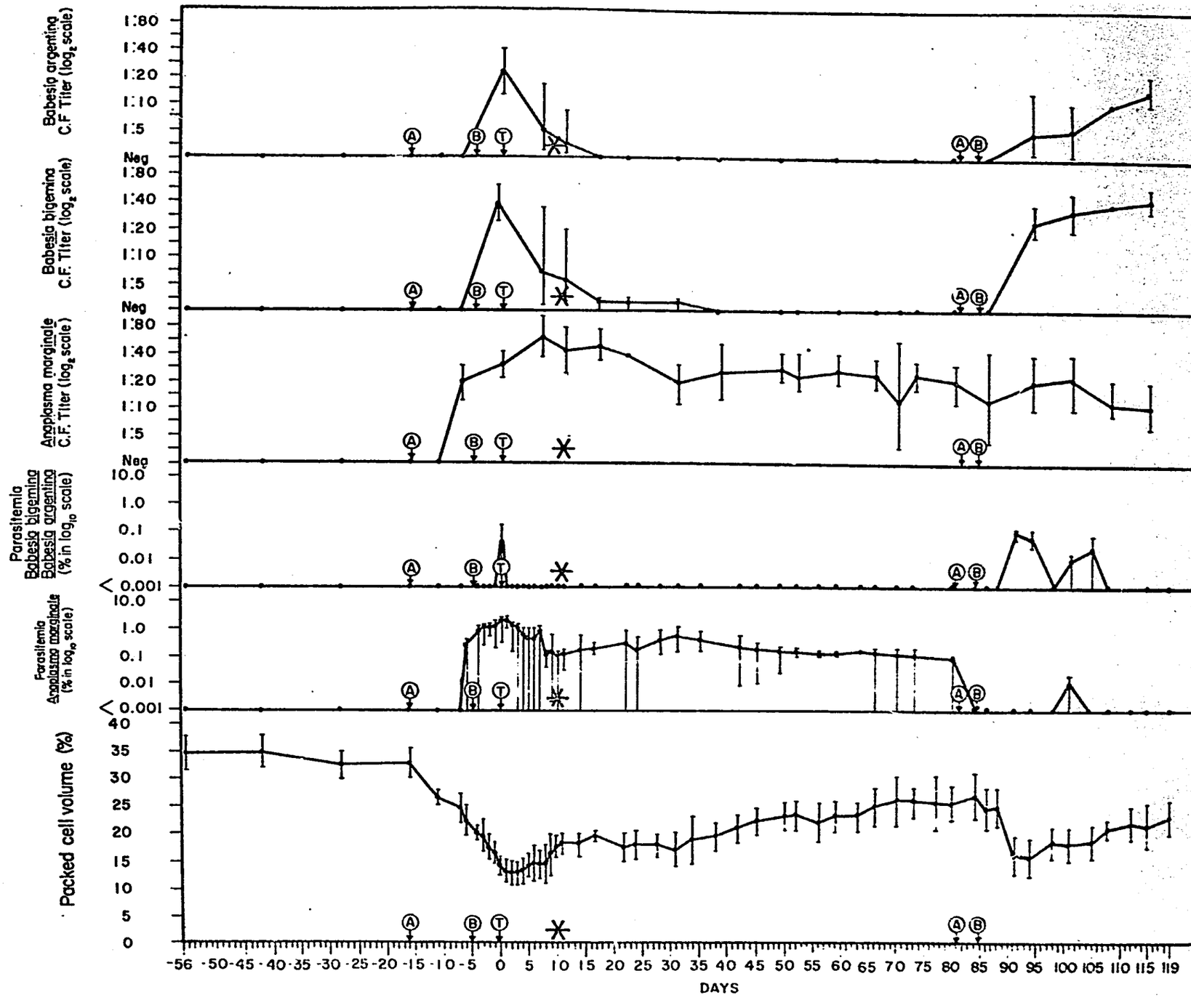
Wood, J. C. (1971). The Activity of Imidocarb Against Babesia Infections of Cattle. Irish Veterinary Journal, 25, 254-257.

Figure 1. Hematologic and serologic results of 6 calves with concurrent anaplasmosis and babesiosis treated intramuscularly with 1.0 mg/kg of imidocarb dihydrochloride. The mean and one standard deviation are represented for each given day. * = death of one calf, A = *Anaplasma marginale* inoculation, B = *Babesia bigemina* and *Babesia argentina* inoculation, and T = day of treatment.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1:80
1:40
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Anaplasma marginale C.F. Titer (log2 scale)
Parasitemia Babesia bigemina Babesia argentina (% in log10 scale)
10.0
1.0
0.1
0.01
< 0.001
Parasitemia Anaplasma marginale (% in log10 scale)
Packed cell volume (%)
40
35
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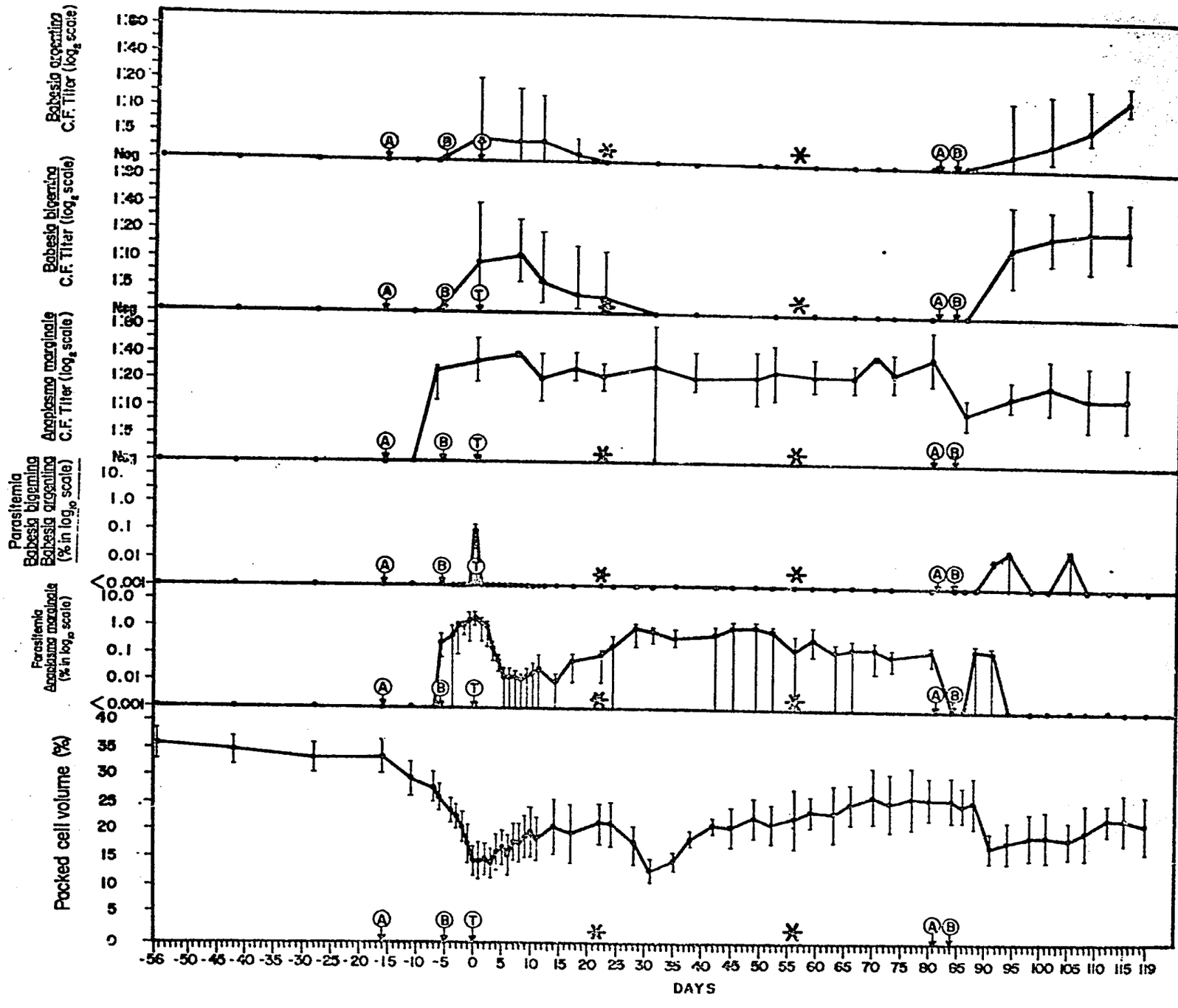
0
A
B
T
-56 -50 -45 -40 -35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 119
DAYS

Figure 2. Hematologic and serologic results of 5 calves with concurrent anaplasmosis and babesiosis treated with 2.0 mg/kg of imidocarb dihydrochloride. The mean and one standard deviation are represented for each given day. * = death of one calf, A = *Anaplasma marginale* inoculation, B = *Babesia bigemina* & *Babesia argentina* inoculation, and T = day of treatment.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Babesia bigemina C.F. Titer (log2 scale)
Anaplasma marginale C.F. Titer (log2 scale)
Parasitemia Babesia bigemina Babesia argentina (% in log10 scale)
Parasitemia Anaplasma marginale (% in log10 scale)
Packed cell volume (%)
DAYS

Figure 3. Hematologic and serologic results of 5 calves with concurrent anaplasmosis and babesiosis treated intramuscularly with 2.5 mg/kg of imidocarb dihydrochloride. The mean and one standard deviation are represented for each given day. * = death of one calf, A = *Anaplasma marginale* inoculation, B = *Babesia bigemina* and *Babesia argentina* inoculation, and T = day of treatment.

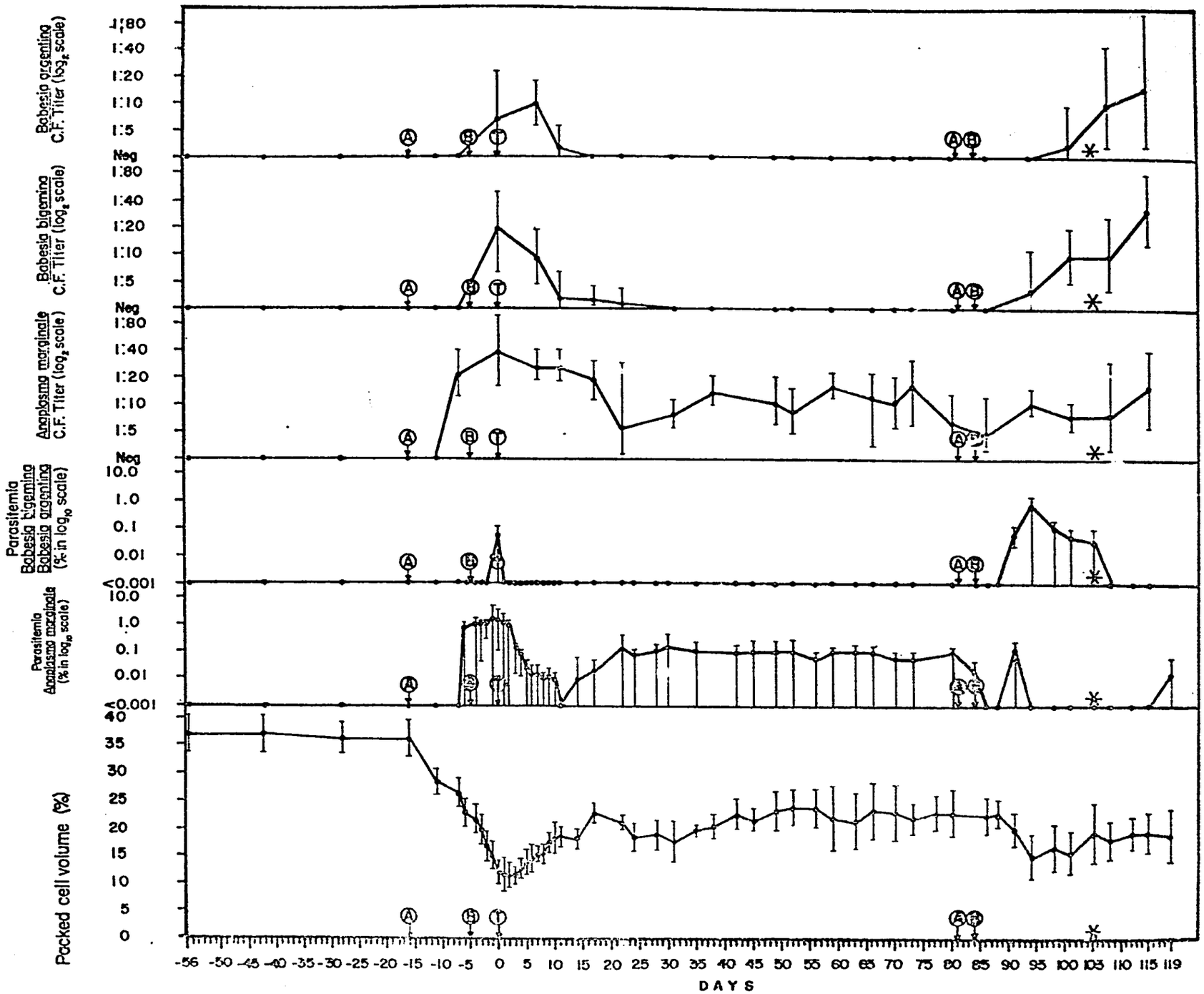
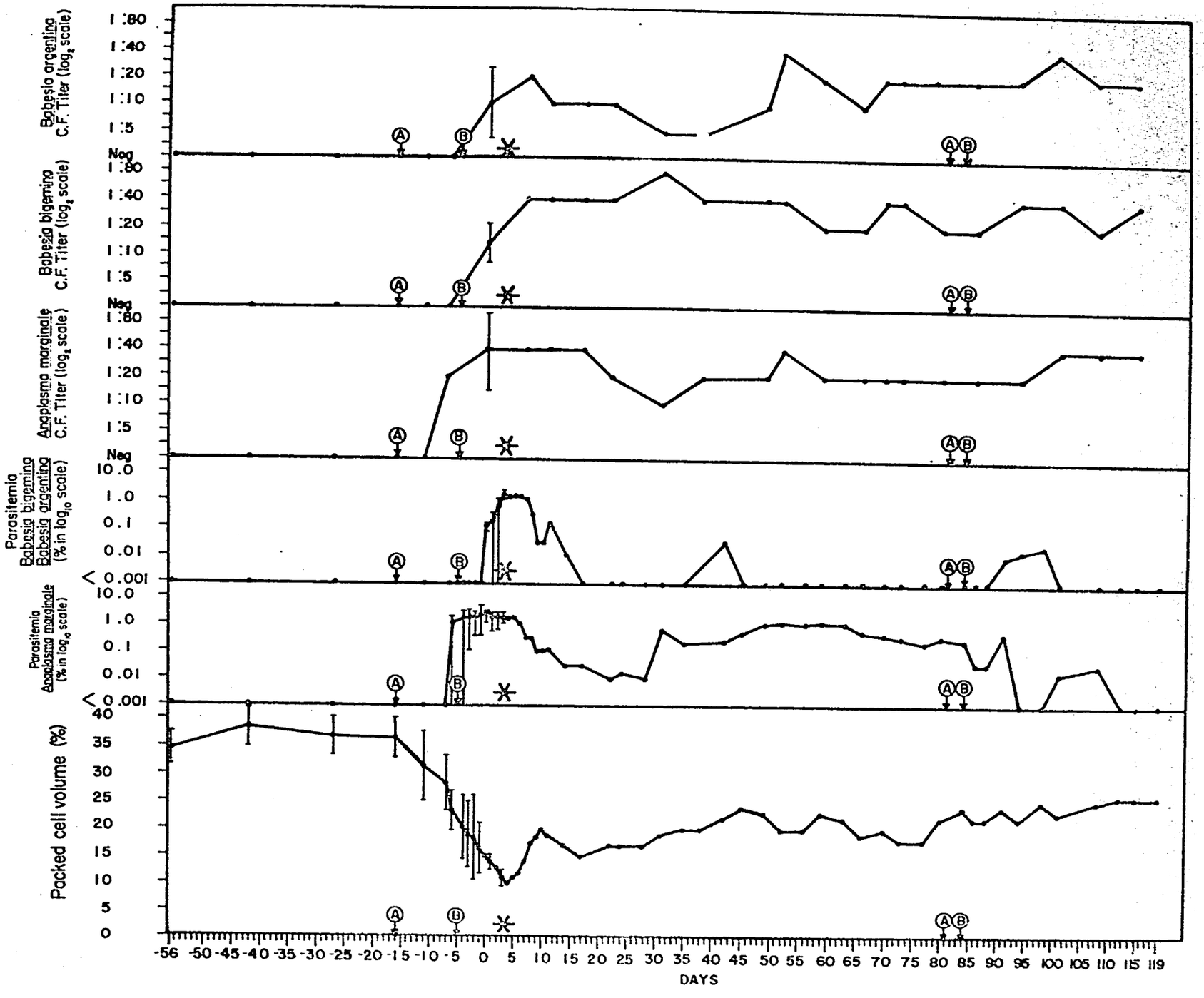

Babesia argentina C.F. Titer (log2 scale)
Babesia bigemina C.F. Titer (log2 scale)
Anaplasma marginale C.F. Titer (log2 scale)
Parasitemia Babesia bigemina Babesia argentina (% in log10 scale)
Parasitemia Anaplasma marginale (% in log10 scale)
Packed cell volume (%)
1:80
1:40
1:20
1:10
1:5
Neg
10.0
1.0
0.1
0.01
<0.001
40
35
30
25
20
15
10
5
0
A
B
T
-56 -50 -45 -40 -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 95 100 105 110 115 119
DAYS

Figure 4. Hematologic and serologic results of 2 untreated calves with concurrent anaplasmosis and babesiosis. The mean and one standard deviation are represented for each given day. * = death of one calf, A = *Anaplasma marginale* inoculation, and B = *Babesia bigemina* and *Babesia argentina* inoculation.


Babesia argentina C.F. Titer (log2 scale)
1:80
1:40
1:20
1:10
1:5
Neg
Babesia bigemina C.F. Titer (log2 scale)
Anaplasma marginale C.F. Titer (log2 scale)
Parasitemia Babesia bigemina Babesia argentina (% in log10 scale)
10.0
1.0
0.1
0.01
< 0.001
Parasitemia Anaplasma marginale (% in log10 scale)
Packed cell volume (%)
40
35
30
25
20
15
10
5
0
A
B
-56 -50 -45 -40 -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 95 100 105 110 115 119
DAYS

Anaplasmosis of Cattle

J. M. Armstrong and R. A. Todorovic

ANAPLASMOSIS OF CATTLE

J. M. Armstrong and R. A. Todorovic*

Anaplasmosis is an infectious disease of cattle, characterized in the acute form by fever, anemia, weakness, constipation, yellowing of the mucous membranes, lack of appetite, depression, dehydration, labored breathing, and, if the female is involved, abortion or temporary infertility. Animals surviving an acute attack often show slow recovery, resulting in the loss of production of either milk or meat. Untreated animals may die. The disease is generally mild in calves up to 6 months of age, but more severe and often fatal in older cattle. Susceptible animals may suffer up to 70 percent losses during a severe outbreak, however, generally mortality is between 5 and 40 percent. Previously exposed or "carrier" animals are resistant to reinfection.

Surveys of economic losses due to anaplasmosis, even though incomplete, indicate that annual losses in the U.S. may be as high as $100,000,000. Losses may be even higher if one includes those due to reduced meat and milk production and the labor connected with treatments and prevention of the disease.

SIGNS OF THE DISEASE

The characteristics of anaplasmosis are influenced by the age of the animal, the virulence of the Anaplasma parasites and the amount of exposure. The infection can cause a rise of body temperature followed by a progressive anemia or loss of red blood cells. One should be on the lookout for an especially rapid form of anaplasmosis in which cattle may die within a few hours after the onset of infection. This is generally called the peracute form. In addition to anemia

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milk flow is suspended, very rapid respiration is noted, and affected animals may exhibit irrational behavior or signs of nervousness.

Most symptoms depend on the stage of the disease. The signs are more pronounced in the acute form of the disease; however, a loss of weight and general emaciation is commonly seen during the recovery phase. These conditions are characterized by pale mucus membranes and jaundice.

The time of incubation of the anaplasmosis organisms varies considerably. Under field conditions, it will generally range from 16 to 40 days, but may extend to 3 months, according to some observers.

Animals that survive the infection become "carriers." The carrier animals are resistant to reinfection, but can be a source of infection to susceptible cattle. Infection can be spread by the tick or insect vectors or by the mechanically transmission of infected blood while bleeding or working cattle.

GEOGRAPHIC DISTRIBUTION

Anaplasmosis is present in most tropical and sub-tropical areas of the world and is also found in many temperate zones of North America. The distribution is generally limited to those areas having adequate vectors, but the wide variety of transmitting agents results in extensive distribution. A recent survey in the United States indicates that anaplasmosis is a serious cattle disease in the Southeast, Northwest, and Pacific Coast areas of the U.S. This is especially true during the summer season or whenever vectors are numerous.

The distribution and incidence of anaplasmosis in Texas is well-indicated by the accompanying map Fig. 1. There appears to be high incidence in area A. This covers the areas along the Gulf Coast in Southeast Texas, starting near

San Patricio County in the south to Orange and Hardin Counties in the northeast. A second area of equal severity is the northeastern corner of Texas, bordering Red River County, south to Harrison County. This comprises a belt of about 50 miles in width, apparently where vector activity is most intense. A secondary area where the disease is of medium incidence (area B) consists of the area of Southeast to Northeast Texas inward for approximately 150 miles. The disease occurs in a low incidence (area C) over approximately the entire eastern half of Texas. Occasional pockets where anaplasmosis can be a severe problem exist in many areas since the incidence of the disease appears to depend on the number of insect and animal carriers. The vector population of a given area will vary from year to year, depending upon climatic factors. It should be mentioned that there is an area, principally in the Hill Country of Texas, where apparently the winter tick is a transmitter of anaplasmosis during the colder months.

TRANSMISSION

A large variety of insects and ticks may serve as transmitting agents. These vectors in some instances are true biological carriers; and in others, transmission occurs by mechanical means. Any transfer of infected blood to susceptible cattle, even in minute amounts, can produce infection.

There are at least 20 species of ticks that have been shown to transmit infection, including *Boophilus*, *Hyalomma*, *Rhipicephalus*, *Dermacentor*, *Ixodes*, and *Haemaphysalis* ticks. Ticks in Texas which can transmit anaplasmosis are the following: *D. variabilis* (American dog tick), *D. albipictus* (winter tick), *Ixodes scapularis* (black-legged tick), *B. annulatus* (Texas fever tick) and *B. microplus* (tropical cattle tick). More research is needed to determine the role played by other species of ticks in the transmission of anaplasmosis.

There are several species of biting flies that might be capable of transmitting anaplasmosis in Texas, principally the tabanids (horse and deer flies). However, other insects are potential vectors such as the horn fly (H. irritans), stable fly (S. calcitrans), black flies or buffalo gnats (family Simulidae), biting midges and mosquitoes.

It is reported that transmission of anaplasmosis by biting flies will occur only within a few minutes after a blood meal has been obtained from an infected animal. The situation is much different with ticks, as the organism can maintain itself in this host for an extended period of time.

Livestockmen should understand that transmission of anaplasmosis can occur when infected blood from one animal is carried mechanically to a susceptible animal. Infected needles for bleeding or inoculating, dehorning saws, nose tongs, tattooing instruments, ear notchers or knives for castration can all be implicated in the spread of the disease.

HOSTS

The principal host for anaplasmosis in Texas is cattle. Sheep, goats and white-tailed deer (Odocoileus virginianus) can be infected artificially, but the role of these animals in the transmission of anaplasmosis to cattle is undetermined.

DIAGNOSIS

In the field acute cases of anaplasmosis are characterized by pale mucous membranes, rapid breathing, weakness, muscle tremor, jaundice and elevation of body temperature as high as 107 degrees F. A firm diagnosis of anaplasmosis requires laboratory confirmation. There are usually no recognizable signs of chronic anaplasmosis, and the diagnosis of this type infection is dependent on blood serum tests.

A number of blood tests are available for the diagnosis of anaplasmosis. The oldest and still a standard is the complement-fixation test (CF). A capillary-tube agglutination test has been used, and, in recent years, a card agglutination test has been developed. Both of these procedures compare favorably with the CF test, all showing a high degree of correlation. The card agglutination test was developed primarily for field use and can be conducted on the ranch within minutes of taking the blood sample.

DIFFERENTIAL DIAGNOSIS

Anaplasmosis may be confused with other diseases such as Texas fever, leptospirosis, or other infections which may result in anemia. Young animals are more resistant to anaplasmosis and more severly affected with leptospirosis than are older animals, whereas age is not so much a factor with other infectious diseases.

Identification of the anaplasma organism, confirmed by a positive serologic test response, is essential for definite diagnosis.

TREATMENT

Many drugs have been employed in an effort to cure or prevent anaplasmosis, but only a few have been found really effective. To date, only the tetracyclines (chlortetracycline, tetracycline hydrochloride, and oxytetracycline) have been found to have a useful effect on the rate of multiplication of Anaplasma parasites and on the course of the disease. However, it should be remembered that tetracycline medication must be applied in the early stages of the disease for the best results. If treatment is not started until considerable loss of blood has occurred, the changes of recovery are diminished. The tetracycline compounds can be given in injections or by feeding. The use of 3 to 5 mg. of tetracyclines per pound of body weight given in one to three injections over a period of days has proven beneficial in the treatment of acute anaplasmosis.

Tetracyclines will prevent animals from becoming infected when fed daily at the level of 0.5 grams per 1,000 lbs. of body weight through the heavy insect season. In order to avoid misuse of the drug or improper application, the local veterinary practitioner should be consulted. Drug treatment should be carried out under his supervision. This is particularly important in dairy cattle to prevent drug residues in market milk.

Although the tetracyclines have proven effective, other supportive treatments are useful such as proper feeding, nursing, and the use of tonics and cathartics for digestive disorders. Due to the anemia caused by red blood cell destruction, animals should be handled carefully with a minimum of exertion or restraint.

Because of the high cost and other disadvantages of antibiotic therapy, investigators have recently tested two new compounds having a specific therapeutic effect against anaplasmosis. These compounds, known as Gloxazone and Imidocarb, are not available for use on the open market. Before they can be approved for commercial use, they must be tested thoroughly to satisfy USDA requirements of toxicity and side effects. When and if they become available commercially, they may prove a more practical and economical approach for treatment of anaplasmosis.

CONTROL OF ANAPLASMOSIS

Anaplasmosis control programs are complex. In order to prevent losses, four approaches are possible:

1. Control of the disease by drugs.
2. Control based on blood serum diagnosis and the removal of reactor animals.
3. Control by vaccination (killed or live vaccines).
4. Control of the vectors.

The choice of methods depends largely on management systems and epizootiological factors. Where the owner can check his animals daily, he can control anaplasmosis by the use of drug treatment for the elimination of carriers. On large ranches, especially those located in an area of high anaplasmosis incidence, drug therapy may not be practical due to the cost of the drugs and labor needed for repeated treatments. Vector control, if complete, would be effective, but at the present time this is often impossible.

However, degree of vector control will assist any control program. Vaccination with a killed vaccine product can be applied on a large scale to stimulate an immunity against anaplasmosis when the risk of infection is high.

A vaccine is presently available in the United States; the manufacturer recommends that the vaccine be given in two separate doses spaced 6 weeks apart and a booster dose be given yearly. However, there are reports that some calves born of dams previously vaccinated with this vaccine have been affected by a condition called neo-natal isoerythrolysis (N.I.) or "yellow calf syndrome". Research on this problem is presently in progress.

The control of the vectors is necessary whether ranchers use vaccines or drugs for anaplasmosis prevention. Any mechanism reducing or eliminating those vectors which transmit anaplasmosis is helpful. Some recommended methods presently in use are dust bags, back rubbers, sprays and dipping of livestock.

The control of anaplasmosis in the U.S. by means of systematic eradication programs is most desirable. As previously stated, the identification and removal or separation of infected animals from non-infected susceptible cattle is an essential first step. Good diagnostic tests are available to identify the infected carrier. The removal of carrier cattle can be accomplished by the establishement of two herds, one infected and one clean, with strict isolation of the two herds. Infection can be eliminated by the treatment of reacting

cattle to remove the carrier status, or by the sale for slaughter of all carrier animals. Vector control measures are an important adjunct to any control program.

The Institute of Tropical Veterinary Medicine at Texas A & M University is currently involved in several field trials in Texas to test the feasibility of removing carrier animals through a testing and treatment program. Treatment techniques are being tested in the hopes of developing a more practical approach for the elimination of carrier infections. To date, results are encouraging that more efficient means can be developed for the elimination of infection.

RECOMMENDATIONS

Livestock owners are urged to contact their local veterinarian or a representative of the Texas Animal Health Commission if they suspicion a outbreak of anaplasmosis in their herd. It is important to obtain a proper diagnosis in order to start preventive measures necessary to control the disease.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of the following individuals in preparing this fact sheet:

Dr. F. D. Maurer, Associate Dean, College of Veterinary Medicine; Dr. K. L. Kuttler, Professor, Institute of Tropical Veterinary Medicine, The Texas A & M University System.

SUMMARY

A brief description of anaplasmosis, with special emphasis on recent achievements in the field of diagnosis and control was discussed and summarized for Texas A&M University Extension Service publication.

BABESIA BIGEMINA: IMMUNE RESPONSE OF CATTLE INOCULATED WITH IRRADIATED PARASITES[1]

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ABSTRACT

Investigations were undertaken to study the effects of various radiation dosages on the infectivity and immunogenicity of Babesia bigemina. Calves infected with 1×10^{10} B. bigemina parasitized erythrocytes exposed to 24 kRad developed progressive parasitemias. Some calves receiving 1×10^{10} parasitized erythrocytes irradiated at 36 kRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 and 60 kRad. A degree of acquired resistance to infection with B. bigemina developed in calves after inoculation with parasites irradiated at 48 and 60 kRad. The resistance developed was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections due to challenge with nonirradiated parasites. Irradiated parasites were frozen without apparent loss of immunizing properties.

INDEX DESCRIPTORS

Cattle; Babesiosis; Babesia bigemina; Irradiation; Attenuation; Immunity; Serology; Hematology; Growth.

INTRODUCTION

Bovine babesiosis is a tick-borne hemaprotozoan disease which occurs in the warmer areas of all continents. Attempts to produce immunity against bovine babesiosis have been undertaken by several investigators with varying degrees of success. Cattle can be artificially immunized against bovine babesiosis by injecting infected blood followed by chemotherapy in animals which show clinical signs. Although cattle infected in this way are relatively immune to natural infections, they carry prolonged latent infections and may serve as reservoirs of the parasite for continued tick dissemination of the disease.

Cattle can be partially protected against homologous challenge by immunization with killed Babesia; however, large amounts of antigen are required to produce only a marginal protection. The inconclusive results obtained with killed vaccines may be attributed to the loss or denaturation of functional antigens during the preparation of the vaccines or to the rapid elimination of parasites in the host. Therefore, a major problem confronting researchers is how to produce a strong protective immunity without the pathogenic effects resulting from replicating Babesia.

Irradiation of protozoa interferes with their physiologic processes and frequently inhibits their normal development and multiplication (Giese, 1967). Studies on the effects of ionizing radiations on parasitic protozoa have repeatedly shown that while an extremely high radiation dose is necessary to cause immediate death of the parasite, much lower doses can interfere with infectivity (Kimball, 1955). The immunogenicity of irradiated Babesia rodhaini (Phillips, 1970 & 1971), Plasmodium spp. (Corradetti, et al., 1968); Nussenzweig, et al., 1969; Sadun, et al., 1969) and Trypanosoma spp. (Duxbury & Sadun, 1969 & 1970; Sanders & Wallace, 1966) in experimental

animals (mice, rats, and owl monkeys) has been investigated. Parasites irradiated with a dose which abolished their reproductive potential and ability to produce patent infections may retain their capacity to produce an immune response in a susceptible experimental animal. Therefore, the use of irradiated Babesia as vaccines might provide the special immunological properties of living protozoa while suppressing the pathogenic effects.

The following series of investigations were undertaken to study the effect of various radiation dosages on the infectivity and immunogenicity of erythrocytic stages of Babesia bigemina. The effect of freezing on the immunogenicity of irradiated B. bigemina was also studied. The studies were conducted toward the ultimate goal of producing irradiated vaccines against babesiosis.

MATERIALS AND METHODS

Experimental Animals

Three-day-old male Holstein calves were obtained from near Facatativá, Colombia and were hand-raised to 3 months of age on the Tibaitatá Instituto Colombiano Agropecuario (ICA) experiment station, in an area free of Boophilus microplus. The calves were housed for the duration of the experiment in a tick-free environment at the ICA Laboratorio de Investigaciones Médicas Veterinarias (LIMV) in Bogotá, Colombia.

Preparation of Irradiated and Challenge Inocula

Blood containing B. bigemina parasitized erythrocytes was collected from splenectomized calves during the acute stage of babesiosis after inoculation with 50 ml of stabilate of B. bigemina. The isolation, separation and preservation of the B. bigemina stabilate used throughout this

study has been previously described (Bishop, et al., 1973). Although calves were inoculated and challenged at different times, each inoculum was prepared from splenectomized calves previously inoculated with 50 ml of the same stabilate of B. bigemina.

For irradiation treatment, 120 ml of inoculum was divided equally between 2 plastic Petri dishes, 1.3 cm. deep and 9.1 cm. in diameter. The depth of inoculum in each Petri dish was approximately 1.0 cm. The inoculum in one Petri dish was exposed to the desired amount of radiation using a 3129-c cobalt-60 teletherapy unit* which delivered a dose rate of approximately 200 Rads/min at an exposure distance of 50 cm. The dose rate was determined by ferrous chemical dosimetry and periodic verification of the dose rate was carried out by means of a dosimeter.** The inoculum in the other Petri dish was placed in an adjacent room for the duration of the irradiation and used as a nonirradiated control.

Experimental Procedures

Four experimental groups of 4 calves each were given 1 intravenous inoculation of blood containing 1×10^{10} B. bigemina parasitized erythrocytes previously exposed to radiation doses of 24, 36, 48 and 60 kRad. A fifth experimental group of 4 calves was given an intravenous inoculation of blood containing 1×10^{10} B. bigemina parasitized erythrocytes previously exposed to 60 kRad and frozen (Bishop, et al.,1973) for 48 hours. All calves in the 5 experimental groups were challenged intravenously with blood containing 1×10^{10} B. bigemina parasitized erythrocytes 4 weeks after inoculation.

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*Eldorado 8, Atomic Energy of Canada Limited: Commercial Products, P.O. Box 93, Ottawa, Canada.

**Victoreen r-Meter Mod. No. 570. Victoreen Instrument Division, 10101 Woodland Ave., Cleveland, Ohio 44104.

The first control group of 3 calves was given an intravenous inoculation of nonirradiated blood containing 1×10^{10} B. bigemina parasitized erythrocytes. To evaluate the possibility that some reduction in parasitemia might be the result of reduction in the number of viable parasites injected rather than an attenuation of the parasites injected, the second and third control groups of 3 calves each received an intravenous inoculation of nonirradiated blood containing 1×10^{7} and 1×10^{5} B. bigemina parasitized erythrocytes, respectively. To test the hypothesis that living erythrocytic stages of B. bigemina exposed to a radiation dose sufficient to prevent progressive parasitemias are more immunogenic than non-living parasites, the fourth control group of 3 calves was given an intravenous inoculation of blood containing 1×10^{10} B. bigemina parasitized erythrocytes previously heat inactivated in a water bath at 56°C for 30 minutes. The fifth control group of 3 calves received an intravenous inoculation of a similar volume of nonparasitized irradiated blood. All calves in the 5 control groups were challenged intravenously with blood containing 1×10^{10} B. bigemina parasitized erythrocytes 4 weeks after inoculation.

Assessment of Reactions

The calves were examined daily for the presence of parasitized erythrocytes by the use of combination thin and thick blood films. The method used for the preparation of the combination thin and thick films has been previously described (Bishop & Adams, 1973).

Subinoculations were performed the day of challenge from groups which failed to demonstrate detectable parasitemia to determine whether subpatent parasitemia had resulted. Subinoculations were accomplished by collecting 100 ml of blood from each calf in each group by venipuncture

and injecting the pooled blood from each group into splenectomized calves.

Daily packed cell volumes were determined by the microhematocrit method and rectal temperatures were measured each morning throughout the experiment. All calves were weighed weekly following a 12-hour withdrawal from feed.

Determination of babesial complement fixing (CF) serum antibody levels was performed twice a week by a microtiter procedure similar to that described by Hidalgo and Dimopoullos (1967). Babesia bigemina antigens for the CF microtiter procedure were prepared from blood containing 25% B. bigemina parasitized erythrocytes by a method described by Mahoney (1967). Antigens were titrated against a group of 8 positive reference serums according to a scheme previously described (Anon. 1968). The positive serums were taken from cattle 1 to 4 months following recovery from blood-induced infections. A unit of antigen was defined as the highest dilution causing complete complement fixation in the presence of the weakest positive reference serum diluted 1:5.

RESULTS

The results summarized in Table I show that calves inoculated with 1×10^{10} B. bigemina parasitized erythrocytes exposed to 24 kRad developed progressive parasitemias with prolonged prepatent periods and lower maximum parasitemias than control calves inoculated with 1×10^{10} nonirradiated parasitized erythrocytes. Three of 4 calves receiving parasitized blood irradiated at 36 kRad did not develop progressive parasitemias. Progressive infections were prevented by exposure to 48 and 60 kRad and subinoculations into susceptible splenectomized calves failed to produce active infections. Control calves that had been inoculated with 1×10^{7} nonirradiated parasitized erythrocytes also had prolonged prepatent periods and lower maximum

parasitemia when compared to control calves inoculated with 1×10^{10} parasitized erythrocytes.

The results summarized in Table II show that all calves developed progressive parasitemias with similar prepatent periods following challenge with 1×10^{10} nonirradiated E. bigemina parasitized erythrocytes. The average maximum parasitemias were 0.8 and 0.6% following challenge for calves inoculated 4 weeks previously with 1×10^{10} heat inactivated parasitized erythrocytes and 10 ml nonparasitized irradiated blood, respectively. The average maximum parasitemias were 0.05 and 0.06% following challenge for calves inoculated 4 weeks previously with 1×10^{10} parasitized erythrocytes irradiated at 60 kRad and 60 kRad (frozen), respectively.

The average maximum morning rectal temperatures were 41.1 and 41.[illegible] following challenge for calves inoculated 4 weeks previously with 1×10^{10} heat inactivated parasitized erythrocytes and 10 ml nonparasitized irradiated blood, respectively. The average maximum morning rectal temperatures were 39.9 and 40.0°C following challenge for calves inoculated 4 weeks previously with 1×10^{10} parasitized erythrocytes irradiated at 60 kRad and 60 kRad (frozen), respectively.

The average minimum packed cell volumes were 19 and 10% following challenge for calves inoculated 4 weeks previously with 1×10^{10} heat inactivated parasitized erythrocytes and 10 ml nonparasitized irradiated blood, respectively. The average minimum packed cell volumes were 25 and 23% following challenge for calves inoculated 4 weeks previously with 1×10^{10} parasitized erythrocytes at 60 kRad and 60 kRad (frozen), respectively.

Three calves (one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 24 kRad, one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 60 kRad and one previously inoculated

with 10 ml nonparasitized irradiated blood) suffered intense respiratory distress for 15 minutes following intravenous challenge. Three other calves (one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 36 kRad, one previously inoculated with 1×10^{4} nonirradiated parasitized erythrocytes and one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 60 kRad and frozen) suffered intense respiratory distress following intravenous challenge. Coughing produced cream-colored frothy fluids mixed with blood. These calves died within 24 hours following challenge. Post-mortem examination revealed lesions which were confined largely to the respiratory system, which included severe intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage of the lung. The trachea and major bronchi contained a hemorrhagic cream-colored frothy fluid. One other calf died following the development of a severe systemic toxic reaction after intravenous inoculation with B. bigemina parasitized blood previously exposed to 60 kRad and frozen.

DISCUSSION

The results from studies designed to determine the effect of various radiation dosages on the infectivity of B. bigemina indicate that calves infected with B. bigemina parasitized erythrocytes exposed to 24 kRad developed progressive parasitemias whereas progressive infections were prevented by exposure to irradiation at 48 kRad. These results confirm and extend those reported recently from cattle inoculated with irradiated B. bigemina (Castro & Cambez, 1968), Babesia major (Brocklesby et al., 1972) and from mice and rats inoculated with irradiated B. rodhaini (Phillips 1970 & 1971).

The results also showed that in addition to a lower infection rate brought about by irradiated parasites, calves that did become infected had prolonged prepatent periods and lower maximum parasitemias than control calves receiving nonirradiated inocula. The results could be due to the death or developmental arrest of some of the parasites, or to an overall retarded development leading to more slowly developing [illegible]. Control calves that had been inoculated with 1×10^7 nonirradiated parasitized erythrocytes also had prolonged prepatent periods and lower maximum parasitemias when compared to control calves inoculated with 1×10^{10} nonirradiated parasitized erythrocytes. Therefore, the prolonged prepatent periods and lower maximum parasitemias in calves that had received irradiated parasites could have been due in part to a reduction in the number of viable parasites injected.

The finding that calves inoculated with 1×10^7 nonirradiated parasitized erythrocytes had prolonged prepatent periods and lower maximum parasitemias (Table 1) indicate that such a standardized inoculum might be of use as a vaccine to produce an attenuated infection. Callow and Mellors (1966) have developed a similar monovalent vaccine for B. [illegible] prepared in splenectomized calves. Kamron et al., (1964) and Pipano (1969) have also reported that the length of incubation period was inversely related and the severity of the reaction directly related to the number of parasites in the inoculum.

A degree of acquired resistance to infection with B. bigemina developed in calves after inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The resistance developed was sufficient to suppress multiplication of the Babesia and to permit calves to survive

to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction and a smaller increase in rectal temperatures following challenge for calves previously inoculated with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The irradiated parasites were apparently responsible for the development of this resistance since irradiated nonparasitized blood did not produce a discernible acquired resistance to B. bigemina. These results are in agreement with and extend those recently reported from mice and rats immunized with irradiated B. rodhaini (Phillips, 1970 & 1971)

The failure of the immunized calves to develop patent infections following inoculation of irradiated parasites indicate that immunity could not be considered to have been developed in response to a long standing chronic blood infection. The observation that inoculation of calves with irradiated nonreplicating B. bigemina may induce protective immunity, suggests that the presence of replicating Babesia in the host is not necessary for the development of acquired resistance. This is in agreement with the concept of "sterile immunity" described for B. argentina (Callow, 1968), B. bigemina (Callow, 1964 & 1967), B. divergens (Joyner & Davies, 1967), B. microti (Cox & Young, 1969) and B. rodhaini (Cox & Young, 1969; Phillips, 1969 & 1971).

The acquired resistance to infection with B. bigemina developed in calves inoculated with 1×10^{10} B. bigemina irradiated at 48 and 60 kRad was much greater than the acquired resistance to infection developed in calves inoculated with 1×10^{10} heat killed B. bigemina. Thus, it seems likely that immunization with irradiated nonreplicating Babesia may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite.

Although CF antibodies are probably not functional in immunity (Mahoney, 1967a), it was of interest for theoretical and diagnostic considerations to determine whether detectable CF antibodies were produced in calves after one inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The results of the CF microtiter tests indicated that 3 of 8 calves were able to produce detectable levels of antibodies with maximum titers ranging from 1:5 to 1:20 even when the normal development and multiplication of Babesia were interrupted by irradiation. There was no discernible difference in acquired resistance to infection between those calves which did or did not develop detectable CF antibodies.

The success obtained in immunizing calves with B. bigemina which had been irradiated, frozen and stored in a dry ice cabinet may be of practical significance in future studies. The Babesia parasites could be irradiated and frozen, without apparent loss of immunizing properties, for use in distant parts of the world.

It is of interest to note a consistent temperature rise in calves following intravenous inoculations with B. bigemina even when the normal development and multiplication of Babesia were interrupted by irradiation or heat inactivation. Vaccines which contain foreign protein can act as pyrogens that cause body temperature rise following injection (Guyton, 1961). It is also possible that plasma proteins and other proteins in blood which are not normally pyrogenic or toxic could be changed chemically by hemolysis, degradation, or denaturization and thereafter cause systemic reactions (Guyton, 1961). One calf died following the development of a severe systemic toxic reaction after intravenous inoculation with previously frozen B. bigemina parasitized blood exposed to 60 kRad.

Six calves also developed severe systemic reactions following intravenous challenge with blood containing 1×10^{10} nonirradiated B. bigemina parasitized blood. Aitken and Sanford (1969) have recently described similar anaphylactic reactions in calves given 1 intravenous injection of a foreign protein following challenge by the same route after a latent period of at least 7 days. Anaphylactic shock of this type, however, usually only follows the intravenous injection of the challenge dose of antigen and could probably be avoided by using other routes in which the antigen would reach the circulation more slowly.

Standardized infecting and immunizing doses in the present study were given intravenously because previous studies have indicated that Babesia may be more immunogenic when given by the intravenous route (Mahoney, 1967b). The same standardized inocula were given intravenously for challenge since the infections produced by these inocula given by the intravenous route had already been characterized. In view of the above observations, a recharacterization of challenge inoculum using a subcutaneous route of inoculation would be warranted for future studies using infected blood for challenge. Experimental or natural field tick challenge would also most likely avoid the problem of anaphylactic reactions following challenge in calves previously inoculated with irradiated B. bigemina parasitized blood.

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Table I. Characteristics of *Babesia bigemina* Infections Produced with Infected Blood Subjected to Different Radiation Doses.

Radiation Dose of Inoculum in Kilorads	No. of Parasitized Erythrocytes Inoculated	Reaction After Inoculation of Irradiated B. bigemina Parasitized Erythrocytes[a]							
		No. Calves with Parasitemia / No. Calves Inoculated[b]	Prepatent Periods in Days	Maximum % Parasitized Erythrocytes	Maximum Temperature in °C	Minimum Packed Cell Volume in %	Maximum Complement Fixation Titer	Average Daily gain in g/day	Deaths
0 (controls)	1×10^{10}	3/3	2.0(2-2)[c]	3.2(0.4-8.0)	41.5(41.3-41.6)	18(13-28)	1:320(1:160-1:640)	423(71-821)	0
0 (controls)	1×10^{7}	3/3	5.7(5-6)	0.03(0.008-0.06)	40.5(40.3-40.7)	27(24-32)	1:201(1:160-1:320)	798(643-911)	0
0 (controls)	1×10^{4}	0/3	NONE	NONE	39.7(39.2-40.0)	27(24-28)	Neg.(Neg.-Neg.)	862(643-1026)	0
24	1×10^{10}	4/4	6.5(6-8)	0.11(0.008-0.40)	40.3(39.5-40.7)	26(22-30)	1:135(1:80-1:320)	661(500-786)	0
36	1×10^{10}	1/4	13.0(13-13)	0.002(0.002-0.002)	40.1(39.7-40.3)	30(26-33)	1:13(Neg.-1:80)	790(536-1000)	0
48	1×10^{10}	0/4	NONE	NONE	40.1(39.6-41.0)	31(29-34)	1:2(Neg.-1:5)	875(661-952)	0
60	1×10^{10}	0/4	NONE	NONE	40.6(40.0-40.9)	30(28-31)	1:3(Neg.-1:20)	857(706-1018)	0

a Characteristics of reactions based on data collected for 28 days post inoculation.

b Detected by either thick blood films or subinoculations.

c Range of means.

Table 2. Immune Response of Calves Inoculated Case with Irradiated *B. bigemina* and Challenged 4 weeks Later with Nonirradiated *B. bigemina*

Treatment of Inoculum	Size of Inoculum	Reaction After Challenge with 1×10^{10} Unirradiated *B. bigemina* Parasitized Erythrocytes[a]								
		No. Calves with Parasitemia / No. Calves Challenged	Incubation Period in Days	Maximum Parasitized Erythrocytes	Maximum Temperature in °C	Minimum Packed Cell Volume in %	Maximum Complement Fixation Titer	Average Daily Gain in g/day	Deaths
0 kRad (control)	1×10^{10} *B. bigemina*	3/3	1.0(1-1)[b]	0.006(0.001-0.008)	40.2(39.9-40.5)	26(20-31)	1:201(1:80-1:320)	837(786-964)	0
0 kRad (control)	1×10^{7} *B. bigemina*	3/3	1.7(1-2)	0.004(0.002-0.006)	40.2(40.1-40.3)	28(26-30)	1:127(1:80-1:160)	976(891-1026)	0
0 kRad (control)	1×10^{4} *B. bigemina*	2/2	1.0(1-1)	0.5(0.5-0.5)	41.3(40.9-41.7)	11(10-12)	1:160(1:160-1:160)	813(698-928)	1[c]
Bacterial (control)	1×10^{10} *B. bigemina*	3/3	1.0(1-1)	0.6(0.4-1.0)	41.1(39.8-41.8)	19(16-22)	1:640(1:640-1:640)	815(582-1107)	0
26 kRad (control)	10 ml Normal Blood	3/3	1.0(1-1)	0.6(0.1-1.0)	41.4(41.0-41.7)	10(3-14)	1:327(1:160-1:320)	982(786-1179)	1[d]
24 kRad	1×10^{10} *B. bigemina*	4/4	1.0(1-1)	0.001(0.002-0.005)	39.2(38.6-40.0)	27(25-30)	1:134(1:80-1:160)	1081(1003-1264)	0
26 kRad	1×10^{10} *B. bigemina*	3/3	2.0(1-4)	0.03(0.003-0.04)	40.2(39.4-40.5)	26(25-27)	1:201(1:160-1:320)	1060(917-1214)	1[c]
48 kRad	1×10^{10} *B. bigemina*	4/4	1.0(1-1)	0.1(0.01-0.3)	39.8(39.5-40.4)	27(21-27)	1:320(1:320-1:640)	947(835-1026)	0
60 kRad	1×10^{10} *B. bigemina*	4/4	1.5(1-2)	0.03(0.01-0.05)	39.8(39.7-40.1)	27(23-27)	1:203(1:160-1:640)	938(759-1140)	0
60 kRad (freeze)	1×10^{10} *B. bigemina*	2/2	1.0(1-1)	0.06(0.05-0.07)	40.3(40.0-40.6)	23(21-25)	1:640(1:640-1:640)	822(768-875)	1[c]

[a] Characteristics of reactions based on data collected for 28 days post challenge.
[b] Range of means
[c] Death due to acute anaplasmosis followed challenge.
[d] Death due to acute babesiosis followed challenge.

COMBINATION THIN AND THICK BLOOD FILMS FOR THE DETECTION OF *BABESIA* PARASITEMIA

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Summary

A method for preparing and examining combination thin and thick blood films for the detection of Babesia spp. parasitemias was developed. A technique for staining the combination thin and thick films, using a phosphate-buffered Giemsa stain solution containing alkyl phenoxy polyethoxy ethanol was also described.

Introduction

During acute bovine babesiasis, the parasitemia can be detected by examining stained thin blood films. In cattle surviving the acute stage of the disease and becoming carriers, however, Babesia spp. are not readily demonstrable in thin blood films. A thick blood film method for detecting Babesia parasitemia was described by Mahoney and Saal[2] that facilitated the examination of a greater quantity of blood.

For the diagnosis of malaria in man, a method for the preparation and staining of combination thin and thick blood films was developed.[1] It seemed that a similar method might prove advantageous for the detection of the parasitemia in cattle affected with acute and chronic babesiasis. The method which evolved is described and some results of this application reported.

Materials and Methods

Preparation of Combination Thin and Thick Blood Films - The blood films were made with samples of bovine blood obtained from the jugular vein, using 1.3 g./l. of disodium ethylenediamine tetraacetate as anticoagulant. For the preparation of the thin blood film, a small drop of blood was placed on one end of a microscopic slide and extended over 1/2 the length of the slide and rapidly air dried. The method used for the preparation of the thick blood

film was based on a technique described by Mahoney and Saal.[2] A loop (2.5 mm., external diameter) was used to deliver a standard drop of blood on the end of the same microscope slide opposite the thin blood film. The loop was made by twisting 30-gauge wire (taken from flyscreen gauze) tightly around a 14-gauge hypodermic needle. After the remaining blood on the loop was blotted on a clean paper towel, the loop was used to spread the drop evenly over an area of 28 square mm. which was outlined by a circle 6 mm. in diameter on a paper underneath the microscope slide. Before use on the next blood sample, the loop was rinsed in water and then in absolute methanol which was allowed to evaporate. The thick blood films were then dried in a horizontal position. Initial drying was hastened by placing the slides near a 150-watt incandescent lamp.

The thin blood films were then fixed with absolute methyl alcohol. The slides were held in a slanted position with the thin blood film down and a few drops of absolute methanol placed on the thin blood film only. To prevent alcohol or alcohol fumes from contacting the thick blood film, the slides were placed in a vertical position with the thick blood film up until the methanol evaporated. The slides were then placed in a staining rack[a] and kept at this state for further processing.

Before staining, the slides were placed in an incubator at 65 C. for 45 minutes for final drying of the thick blood films. Final drying was required to ensure that the thick blood films did not detach during the staining process. To prevent deterioration of the thick blood films, the combination thin and thick blood films were stained soon after final drying.

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[a]Staining Rack Model S7680. Scientific Products, Division of American Hospital Supply Corporation, Evanston, Illinois 66201.

Staining of the Combination Thin and Thick Blood Films - The method used for the staining of the combination thin and thick blood films was based on a staining technique described for *Plasmodium spp.* in man. The thin and thick blood films, prepared and placed in staining racks, as described in the preceding topical paragraph, were stained in a 1:50 solution of Giemsa stain[b] for 45 minutes. Phosphate-buffered water (pH 7.0-7.1) containing 0.01% alkyl phenoxy polyethoxy ethanol[c] (APPE) was used to prepare the stain solution. Stock solutions of 0.067M Na_2HPO_4, 0.067M Na_2HPO_4, and 10% APPE were prepared and kept in separate glass stoppered bottles. Fresh buffered water (pH 7.0-7.1) containing 0.01% APPE was prepared each week by using 39 ml. of 0.067M Na_2HPO_4, 61 ml. of 0.067M $NaHPO_4$, 1 ml. of 10% APPE, and 899 ml. of distilled water. An electric pH meter[d] was used to determine the pH of the freshly prepared buffered water containing APPE.

Immediately following staining, the thin and thick blood films were rinsed briefly by dipping the slides in a staining dish filled with buffered water (pH 7.0-7.1) containing 0.01% APPE. A rubber band was placed around the staining rack to hold the slides in place. The staining rack was placed in a vertical position with only the thick blood film immersed in the same buffered water containing 10% APPE for an additional 3 to 5 minutes. The slides were then air dried in the vertical position with the thick film down.

Results and Discussion

The use of a 1:50 solution of Giemsa stain provided osmotic pressures

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[b]Giemsa Stain. Gradwohl Laboratories, St. Louis, Mo.

[c]Triton X-100. Rohm and Haas, Philadelphia, Pa. 19105.

[d]Beckman Expandomatic Model 76. Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, California, 92634.

that caused lysis of the unfixed erythrocytes in thick blood films during the 45 minutes' staining. Therefore, the usual morphologic criteria for differential identification of parasites based on differences in size and in position in the erythrocyte are not applicable to thick blood films. The use of the surface-active agent APPE in the staining and washing solutions resulted in brighter and cleaner preparations with only parasites, leukocytes, and platelets remaining in the thick blood films.

The use of buffered (pH 7.0-7.1) Giemsa stain solution provided the most dependable differential staining of the parasite (deep blue cytoplasm and a brilliant red nucleus) For confirmation of Babesia parasitemia on thick blood films, it was necessary to resolve the objects under examination into a red nucleus and blue cytoplasm and to find some paired parasites that were typically pyriform. The depth of the thick blood film allowed organisms to lie in any plane which caused some distortion from the typical appearance of the parasites seen in thin films. The typical pairs could, however, be readily recognized even when some distortion occurred. Photographs of Babesia bigemina on thin and thick blood films are shown (Fig. 1 and 2).

An estimate of the number of parasites per cubic millimeter of blood on thick blood films could be made from the number of parasites seen in 20 microscopic fields (using X10 ocular and X100 oil objective) and from the number of leukocytes per cubic millimeter of blood. The number of parasites per cubic millimeter of blood was then converted to the percentage of erythrocytes from the number of erythrocytes per cubic millimeter of blood.

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1. Anonymous: Manual for the Laboratory Diagnosis of Parasitic Diseases. II. Blood Parasites 8010-C. Parasitology Training Unit, National Communicable Disease Center, Atlanta, Georgia, (1970):32-34.

2. Mahoney, D. F. and Saal, J. R.: Bovine Babesiosis: Thick Blood Films for the Detection of Parasitemia. Aust. Vet. J., 37, (Feb., 1961): 44-47.

Fig. 1 - Babesia bigemina parasitized bovine erythrocytes in a thin blood film. Giemsa; X1330.

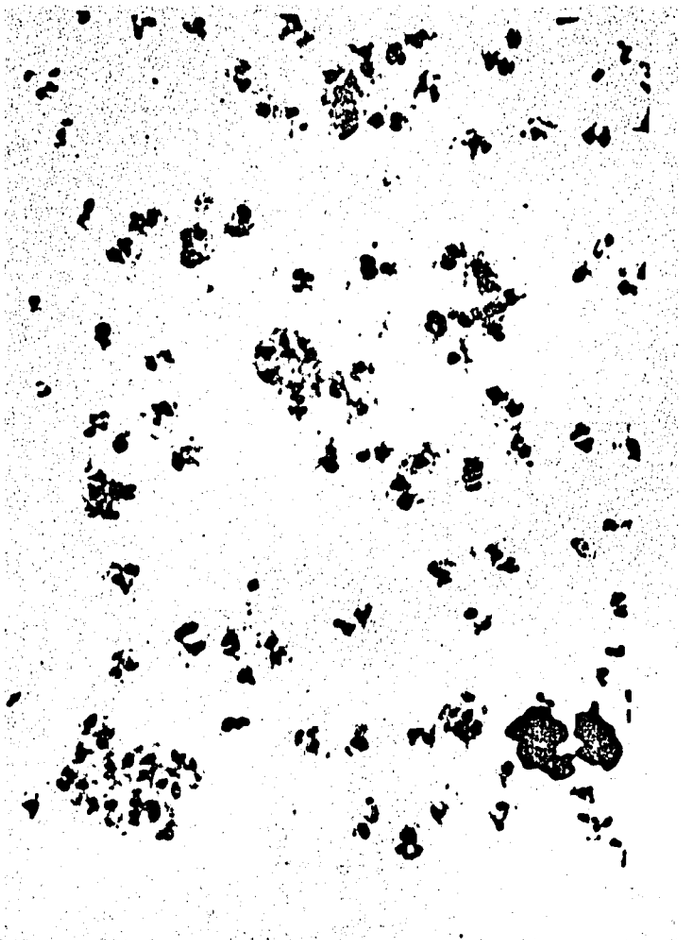

Fig. 2 - Babesia bigemina parasites in a thick bovine blood film. Giemsa; X1330.

THE ISOLATION, SEPARATION AND PRESERVATION OF *BABESIA BIGEMINA*

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SUMMARY

Experiments were performed to separate Babesia bigemina from contaminating organisms. Babesia bigemina was passaged serially through 5 splenectomized calves. The first calf was inoculated with blood carrying several different organisms, and subsequent subinoculations were done soon after blood smears from each calf were found to be positive for B. bigemina. Five blood passages were carried out in 6.5 days. Babesia argentina, B. major and A. marginale were eliminated as contaminants the B. bigemina isolated after four passages. A frozen stabilate of the isolated B. bigemina was established.

INTRODUCTION

The history of the recognition of bovine babesiosis in Colombia and the identification of the 3 species responsible was recounted by Velásquez (1938). The 3 species, Babesia bigemina, Babesia argentina and Babesia major, may occur together as a mixed infection and the incidence of infection appears to be related to the occurrence and activity of the tick-vectors at the various altitutes (Todorovic, Adams and Roberts, 1969).

In an attempt to add to our knowledge of the mechanisms involved in acquired resistance to B. bigemina, a series of investigations were undertaken. As a starting point, it was necessary to have an isolate of B. bigemina free from contaminating organisms. However, B. bigemina carriers in Colombia are almost invariably infected with at least one other hemoparasite. Babesia argentina, B. major, Anaplasma marginale, Trypanosoma vivax, Trypanosoma theileri, Eperythrozoon wenyoni, Eperythrozoon teganodes and Eperythrozoon tuomii are

also parasites of cattle in Colombia in areas where arthropod vectors are present (Kuttler, Adams and Zaraza, 1969; Todorovic, et al., 1969; Wells, Betancourth and Page, 1970; Adams, Craig, Platt and Wyss, 1972). Kuttler (1969) separated Babesia spp. and A. marginale from a cow from the Llanos Orientales (Eastern Plains) of Colombia. A pure Babesia spp. carrier was established by the simultaneous inoculation of mixed infected blood and with alpha-ethoxyethylglyoxal disemithiocarbazone* which prevented an A. marginale infection.

The organisms originally contaminating our isolate of B. bigemina were B. argentina, B. major and A. marginale. This paper describes the isolation, separation and preservation of a B. bigemina stabilate used at the Laboratorio de Investigaciones Médicas Veterinarias (LIMV), Instituto Colombiano Agropecuario (ICA), Bogotá, Colombia.

MATERIALS AND METHODS

Three-day-old male Holstein-Friesian calves were obtained from near Facatativá, Colombia and were hand-raised on the Tibaitatá ICA experiment station (altitude 2600 m.) in an area free of Boophilus microplus.

The isolation of B. bigemina was made from a natural case of babesiosis. A four-month-old splenectomized calf was transported by airplane from the ICA-LIMV laboratories in Bogotá to the northern coastal area of Colombia and placed on a tick-infested pasture at the Turipaná ICA experiment station (altitude 24 m.) near Montería.

Daily thin films were made with blood obtained from the jugular vein using 1.3 g/l of disodium ethylenediamine tetraacetate (EDTA) as an anticoagulant.

* Gloxazone. Burroughs-Wellcome and Co., Inc, Tuckahoe, N. Y.

The films were fixed in absolute methanol and stained with a 1 to 20 solution of Giemsa* for 20 minutes, using phosphate buffered water (pH 7.0-7.1) containing 0.01% alkyl phenoxy polyethoxy ethanol** (APPE) to prepare the stain solution. Stock solutions of M/15 Na_2PO_4, M/15 NaH_2PO_4, and 10% APPE were prepared and stored in separate glass stoppered bottles. Fresh buffered water containing 0.01% APPE was prepared weekly by using 39 ml of M/15 NaH_2PO_4, 61 ml of M/15 Na_2HPO_4, 1 ml of 10% APPE and 899 ml of distilled water. An electric pH meter* was used to determine the pH of the freshly prepared buffered water containing APPE. The films were examined microscopically, using a microscope with an oil immersion objective, for 30 minutes in cases when the first appearance of Babesia was thought to be imminent. The films were examined about 3 to 6 mm from the end of the film and transversed from one side of the film to the other to give a constant and representative sample. After B. bigemina was found in blood smears, 180 ml of blood were withdrawn from the jugular vein of the splenectomized calf, using 1.3 g/l of dipotassium EDTA as an anticoagulant. The blood was transported by airplane to the ICA - LIMV laboratories in Bogotá for the separation of B. bigemina from B. argentina, B. major and Anaplasma marginale.

The method of separation of B. bigemina from B. argentina, B. major and A. marginale involved rapid passage through 5 splenectomized calves and was based on that used by Sergent, Donatien, Parrot, Lestoquard and Plantureux (1927) and Callow and Hoyte (1961a). The first calf was inoculated with blood which con-

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* Giemsa Stain. Gradwohl Laboratories, 3513 Lucas Avenue, St. Louis, Mo., 63155

** Triton X-100, Rohm and Hass Company, Independence Mall West, Philadelphia, Pa. 19105

*** Beckman Expandometic Model 76. Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, California 92634

tained several different organisms, and subsequent subinoculations were done soon after blood smears from each calf were found to be positive for B. bigemina. Blood for subinoculation was collected from the jugular vein without using an anticoagulant and each passage was carried out immediately by injecting the blood into the jugular vein of the next splenectomized calf.

The drugs used to treat the Babesia infections were trypan blue and 4,4'-diamidino-diazoaminobenzene diaceturate*. Trypan blue was used for suppressing the B. bigemina infection without interfering with the potential B. argentina infection.

A frozen stabilate of B. bigemina was made using a modification of the method reported by Pipano and Senft (1966). Blood containing 2.5% B. bigemina parasitized erythrocytes was collected from the fifth splenectomized calf at the acute stage of the disease by venipuncture using 1.3 g/l disodium EDTA as an anticoagulant. Glycerol at a final concentration of 11.6% was used as a cryoprotective agent. Fifty ml aliquots of the blood-glycerol mixture were dispensed in 60 ml plastic narrow mouth bottles with screw caps** and incubated for 30 minutes at 4°C and stored in a dry ice cabinet at -79°C.

RESULTS

The splenectomized calf placed on a tick-infested pastures at the Turipaná ICA station was found to be heavily infested with B. microplus ticks in the larval stage within 3 days, ticks in the nymphal stage within 8 days and ticks in the adult stage within 14 days. After 16 days, the rectal temperature of

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* Ganaseg. Squibb, Cali, Colombia.
** Kimble, Owens, Illinois, Toledo, Ohio 43601

the calf was 40.4°C and B. argentina was found in thin blood smears stained with Giemsa. Two days later, B. bigemina was found in blood smears. The results of the rapid passages of B. bigemina through 5 splenectomized calves to separate B. bigemina from contaminating organisms are summarized in Table I.

Babesia bigemina was found in blood smears from the first calf after 36 hours. The first calf, 4 days after the inoculation, had a B. argentina parasitemia which was treated with 4,4'-diamidino-diazoaminobenzene diaceturate at 3 mg/kg intramuscularly for 5 days. Anaplasma marginale was present in blood smears from the first calf 44 days after inoculation.

Babesia bigemina was found in blood smears from the second calf after 30 hours. The second calf, 4 days after the inoculation, had a B. argentina parasitemia which was treated with 4,4'-diamidino-diazoaminobenzene diaceturate at 3 mg/kg intramuscularly for the following 2 days. The second calf died from babesiosis 1 day after the last treatment.

The third calf was positive for B. bigemina 24 hours after inoculation with 12 ml of blood from the second calf. The third calf was subsequently treated with an intravenous injection of 20 ml of 1% trypan blue, which supressed the B. bigemina infection. The third calf, 15 days after inoculation with blood, had B. argentina and B. major which were treated intramuscularly the following day with 4,4' diamidino-diazoaminobenzene diaceturate at 3 mg/kg. The third calf died from babesiosis 1 day after treatment.

The fourth calf was positive for B. bigemina 37.5 hours after inoculation with 12 ml of blood from the third calf. The procedure of treating the fourth calf with trypan blue was repeated.

Babesia bigemina was found in blood smears from the fifth calf after 57.5 hours, and 12 ml of blood was passaged intravenously into an intact calf. A frozen stabilate of B. bigemina was made from the fifth calf 4 days after inoculation when the parasitemia was 2.5%. The day following freezing, 50 ml of the frozen stabilate was inoculated intravenously into a second intact calf.

The first intact calf was positive for B. bigemina 2 days after inoculation, and the second intact calf was positive 5 days after inoculation. The intact calves required no treatment with trypan blue. Babesia argentina, B. major and A. marginale were not diagnosed in the 2 intact calves nor in any smear from the fourth and fifth splenectomized calves for 2 months after inoculation. Babesia argentina and B. major were eliminated as contaminanting organisms after 4 passages. The second and third splenectomized calves died of babesiosis which made it impossible to estimate at which point A. marginale failed to be passaged.

DISCUSSION

The results of the experiment, designed to obtain B. bigemina free from contaminating organisms, show that B. argentina and B. bigemina were found in blood smears from the splenectomized calf 16 and 18 days, respectively after the calf had been placed on a tick-infested pasture. Callow and Hoyte (1961b) observed the experimental transmission of B. bigemina by B. microplus and recorded patent infection with B. bigemina 11 to 17 days following the placement of larvae on cattle and that transmission did not take place until the ticks became nymphs. Callow and Hoyte (1961b) also reported that B. argentina

could be transmitted by larvae, and therefore, patent infections with B. argentina could be detected earlier than patent infections with B. bigemina following placement of larvae on cattle. The observations on the natural transmission of B. bigemina in the present experiment are in agreement with the observations of the experimental transmission of B. bigemina by B. microplus recorded by Callow and Hoyte (1961b).

The ability of B. bigemina to multiply so rapidly that it was found in thin blood smears from 24 to 57.5 hours after calves had been inoculated with relatively small numbers of parasites, made it possible to separate B. bigemina from other blood parasites. The prepatent periods for B. bigemina observed were in agreement with those prepatent periods reported by Callow and Hoyte (1961b).

The apparent lability of newly isolated Babesia spp. emphasized the need for the establishment of stabilates (Irvin and Brocklesby, 1969; Frerich, Holbrook and Johnson, 1969). Many factors, such as clonal selection, methods of transmission, type of host animal and antigenic variation are probably involved in the lability of Babesia spp. (Phillips, 1969). A frozen stabilate of the B. bigemina isolate in this study was established.

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Table I. The Separation of *B. bigemina* from *B. argentina*, *B. major* and *A. marginale* by Rapid Passage through 5 Splenectomized Calves.

Passage	Inoculum (ml. intravenously)	Prepatent Period (hours)	Parasite	Time of Subinoculation (hours)
1	180 from naturally infected calf.	36	*B. bigemina*	37.5
2	12 from No. 1.	30	*B. bigemina*	31
3	12 from No. 2	24	*B. bigemina*	24
4	12 from No. 3.	37.5	*B. bigemina*	37.5
5	12 from No. 4.	37.5	*B. bigemina*	———

Observations During Concurrent Anaplasmosis and Babesiosis in Experimentally Infected Calves

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SUMMARY

The clinical, serological and pathological manifestations of disease in intact calves concurrently infected with Anaplasma marginale and Babesia bigemina were investigated. Clinical signs were more severe in the concurrently infected calves than in singularly infected controls. Decreases in packed cell volume, albumin:globulin ratio, myeloid:erythroid ratio and increases in the number of reticulocytes, total serum proteins and serum gamma globulins were more pronounced in the concurrently infected calves. The concurrent infections had no apparent effect on the production of complement fixing antibodies. Gross lesions observed in the concurrently infected calves included: pleural and peritoneal transudates; splenomegaly; hepatomegaly; and moderate lymph node enlargement. Histological lesions included: moderate hepatocellular degeneration; lymphoid hyperplasia in the spleen and lymph nodes; and hemosiderosis of the spleen, lymph nodes, liver and kidneys. The relationship of A. marginale and B. bigemina during the concurrent infections appeared to be one of independency. The increased severity of the clinical and pathological signs of disease in the concurrently infected calves was attributed to the concurrent infections being additive.

Introduction

The common distribution of anaplasmosis and babesiosis and the high incidence of concurrent infection of cattle with both Anaplasma and Babesia species has been noted by numerous investigators.[5,6,14,15,17,24,26] In many tropical and subtropical regions, the high incidence of concurrent infection has resulted in the standard procedure of premunizing cattle against both of the diseases.[14,15,26,29] Descriptions of the clinical course and pathological manifestations of concurrent infection have been few. Simultaneous premunization

against both anaplasmosis and babesiosis was reported to produce an intense anemia.[6] The severity of the anemia was attributed to the additive effect of the individual anemias caused by each of the hemoparasites. Other investigators, studying concurrent infection in experimentally infected splenectomized calves reported that only 1 of the hemoparasites established the normal expected parasitemia.[16] The invasion of erythrocytes by the second hemoparasite, though initially substantial, decreased and occasionally ceased prematurely. It was suggested that a non-antibody inhibiting factor may have been produced which allowed the hemoparasite with the highest established parasitemia to prevail while further infection of erythrocytes by the second organism was inhibited. Splenectomized calves infected with B. bigemina during the convalescent stage of anaplasmosis were reported to have abnormally low Babesia parasitemias and a prepatent period which was from 16 to 21 days longer than that normally observed for babesiosis.[27]

The common distribution of anaplasmosis and babesiosis and the high incidence of concurrent disease has indicated that a clearer understanding of the relationship of the 2 disease agents during concurrent infection is desirable. The purpose of this investigation was to describe the clinical course, serological response and pathological manifestations of concurrent infection in intact calves experimentally infected with A. marginale and B. bigemina; and to study the relationship of the 2 organisms during the concurrent infection.

Materials and Methods

Twenty-two, male, non-splenectomized, Holstein-Friesian calves 7 months of age and free of previous or current Anaplasma and Babesia infection were used as experimental animals. The calves were randomly divided into 4 groups. Groups

I and II each contained 3 calves and served as control groups for anaplasmosis and babesiosis, respectively. Groups III and IV each contained 8 calves. The calves of Group III were infected on day 0 with A. marginale. Following recovery from acute anaplasmosis, the calves were infected with B. bigemina and served to study the effect of a secondary concurrent infection with B. bigemina in calves recovering from anaplasmosis. The calves of Group IV were infected on day 0 with B. bigemina. Following recovery from acute babesiosis, the calves were infected with A. marginale and served to study the effect of a secondary concurrent infection with A. marginale in calves recovering from babesiosis.

Stabilates prepared from purified Colombian isolates of A. marginale and B. bigemina were used in the investigation. Each calf was infected by the subcutaneous inoculation of stabilate which was thawed in a water bath at 38 C for 1 minute. The Anaplasma inoculum contained approximately 9.5×10^8 marginal bodies and the Babesia inoculum 10.5×10^8 organisms.

The body weight of each calf was determined on day 0 and at the termination of the investigation and the mean total weight gains and average daily gains for each group of calves was determined. Blood and serum samples were collected and rectal temperatures determined twice weekly from each calf for 2 weeks prior to infection and for 17 weeks following infection.

Packed cell volumes (PCV), reticulocyte counts and total and differential leukocyte counts were determined by standard laboratory procedures.[23] The percentage of parasitized erythrocytes was determined by the microscopic examination of Giemsa stained thin blood smears. Total serum protein (TSP) concentrations were ascertained using a hand held refractometer.[3] Serum protein electrophoretic patterns were determined by a technique using cellulose acetate membranes.[4] Serum glutamic oxalacetic transaminase (SGOT) activity was measured by a kit procedure[a] modified from the original method.[19] Serum

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[a]Sigma Chemical Company, St. Louis, Missouri.

sorbitol dehydrogenase (SD) activity was measured by a modified kit procedure[b] adapted from an original method.[7] Serum bilirubin concentrations were determined by a modification of the Van den Bergh procedure.[8]

Serum samples were screened for the presence of complement fixing (CF) antibodies for A. marginale by the standardized CF test for anaplasmosis.[2] The CF test employed to detect antibodies against B. bigemina was a previously described modification[25] of an original method.[12] All serum samples found to be positive by the screening procedures were titrated by a microprocedure technique.[9] Antigen used in the CF test for anaplasmosis was obtained from the United States Department of Agriculture. Antigen used in the CF test for babesiosis was obtained by a previously reported technique using a distilled water extract of the parasite suspension.[13]

Rib bone marrow specimens were collected by biopsy from 2 calves from each of the 4 groups of calves on days 0, 50, 90 and 120 of the investigation. Myeloid:erythroid (M:E) ratios were determined.

Two concurrently infected calves from each of Groups III and IV and a non-infected control calf were euthanatized by electrocution and submitted to gross and histopathological examinations.

Results

The clinical manifestations of infection were most severe in the concurrently infected calves, less severe in the Anaplasma controls and least severe in the Babesia controls. The clinical signs of infection consisted of increases in rectal temperature, inactivity, dull haircoats and poor body condition. Me total weight gains and average daily gains were moderately lower in the concurrently infected calves than in the Anaplasma controls and

[b]Sigma Chemical Company, St. Louis, Missouri.

markedly lower than in the Babesia controls (Table 1).

Decreases in the mean PCV were more marked in the concurrently infected calves than in the control calves (Fig. 1). Mean low PCVs of 13% and 20% were observed in the calves concurrently infected with B. bigemina and A. marginale, respectively, as compared to mean low values of 27% and 22% in the Babesia and Anaplasma control groups.

Significant increases in the mean number of infected erythrocytes were observed in the concurrently infected calves (Fig. 2). Mean maximum parasitemias of 1.0% and 2.2% were observed in the calves concurrently infected with B. bigemina and A. marginale, respectively, while the mean maximum parasitemias observed in the Babesia and Anaplasma controls were 0.06% and 1.4%, respectively.

No differences occurred in the total number of leukocytes nor in the differential leukocyte counts between the concurrently infected and control calves. A moderate monocytosis occurred in each of the 4 groups of calves.

The changes which occurred in the SGOT, serum SD and serum bilirubin concentrations were not significantly different between the concurrently infected and the control calves.

The M:E ratio steadily decreased in each of the 4 groups of calves and was substantially lower, at the termination of the investigation, in the concurrently infected calves than in the control calves. The number of reticulocytes was observed to increase moderately higher in the concurrently infected than in the control calves.

Total serum protein concentrations increased substantially higher in the concurrently infected and in the Anaplasma control calves than in the Babesia controls. The average serum albumin concentration and the A:G ratio decreased in each of the 4 groups of calves with little difference occurring between the concurrently infected and the control calves.

Serum globulin concentrations increased in each of the 4 groups of calves

and were substantially higher in the concurrently infected and in the Anaplasma controls than in the Babesia controls (Fig. 3).

Significant differences in the mean maximum CF antibody titers between the concurrently infected and the control calves were not observed (Fig. 4). Mean maximum CF titers of 1:160 and 1:140 were observed in the Anaplasma controls and calves concurrently infected with Anaplasma, respectively. The mean maximum CF antibody titers observed in the Babesia controls and the calves concurrently infected with Babesia were 1:150 and 1:170, respectively.

Gross lesions observed in the concurrently infected calves which were euthanatized for dissection included: an excessive amount of clear yellow fluid in the peritoneal and pleural cavities; moderate splenomegaly with prominent malpighian follicles; moderate hepatomegaly and lymph node enlargement; and the occasional serous atrophy of depot fat.

Tissue sections from the concurrently infected calves were grouped together by organ and examined microscopically:

Liver. Slight to moderate centrolobular degeneration of the hepatocytes was observed. A slight to moderate infiltration of lymphocytes and reticuloendothelial cells was observed in the periportal areas. The hepatic sinusoids contained an excessive number of mononuclear inflammatory cells and immature nucleated erythrocytes. Kupffer cell hypertrophy, erythrophagocytosis and phagocytized granules of hemosiderin were occasionally observed.

Kidney. An eosinophilic proteinaceous material was observed in Bowman's capsule and in the lumens of the renal tubules. A slight infiltration of lymphocytes and plasma cells was occasionally observed in the interstitial tissues. Phagocytized hemosiderin granules were occasionally seen in macrophages in the glomerular tufts and in the interstitium.

Spleen. A moderate to marked lymphoid hyperplasia of the malpighian corpuscles was commonly observed. Hyperplasia of reticuloendothelial cells

in the red pulp was observed in some sections. Granules of phagocytized hemosiderin were observed in the sinuses of all sections.

Lymph Nodes. A histological examination was conducted on tissue sections prepared from the suprapharyngeal, prescapular, prefemoral, sternal, bronchial, renal and mesenteric lymph nodes. The microscopic abnormalities observed were similar irregardless of which of the lymph nodes was examined. Moderate hyperplasia of the lymphoid follicles was observed in all sections, with marked hyperplasia being observed in several sections. Hyperplasia of reticuloendothelial cells was occasionally observed. Granules of phagocytized hemosiderin were present in several sections.

Sections of tissues from the lung, cerebrum, cerebellum, adrenal and thyroid glands were also examined. The microscopic abnormalities observed were slight and inconsistent and were not considered to have resulted from infection with *A. marginale* or with *B. bigemina*.

Discussion

The increased severity of the clinical signs and the more pronounced reductions in total weight gains and average daily gains observed in the concurrently infected calves were apparently due to the combined effects of the *Anaplasma* and *Babesia* infections. The mild clinical signs and small reductions in weight gains which occurred in the *Babesia* control group suggested the severity of the clinical disease observed in the concurrently infected calves was predominately due to infection with *A. marginale*.

The severity of the anemic condition which occurred in the concurrently infected calves was considered to have resulted from the combined effects of the *Anaplasma* and *Babesia* infection with the *Anaplasma* organism being predominately responsible.

The significant increase in the number of infected erythrocytes in the concurrently infected calves was an unexpected observation. Several authors reported that exacerbations of anaplasmosis may result from intercurrent infections placing additional stress on the reticuloendothelial system of the host.[11,18] The ability of the reticuloendothelial system to remove infecting organisms may have been moderately reduced in the concurrently infected calves allowing the secondary infecting organism to reach substantially higher levels in the peripheral blood.

The moderate monocytosis observed in each of the 4 groups of calves was considered to have been associated with the removal of damaged erythrocytes, erythrocytic debris and infecting organisms.

The presence of reticulocytes in the peripheral blood and of nucleated erythrocytes in the hepatic sinusoids along with non-significant changes in the total and differential leukocyte counts indicated the decrease observed in the M:E ratio was due to an increase in the number of cells of the erythroid series. The more prolonged and severe anemic condition of the concurrently infected calves probably acted as a more intense stimulus resulting in the M:E ratio being substantially lower in the concurrently infected than in the control calves.

The elevation in TSP observed in each of the 4 groups of calves was attributed predominately to an increase in the concentration of the serum gamma globulin fraction. Increases in TSP and serum globulin concentrations were higher in the *Anaplasma* controls than in the *Babesia* controls suggesting the substantial increase in TSP and serum globulins which occurred in the concurrently infected calves was due to an additive effect of both infections with the *Anaplasma* infection being predominately responsible.

The decrease in A:G ratio which occurred in each of the 4 groups was attributed to the increase in the gamma globulin concentration associated with antibody

production and to the decrease in serum albumin concentration.

The concurrently infected calves developed CF antibody titers against A. marginale and against B. bigemina which were considered to be independent of the concurrently infecting organism.

The gross lesions observed in the concurrently infected calves were the same as those described as occurring during nonacute anaplasmosis[11,21,22] or during nonacute babesiosis.[11,20] The presence of transudates in the major body cavities was considered to have been associated with a decrease in the osmotic pressure of the blood due to the loss of albumin via the kidneys. The decrease in serum albumin concentration and the presence of proteinaceous material in Bowman's capsule and in the renal tubules indicated the loss of albumin via the renal pathway. Splenomegaly, lymph node enlargement and the moderate to marked lymphoid hyperplasia observed in the malpighian corpuscles of the spleen and lymphoid follicles of the lymph nodes were considered to be indicative of a responding reticuloendothelial system.

The moderate degenerative changes observed in the hepatocytes in the centrolobular areas of the liver were attributed to hypoxia associated with the anemic condition of the calves. The degeneration of hepatocytes during anaplasmosis was previously reported to be the result of a hypoxic condition resulting from anemia.[1,10,22]

The presence of hemosiderin granules within macrophages in the spleen, glomerular tufts, lymph nodes and in the Kupffer cells of the liver was attributed to the excessive destruction of erythrocytes and the subsequent accumulation of erythrocytic debris.

Conclusions

The clinical and pathological manifestations of disease were considered to have been more severe in the concurrently infected calves than those observed in

the singularly infected controls. Infection with A. marginale apparently contributed more to the severity of the concurrent infection than did infection with B. bigemina. Neither an inhibitory nor a synergistic effect between the infecting organisms was evident during the investigation and the relationship of the organisms during the concurrent infection was one of independency. The increased severity of the concurrent infection was attributed to the concurrent infections being additive.

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Chemotherapy for the Elimination of *Anaplasma marginale* Infection

K. L. Kuttler

CHEMOTHERAPY FOR THE ELIMINATION
OF ANAPLASMA MARGINALE INFECTION*

During the past few years, a total of 57 cattle infected with anaplasmosis have been used to test various chemotherapeutic compounds and combinations of these compounds for efficacy and safety. Some animals were reinfected after A. marginale had been eliminated and used a second time after at least a 4-month interval. When treatment failed to eliminate infection, a second different treatment was sometimes administered during the relapse. A total of 87 different treatments were administered, 13 being given to adult non-splenectomized cows, 5 to non-splenectomized calves, and the remaining 69 to splenectomized calves. Anaplasma infection in splenectomized calves produces an effect similar to that seen in adult cattle. For this reason, and in view of the relative lower cost of splenectomized calves, they have been used in most instances. The results of these trials are listed in summary form. In general, Imidocarb (4A65) used alone is effective when given at the level of 3, 4, 5, and 6 mg/kg 3 times at 1- or 2-day intervals, or 2 times at 14-day intervals (5 mg/kg). It was about 64% effective in these instances. When the total Imidocarb injected was 15 mg/kg or over fatal toxicosis was encountered in about 25% of the animals so treated. Imidocarb used in conjunction with Gloxazone (356C61) was effective at levels below 15 mg/kg. The injection of 3 mg/kg 356C61 and 3 mg/kg 4A65 3 times at 1- or 2-day intervals was effective in eliminating infection in 4 of 4 trials. Three injections of 5 mg/kg 356C61 and 2 mg/kg 4A65 was also effective. The reverse of this schedule, 2 mg/kg 356C61 and 5 mg/kg 4A65 given 3 times at 1- or 2-day intervals, was less effective with elimination of infection in only 1 of 3 calves.

The efficacy of 356C61 and oxytetracycline has been previously reported, and this combination continues to work when at least 5-10 mg/kg 356C61 and

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*Data prepared for publication

11-22 mg/kg oxytetracycline are given 3 times at 1- to 2-day intervals. Efficacy drops rapidly when variations from these schedules are made. Some promise was noted for the 14-day interval when 5 mg/kg 356C61 and 11 mg/kg oxytetracycline were given only twice.

Oral aureomycin given splenectomized calves was relatively ineffective. A combination of 356C61, 4A65, and oxytetracycline when given 1 time failed to eliminate infection even though the total amounts were equal to those previously effective when given in divided doses.

Adult cattle responded very much like the calves. Toxicity was encountered when the total drug level was 10 mg/kg or over. The combination 356C61 and oxytetracycline was more effective than 4A65 given alone. No treatments involving the simultaneous treatment with 4A65 and 356C61 were tested.

Anaplasma infection was determined on the basis of a demonstrable parasitemia, a positive CF titer, and a history of an acute hemolytic response to infection. Success or failure of treatment was determined by a disappearance of A. marginale parasitemia, CF titer, and a return to normal of PCV. The presence of carrier infections was further determined by the sub-inoculation of 200 ml blood from the treated calf into a susceptible splenectomized calf. In some instances following this procedure the treated calf was reinfected to establish susceptibility. Sub-inoculations were made 90 days or longer after treatment, and reinfection 120 days or longer after treatment.

TABLE 1

TREATMENT TABULATIONS

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)		(Days)	
SC 68	1:320	7.0	31	356C61	5	2	14	22	>60	Successful
				oxytet	11	2	14			
SC 132	1:5	2.0	24	356C61	2	2	14	20	30	Failed
				4A65	2	2	14			
IC 327	1:80	0	24	4A65	5	2	14	28	>90	Successful (splenectomized and checked for infectivity)
SC 368	1:5	0.4	24	356C61	5	3	1	29	>90	Successful (checked for infectivity and rechallenged)
				oxytet	11	3	1			
IC 370	1:5	0	22	356C61	2	2	14	24	>90	Failed (checked for infectivity)
				4A65	2	2	14			
SC 503	1:5	1.0	22	4A65	1	2	7	22	30	Failed
SC 503	1:5	0.1	22	356C61	2	2	14	27	28	Failed
				4A65	2	2	14			
SC 507	1:20	3.0	22	356C61	2	2	14	21	23	Failed
				4A65	2	2	14			
AC 539	1:5	0	25	4A65	4.4	1	-	23	45	Failed
AC 542	1:320	0	23	4A65	4.4	2	16	19	49	Failed

SC - splenectomized calf
IC - intact calf
AC - adult cow

TABLE 1 - CONT'D.

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME (Days)	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)			
AC 543	1:80	5.0	19	4A65	4.2	1	-	24	41	Failed
AC 547	1:320	0.5	26	4A65	4.2	1	-	29	42	Failed
AC 549	1:160	0.8	19	4A65	4.2	1	-	19	28	Failed
SC 554	1:320	13.0	14	356C61-DMSO	5	1	-	27	43	Failed
				oxytet	11	1	-			
SC 245	1:10	4.0	22	356C61 (I/M)	3	3	2	32	>90	Successful (checked for infectivity)
				4A65 (I/M)	3	3	2			
SC 328	1:640	0.8	22	356C61	2	3	2	28	>90	Successful (checked for infectivity)
				4A65	2	3	2			
SC 340	1:5	0.2	14	356C61	5	3	1	27	48	Failed
				oxytet	11	3	1			
SC 373	1:40	0.8	24	356C61	5	3	1	32	43	Failed
				oxytet	11	3	1			
AC 417	1:40	0.5	17	356C61	5	3	2	26	56	Failed
				oxytet	11	3	2			
AC 417	1:5	0	24	4A65	5	3	7	18	149	Failed
SC 478-544	1:40	4.0	23	356C61	3	3	2	31	>90	Successful (checked for infectivity)
				4A65	3	3	2			

TABLE 1 - CONT'D.

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)		(Days)	
IC 129	1:160	0	23	4A65 (I/M)	5	1	-	27	38	Failed
SC 135	1:160	1.0	12	356C61 oxytet	5 22	3 3	2 2	26	>90	Successful (checked for infectivity)
SC 135	1:320	1.0	12	356C61 4A65	15 6	1 1	- -	18	54	Failed
SC 201	1:80	66.0	10	356C61 oxytet	5 11	3 3	1 1	27	>90	Successful (checked for infectivity)
SC 201-478	1:40	0.1	22	4A65	4	3	1	27	>90	Successful (checked for infectivity)
IC 217	<1:5	0.1	21	356C61 oxytet	5 11	3 3	2 2	25	>90	Successful (checked for infectivity)
SC 217	1:5	2.0	16	356C61 4A65 oxytet	20 2 44	1 1 1	- - -	27	50	Failed
SC 217	1:40	0	22	356C61 4A65	5 2	3 3	1 1	22	>90	Successful (checked for infectivity)
SC 219	1:20	0.2	12	4A65	2	3	2	18	34	Failed
SC 219	1:5	5.0	14	356C61 oxytet	5 11	3 3	1 1	28	>90	Successful (checked for infectivity)
SC 219	1:40	<0.1	16	356C61 4A65	15 6	1 1	- -	22	42	Failed

TABLE 1 - CONT'D.

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)		(Days)	
SC 245	1:40	3.0	15	356C61 oxytet	10 11	2 2	2 2	16	38	Failed
SC 245	1:20	7.0	16	356C61 oxytet	5 11	3 3	2 2	28	49	Failed
SC 245	1:20	0.1	23	4A65	4	3	1	25	>90	Successful (checked for infectivity)
SC 248	1:10	2.0	21	356C61 oxytet	10 11	2 2	2 2	14	41	Failed
SC 248	1:10	0.1	19	356C61 oxytet	10 11	3 3	1 1	28	>90	Successful (checked for infectivity)
SC 248	1:20	2.0	18	356C61 4A65	5 2	3 3	1 1	22	>90	Successful (checked for infectivity)
SC 265	1:80	0.2	14	356C61 oxytet	2 11	3 3	2 2	27	45	Failed
SC 265	1:40	10.0	15	4A65	6	3	1	28	>90	Successful (checked for infectivity)
SC 272	1:5	0.2	19	4A65	2	3	1	22	67	Failed
SC 275	1:40	0.4	20	356C61 oxytet	5 11	3 3	3 3	28	>90	Successful (checked for infectivity)
SC 277	1:80	0.5	16	356C61 oxytet	10 11	3 3	2 2	28	>90	Successful (checked for infectivity)
SC 279	1:10	4.0	13	4A65	4	3	1	28	46	Failed

TABLE 1 - CONT'D.

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)		(Days)	
SC 279	1:160	0.1	16	Aureo-mycin	1.1	60	1	30	>90	Successful (checked for infectivity)
SC 280	1:160	0	24	356C61 oxytet	5 11	3 3	3 3	26	>90	Successful (checked for infectivity)
SC 280	1:5	1.0	16	4A65	5	3	30	17	36	Failed
IC 401	1:5	0	25	4A65	5	3	1	27	>90	Successful (checked for infectivity)
SC 403	1:20	0	28	4A65	5	3	1			Died - drug toxicosis 6 days after treatment
SC 404	1:40	0	20	4A65	5	2	14	20	30	Failed
SC 404	1:20	4.0	21	356C61 4A65	2 5	3 3	2 2	26	76	Failed
SC 405	1:80	0.3	15	356C61 4A65 oxytet	15 4 44	1 1 1	- - -	28	42	Failed
SC 405	1:80	1.0	25	4A65	5	3	2	27	>90	Successful
SC 408	1:160	5.0	7.0	4A65	5	3	2			Died - acute anaplasmosis 4 days after treatment
AC 409	1:80	0.5	17	356C61 oxytet	5 11	3 3	2 2	33	>90	Successful - not confirmed susceptible 10 mos. later
AC 409	1:80	0.3	22	4A65	5	3	2			Died - drug toxicosis

TABLE 1 - CONT'D.

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)		(Days)	
SC 413	1:640	0	12	4A65	5	3	2	28	>90	Successful (checked for infectivity)
AC 414	1:320	10.0	16	356C61	2	1	-	25	50	Failed
AC 414	1:5	0	28	4A65	6	3	2			Died - drug toxicosis 1 day after last treatment
AC 419S	1:320	0.3	18	356C61 oxytet	5 11	3 3	2 2	26	>90	Successful (checked for infectivity)
AC 419S	1:10	0	23	4A65	5	2	14			Died - drug toxicosis 4 weeks after treatment
SC 424	1:320	0	21	356C61 4A65 oxytet	15 4 44	1 1 1	- - -	30	42	Failed
SC 424	1:80	15.0	17	4A65	15	1	-	24	36	Failed
SC 424	1:40	2.0	13	4A65	4	3	1	30	39	Failed
SC 424	1:40	3.0	13	Aureo-mycin (oral)	11	60	1	30	7	Failed
AC 416-425	1:20	0	25	356C61 oxytet	5 11	3 3	2 2	15	49	Failed
AC 416-425	1:10	2.0	19	4A65	6	3	2			Died - drug toxicosis 17 days after treatment
SC 429	1:640	4.0	19	4A65	5	2	14	30	55	Failed

TABLE 1 - CONT'D.

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)		(Days)	
SC 429	1:80	2.0	16	356C61 4A65	3 3	3 3	1 1	25	>90	Successful (checked for infectivity)
SC 431	1:160	0.6	9	4A65	15	1	-	24	35	Failed
SC 431	1:160	2.0	19	356C61 4A65	2 5	3 3	1 1			Died - drug toxicosis 17 days after treatment
SC 434	1:80	2.0	16	Aureo-mycin	1.1	102	1	32	13	Failed
SC 436	1:40	10.0	15	4A65	5	3	14	24	>90	Successful (checked for infectivity)
SC 437	1:20	0.3	21	4A65	5	3	2	33	>90	Successful (checked for infectivity)
SC 439	1:20	0.7	19	4A65	3	3	1	29	>90	Successful (checked for infectivity)
SC 450	1:80	50.0	21	356C61 4A65	2 5	3 3	1 1	30	>90	Successful (checked for infectivity)
SC 450	1:20	3.0	18	Aureo-mycin (oral)	2.2	70	1	24	31	Failed
SC 450	1:10	7.0	23	4A65	10	3	7			Died - drug toxicosis 19 days after treatment
SC 455	1:320	1.0	21	4A65	4	2	14	17	34	Failed

TABLE 1 - CONT'D.

CALF	TIME OF TREATMENT			TREATMENT				PCV SIX WEEKS POST TREATMENT	RELAPSE TIME (Days)	RESULT OF TREATMENT
	CF	PARAS.	PCV	DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)			
SC 455	1:160	0.2	18	356C61 4A65	3 3	3 3	1 1	27	>90	Successful (checked for infectivity)
SC 457	1:320	0	18	4A65	5	3	1			Died - drug toxicosis 6 days after treatment
SC 458	1:640	16.0	21	4A65	5	3	1	31	53	Failed
SC 458	1:640	2.0	20	4A65	5	3	14	18	19	Failed
SC 458	1:640	7.0	20	356C61 4A65	3 3	3 3	1 1	21	>90	Successful (checked for infectivity)
SC 459	1:40	4.0	14	4A65	5	9	28			Reoccurring parasitemias Died - drug toxicosis 236 days after treatment
SC 463	1:5	0.3	24	356C61 4A65	2 5	2 2	28 28	17	29	Failed
SC 490	1:5	0.1	22	356C61 4A65	2 2	3 3	2 2	22	>90	Successful

TABLE 2

TREATMENT
SUMMARY RESULTS

DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)	NO. OF ANIMALS TREATED	FAILED	INFECTIONS ELIMINATED	FATAL TOXICOSIS	TOTAL DRUG INJECTED PER kg
4A65	1	2	7	1	1	0	0	2
4A65	2	3	1	1	1	0	0	6
4A65	2	3	2	1	1	0	0	6
4A65	3	3	1	1	0	1	0	9
4A65	4	2	14	1	1	0	0	8
4A65	4	3	1	4	2	2	0	12
4A65	5	1	-	1	1	0	0	5
4A65	5	2	14	3	2	1	0	10
4A65	5	3	1	5	2	1	2	15
4A65	5	3	2	4	1	3	0	15
4A65	5	3	14	1	0	1	0	15
4A65	5	3	30	1	1	0	0	15
4A65	5	9	28	1	0	0	1	45
4A65	6	3	1	1	0	1	0	18
4A65	6	3	2	1	0	0	1	18
4A65	10	3	7	1	0	0	1	30
4A65	15	1	-	2	2	0	0	15
356C61	2	3	2	2	0	2	0	6
4A65	2	3	2					6
356C61	2	2	14	4	4	0	0	4
4A65	2	2	14					4
356C61	2	2	28	1	1	0	0	4
4A65	5	2	28					10

DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)	NO. OF ANIMALS TREATED	FAILED	INFECTIONS ELIMINATED	FATAL TOXICOSIS	TOTAL DRUG INJECTED PER kg
356C61	2	3	1	2	0	1	1	6
4A65	5	3	1					15
356C61	2	3	2	1	1	0	0	6
4A65	5	3	2					15
356C61	3	3	1	3	0	3	0	9
4A65	3	3	1					9
356C61	3	3	2	2	0	2	0	9
4A65	3	3	2					9
356C61	5	3	1	2	0	2	0	15
4A65	2	3	1					6
356C61	15	1	-	2	2	0	0	15
4A65	6	1	-					6
356C61	2	3	2	1	1	0	0	6
oxytet.	11	3	2					33
356C61	5	1	-	1	1	0	0	5
oxytet.	11	1	-					11
356C61	5	2	14	1	0	1	0	10
oxytet.	11	2	14					22
356C61	5	3	1	5	2	3	0	15
oxytet.	11	3	1					33
356C61	5	3	2	3	2	1	0	15
oxytet.	11	3	2					33
356C61	5	3	3	2	0	2	0	15
oxytet.	11	3	3					33
356C61	5	3	2	1	0	1	0	15
oxytet.	22	3	2					66
356C61	10	2	2	2	2	0	0	20
oxytet.	11	2	2					22
356C61	10	3	1	1	0	1	0	30
oxytet.	11	3	1					33

DRUG	DOSE mg/kg	NO. OF TREATMENTS	TREATMENT INTERVAL (Days)	NO. OF ANIMALS TREATED	FAILED	INFECTIONS ELIMINATED	FATAL TOXICOSIS	TOTAL DRUG INJECTED PER kg
356C61	10	3	2	1	0	1	0	20
oxytet.	11	3	2					22
oral Aureomycin	11	60	1	1	1	0	0	660
oral Aureomycin	1.1	102	1	1	1	0	0	112.2
oral Aureomycin	1.1	60	1	1	0	1	0	66
oral Aureomycin	2.2	70	1	1	1	0	0	154
356C61	15	1	-	2	2	0	0	15
4A65	4	1	-					4
oxytet.	44	1	-					44
356C61	20	1	-	1	1	0	0	20
4A65	2	1	-					2
oxytet.	44	1	-					44
			ADULT CATTLE					
4A65	4.2	1	-	3	3	0	0	4.2
4A65	4.4	1	-	1	1	0	0	4.4
4A65	4.4	2	16	1	1	0	0	8.8
4A65	5	2	14	1	1	0	1	10.0
4A65	5	3	2	1	0	0	1	15.0
4A65	5	3	7	1	1	0	0	15.0
4A65	6	3	2	1	0	0	1	18.0
356C61	2	1	-	1	1	0	0	2.0
356C61	5	3	2	3	1	2	0	15.0
oxytet.	11	3	2					33.0

Current Status of the Tropical Cattle Fever Tick *Boophilus microplus* and Texas Wildlife

K. L. Kuttler

CURRENT STATUS OF THE TROPICAL CATTLE FEVER TICK *BOOPHILUS MICROPLUS* AND TEXAS WILDLIFE

K. L. Kuttler

The tropical cattle fever tick, *Boophilus microplus*, is one of 2 *Boophilus* ticks found in the western hemisphere and is widespread throughout Central and South America, including the Carribean islands. *Boophilus microplus* is also found in Australia, Asia, and Africa, but is generally limited to the tropics.

The second tick of this genus is *Boophilus annulatus*, or the Texas fever tick, which also occurs in the tropics, but is more common in subtropical climates. This tick was found in Texas and the southeastern U.S. in the early 1900's and was responsible for Texas Fever or piroplasmosis.

The *Boophilus* ticks complete their parasitic stage on 1 animal in about 21-24 days under optimum conditions. These ticks are commonly found on cattle, deer, horses, sheep, and goats. They may be found less commonly on dogs, pigs, rabbits, etc. The *B. annulatus* tick is somewhat more resistant to climatic factors and in 1890 was found as far north as Oklahoma, Arkansas, Tennessee, and the southern part of Virginia. *Boophilus microplus*, on the other hand, was limited to the southern tip of Texas and southern Florida. In Texas, *annulatus* has been the principle problem and is the tick most studied.

There have been periodic outbreaks along the border with Mexico of *B. annulatus* which in the past have been eradicated by conventional means of dipping all cattle in the quarantined zone every 2 weeks for a 5 to 9 month period, or until such time as the ticks are eradicated.

In 1968 an outbreak in Dimmit county was observed in which deer were infected. This outbreak was presumably controlled by cattle dipping. *Boophilus annulatus* ticks recovered from deer in this outbreak were taken to the USDA laboratory in Nuevo Laredo, and there placed on deer where they have undergone 13 generations.

These ticks develop to about 2/3 the normal size on deer, but are capable of undergoing a complete life cycle. When placed on calves, these ticks are capable of regaining their full vitality.

The primary concern associated with B. annulatus ticks is related to their ability to transmit disease. They act as a biological host for piroplasmosis and anaplasmosis. It was this disease aspect that led to the tick eradication programs of the 1920's and 30's, which in turn eliminated piroplasmosis from the U.S. Unfortunately there were other vectors for anaplasmosis, but the Boophilus ticks were the only vectors capable of transmitting piroplasmosis.

Last year a large area around Alice, Texas was quarantined because cattle were found infected with B. microplus. As might be expected, deer have also been found infested in this area. Dr. Bill Kiel (King Ranch biologist) has observed infested deer on a number of occasions. Boophilus microplus has not previously been reported to have survived this far north in Texas. There have probably been instances when microplus ticks have been carried even farther north, but their presence was temporary. It might be that a similar situation will exist with regards to the current microplus outbreak. Dr. Manning Price, entomologist at Texas A&M, believes, however, that based on Australian data it is possible for B. microplus to survive where it is and even extend north and eastward around the gulf coast. We know it was well established in Florida at one time. The exact geographic limitations of B. microplus are not currently known.

The question might be asked how can the tick be eradicated in cattle when it is present on deer. A direct answer to this question is not possible at this time, but past experience has shown that eradication can be accomplished by cattle dipping. As previously mentioned, B. annulatus was eradicated in Dimmit county where deer were known to be infected. It was known that during

the eradication campaign in the 20's and 30's that deer were infested but ticks were nevertheless eradicated. In Texas, the tick was principally B. annulatus. In Florida, both annulatus and microplus were present. The microplus was much more difficult to eradicate, and this was not accomplished until relatively recent times. There are some authorities who even now question the eradication of B. microplus ticks in Florida.

Boophilus microplus ticks while more susceptible to adverse climatic factors are more versatile than annulatus with respect to the variety of animals it can attach to and successfully feed on. It has been shown by Park and others to feed, survive, and undergo a normal life cycle on deer. It is well adapted to this host. It has also been found on a number of other animals including man, but normally does not occur in any great numbers on these animals.

Kistner and Hayes working with microplus on the Virgin Islands observed this tick and arrived at several useful conclusions.

1.) B. microplus can maintain itself on white-tailed deer in the absence of cattle. Two deer were found infested with B. microplus where contact with domestic livestock had not occurred for 20 years.

2.) B. microplus was not, however, found on 5 deer trapped in an area where they were mixing with cattle and where the cattle were being dipped regularly.

This second finding had been previously reported by Travis in the 40's, in Florida, who reported finding microplus ticks on deer trapped in swamps where cattle were not present. No microplus ticks were seen on deer examined in forest areas where they co-mingled with cattle being dipped regularly.

Past evidence suggests that Kistner's conclusions are correct, and that ticks can be eradicated from an area cohabited by both deer and cattle by

regular and prolonged cattle dipping.

Regular dipping of all cattle in the present quarantine area is currently underway. The size of the area involved, the terrain, the ratio of cattle to deer, and the known ability of the tick to survive on deer may alter previously held concepts on tick eradication. It is probable in light of previous experience that a longer quarantine and dipping period will be required to eradicate the microplus tick when compared to annulatus, unless we get some help from climatic conditions. It is even possible that the current procedures will not work, but they did work before and the most reasonable approach at this time appears to be vigorous application of tried and tested techniques for the elimination of this ecto-parasite.

Recent work has been done relative to the infection of deer with babesiosis or piroplasmosis.

Known infected B. microplus ticks were placed simultaneously on susceptible calves and deer. The calves in every instance developed piroplasmosis. The deer failed to develop evidence of illness. Thinking that the deer may have developed a chronic or non-apparent infection, their blood was inoculated into calves at weekly intervals for 4 to 6 weeks in an attempt to pick up the infection. All of the trials were negative in that infection failed to occur. We then took the ticks which developed normally on deer, allowed them to oviposit, and then placed the resulting larvae on splenectomized calves which failed at this point to develop piroplasmosis. This led us to the conclusion that the ticks, while infected when placed on deer, eliminated the infection by undergoing one life cycle on deer. It is probable that the same thing would occur when ticks were placed on other non-bovine animals. Babesia bigemina and B. argentina occur naturally only in cattle. This is probably the reason that recent tick outbreaks in Texas have not been accompanied by piroplasmosis. Should these non-infected ticks have occasion to engorge on

an infected cow, the resulting larvae would be infected and could then theoretically transmit Texas fever. It is estimated that approximately 700,000 cattle are imported from Mexico each year. Not all of these cattle are Babesia carriers but a fairly large percentage probably are. The opportunity exists in the U.S. for a Babesia outbreak when the ticks and infected cattle come in contact.

East Coast Fever
(Theileriasis, Theileriosis, Rhodesian Tick Fever, Rhodesian Red Water)

K. L. Kuttler

East Coast Fever
(Theileriasis, Theileriosis, Rhodesian Tick Fever, Rhodesian Red Water)

East Coast Fever (ECF) is a highly pathogenic, tick transmitted, febrile infection occurring in cattle, caused by the protozoal parasite Theileria parva, which primarily involves the hemopoietic and lymphopoietic systems. This disease is confined principally to areas of East Africa where the principle vector, Rhipicephalus appendiculatus, occurs.

Etiology: The causative organism, Theileria parva, can be identified in the bovine erythrocyte or as schizonts in the lymphocytes. The erythrocytic forms, often referred to as piroplasms or merizoites, are very pleomorphic ranging in shape from rod to rounded forms. Neitz (1957) described the most commonly occurring rod forms (80%) as being 1.5 - 2.0 u X .5 u, the round forms (12%) 1.0 - 1.2 u in diameter, the oval forms (6%) 1.0 - 1.7 u X 0.6 u, and the Anaplasma like forms (2%) as being 0.5 - 0.7 u in diameter. It is doubtful if positive differentiation of blood forms can be made between T. parva and T. mutans, or T. annulata, however the latter two tend to become more rounded and slightly larger. The blood forms of T. cervi, occurring in white-tailed deer of North America, also closely resemble T. parva. In size the rounded or larger Theileria parasites may resemble the small Babesias, and positive identification based solely on morphology is not always certain.

The lymphocytic or schizogonous forms, sometimes referred to as Koch blue bodies, are present in the early phases of infection, and are diagnostic of Theileria infections when seen. Following attachment and feeding of infected ticks on a susceptible host the infectious organism migrates to lymphatic tissue where it undergoes schizogonous development. Initially macro-schizonts (agamonts) develop which eventually rupture discharging numerous individual macro-schizonts or macro-merizoites. These organisms are thought to re-invade

lymphocytic tissue either forming another macro-schizont or a micro-schizont (gamonts). The micro-schizonts have smaller and more numerous granules which after maturing and freed from the schizont are referred to as micro-merizoites or merizoites. It is thought that these merizoites invade the erythrocytes and become the blood forms observed in these cells. The macro-schizont chromatin granules range in size from 0.4 to 2.0 u averaging 1.2 u, whereas the micro-schizont granules range in size from 0.3 to 0.8 with an average of 0.5 u.

The tick vector becomes infected by the ingestion of blood forms which following a cycle in the tick are returned to the vertebrate host as sporozoites by the tick thus completing the cycle.

As early as 1957 and 1958 the parasites were observed to survive for short periods of time in tissue fragments. Hulliger (1964 and 1965) maintained an apparent schizogonous form of the parasites for several months with repeated subculturing in bovine lymphocytes. Even though an infection could be induced in cattle with this material, the surviving animals, while resistant to the organism present in tissue culture, were susceptible to T. parva via a tick challenge. Malmquist et al in 1970 reported the establishment of 3 spleen cell lines derived from 3 calves experimentally infected with T. parva in which the macro-schizogonous phase of the organism has been maintained for up to 10 months. Growth apparently occurs in lymphoblasts which can readily be subcultured. The infectious particles present in these cultures are capable of inducing infections in cattle indistinguishable from ECF.

History: East Coast Fever was probably first recorded in East Africa during the 16th century by missionaries. It was not until 1898 however, that Koch observed the etiologic agent which he thought to be immature or young forms of Babesia bigemina. In 1903, while investigating death losses in cattle thought to be due to red water, Theiler observed intraerythrocytic forms which were entirely unlike the large, pear shaped, Babesia bigemina.

Theiler concluded that this condition was an entirely new disease entity based on morphology as well as his observation that red water immune cattle were susceptible to this new organism. Theiler (1904) in his description of the parasite called it Piroplasma parvum. When Bettencourt, Franca, and Borges (1907) created the new genus Theileria, which included this organism it was named Theileria parva. Prior to 1900 the disease had been enzootic along the east coast of Africa, having been well known for many generations. Because of the relative isolation from cattle in the interior, infection was generally confined to these coastal areas. By 1910 however, the movement and importation of cattle through and from coastal regions spread this infection throughout much of South Africa, Rhodesia and the inland areas of Tanzania, Kenya and Uganda. It was estimated that between 1910 and 1914 900,000 head of cattle died in South Africa alone (Neitz 1964).

Signs: The first indication of infection occurs as an increase in temperature (41.1° to 42.2°C). Koch bodies may occur in the regional lymph nodes several days before fever and erythrocytic forms 2 to 17 days after the rise of temperature. Later there is obvious swelling of superficial lymph glands, inappetence, rumen atony, drop in milk production, excessive salivation and lacrimation, diarrhea, emaciation, serous nasal discharge, acceleration of respiration, and pronounced dyspnea shortly before death. The course of the disease is usually 8 to 25 days with an average of 15 days. Jaundice and anemia are not outstanding features of T. parva infections even though drops in packed cell volumes do occur especially in protracted cases. Generally the morbidity among susceptible cattle will approach 100% with a 95% mortality. Among indigenous cattle and young calves raised in enzootic areas the mortality may be less, with as many as 75% of adult cattle and even a higher percentage of calves recovering.

Incubation Period: According to Neitz (1957) the incubation period is 8-25 days with an average of 13 days following infestation by infected ticks.

Pathologic Changes:

Postmortem Lesions: The carcass is usually emaciated with froth coming from the nostrils. The subcutaneous and intramuscular areas may be yellow and infiltrated with clear serous fluid to give them a gelatinous appearance. The pathologic lesions are dominated by a proliferation and enlargement of the lymphoid tissue, including Peyer's patches. There appear to be liver and kidney infarcts which are really perivascular proliferative foci of lymphocytes. The lungs are generally saturated with clear edematous fluid. There may be marked petechial hemorrhages on serous and mucous surfaces throughout the abdominal and thoracic cavities. Neitz (1948) has reported intramuscular hemorrhages. In cases of central nervous system involvement (Turning-disease Mettam, 1934 and Mettam and Carmichael, 1936) there may be perivascular lymphocytic infiltrations, and lymphocytic embolisms of the cerebral vessels.

Microscopic Pathology: An initial intense lymphocytic hyperplasia is noted, particularly in the local nodes. The hyperplasia is of a lymphoreticular type with a rapid multiplication of reticular cells, and large and medium lymphocytes. De Koch (1957) and Barnett (1960) observed that the early lymphocytic hyperplasia was replaced by regressive changes in the lymphoid tissue beginning about the fourth to sixth day involving depletion of the lymphocytes, disruption and destruction of the lymphocytopoietic centers, and a progressive toxicosis of cells associated with cellular destruction.

Diagnosis:

A. In the Field: A febrile highly fatal disease associated with enlarged lymph nodes and pulmonary edema in an area of *R. appendiculatus* infestation is suggestive of ECF. A history of failure to spray or dip at the usual intervals, and the presence of *R.*

appendiculatus on the animals would further suggest a diagnosis of ECF.

B. Laboratory: A Giemsa-stained smear from a lymph node biopsy, taken preferably from the parotid or pre-scapular nodes, should be examined for Koch blue bodies (macro or micro-schizonts). The presence of these bodies is diagnostic of theileriosis, and depending on the history and symptomatology diagnostic of ECF. The presence of blood forms on Giemsa-stained blood smears are also diagnostic of theileriosis. These simple criteria are generally adequate to differentiate the disease from viral or bacterial infections.

In recent years greater attention has been displayed in the development of specific serologic tests for the diagnosis of ECF. A specific agar gel precipitin test was demonstrated by Gourlay and Brocklesby in 1967, and in 1968 Schindler and Mehlitz reported using a Coons test and the complement-fixation tests. These workers have used these procedures more as tools in studying antigenic similarities between theilerial infections rather than attempting to adapt them as routine diagnostic procedures. Burridge has described an indirect fluorescent antibody test to detect specific Theileria antibodies which has been useful in measuring an immune response to vaccinated cattle.

C. Differential: In addition to T. parva Koch bodies may be found in animals infected with T. mutans, T. annulatus, and T. lawrencei. These bodies are rare however in T. mutans which generally produces only a mild infection with rapid recovery followed by premunition, or a carrier infection. T. mutans is readily transmitted by blood inoculation which is not true of T. parva. Koch bodies are common

in T. annulata infections which generally are milder than infections due to T. parva. Theileria annulata is not known to occur in Africa south of the Sahara, and is generally confined to North Africa, Southern Asia and parts of Southern Europe. Theileria lawrencei is thought to be a primary pathogen for the African buffalo, and on occasion produces a relatively severe infection in cattle (Corridor Disease, Neitz, 1955). Transmission of T. lawrencei from cattle to cattle is however reported by Matson (1967).

Barnett and Brocklesby (1966) reported the serial passage in cattle of T. lawrencei (Kenya) isolated from buffalo in Kenya. After 7 cattle passages of T. lawrencei (Kenya) it was indistinguishable from T. parva based on morphology, pathogenicity and cross immunity trials. These authors conclude T. lawrencei had become indistinguishable from T. parva hence the validity of T. lawrencei is highly questionable.

Serologic and immunologic comparisons of T. parva, T. lawrencei and T. mutans by Schindler, Mehlitz, and Matson (1969) and by Schindler and Mehlitz (1968) have shown antigenic similarity in these organisms. An antigen common to all three Theileria was demonstrated with the suggestion that lawrencei was intermediate between parva and mutans.

Prognosis: East Coast Fever in susceptible stock is frequently severe with mortality approaching 95%. The prognosis in such animals is very poor. The prognosis may be more favorable in indigenous Zebu type cattle, where mortality may be much lower.

Epizootiology:

A. Geographic Distribution: East Coast Fever is epizootic in East Africa. The disease was introduced into Rhodesia by a shipment

of cattle from Tanganyika in the early part of this century. From Rhodesia, it spread southward to Mozambique (formerly Portuguese East Africa), the Transvaal, Sawziland, Natal, and the Eastern Cape Province of South Africa, westward to the Belgian Congo and north to Uganda, Kenya, and the Sudan. The disease has also appeared in Zanzibar, probably exists in Ethiopia and Southern Somalia, and has been suspected but not confirmed in some areas of West Africa.

The Republic of South Africa and Mozambique have reportedly eradicated the disease through intensive tick control, quarantine, and slaughter of all cattle associated with isolated outbreaks.

B. Transmission: Stage to stage transmission of *T. parva* is predominantly accomplished by the 3 host tick *Rhipicephalus appendiculatus*. Transovarial transmission does not occur. Several other species of ticks have been demonstrated as capable of transmitting the infection and these include *Rhipicephalus ayrei*, *R. capensis*, *R. evertsi*, *R. jeanelli*, *R. neavei*, *R. simus*, *Hyalomma anatolicum*, *H. dromedarii*, and *H. truncatum*. Transmission of the parasites by these vectors is on a stage to stage basis.

Martin *et al* in 1965 reported that *R. appendiculatus* became non-infective within 34-40 weeks after moulting, even though capable of feeding for as long as 15 months. Ticks infected with *T. parva* could transmit the parasite to cattle 24 hours after being placed on the host.

Hosts: East Coast Fever is primarily a disease of cattle, however, both the African buffalo and the Indian water buffalo are susceptible. The Indian water buffalo appears to be equally as susceptible as cattle. The African buffalo, while a potential carrier, is relatively resistant to *T. parva*, however it may show moderate to severe response to *T. lawrencei*.

If however, lawrencei is a strain of T. parva, as suggested by Barnett and Brocklesby (1966), then the role of the buffalo in the epidemiology of ECF must be reconsidered. Most wild antelopes in East Africa carry intra-erythrocytic piroplasms that are morphologically indistinguishable from those of T. parva. It is not known if these parasites are pathogenic to the host in which they are found, nor is it known if these piroplasms are transmissible to cattle. One such Theileria has been transmitted from eland to cattle.

Control and Eradication:

A. Preventive Measures: Regular dipping to control the vector, quarantine, and slaughter of infected stock under some conditions, has been successful in eliminating infection in South Africa, and to a more limited extent in Rhodesia. A combination of quarantine and tick control is probably the most practical approach in containing the disease in enzootic areas. Dipping or spraying twice weekly is essential for tick eradication, but in practice a 7 to 10 day dipping interval is usually adequate. The arsenicals, BHC, and toxaphene are used in dips and sprays for this purpose. Newer compounds with longer residual action might reduce the frequency of dipping.

B. Treatment: No effective therapy exists for ECF. Administration of chlortetracycline at the rate of 12-15 mg/kg when given very early in the course of infection may reduce the severity of infection. Good care and management of calves in enzootic areas has reduced mortality.

C. Sanitation and Disinfection: Sanitation and disinfection, aside from that involved in control and elimination of ticks, do not contribute to an abatement of the disease incidence in enzootic areas.

D. Immunization: There is not at present a practical immunization procedure in routine use. There are now indications that vaccines may be developed in the near future considering the advent of new techniques including growth of the organism on tissue culture, radiation, and new methods of isolating infectious particles from ticks to artificially induce infection. Recovery from ECF may confirm a solid, sterile immunity, however in some instances carrier infection may persist (Neitz, 1964). Mild strains of *T. parva* can be used to induce protective immunity, but the lack of a practical delivery system has limited the use of these organisms. In the past ECF, has not been readily transmitted by the needle inoculation of infected bovine tissues, but infected tissue cultures, and infected tick tissues have been shown to readily induce an immunizing infection (Cunningham, 1970). Barnett (1957) and Wilde (1968) noted that the severity of infection is related to the level of exposure, and cattle recovering from mild infections induced by low level exposures are solidly resistant to *T. parva* tick challenge. Controlled infections might therefore, be of value in preventing ECF.

Under experimental conditions the occurrence of antigenic variants appear easily induced with *Theileria*. The possibility of such variants crossing an immunologic barrier is one which must be kept in mind when and if a vaccination program for ECF reaches the point of common use.

APPENDIX 2

Microbiological Procedures:

Piroplasma and Koch bodies from blood, lymph nodes and spleen.

Histological Examination:

Lymphatic hyperplasia, small *piroplasma* in red cells and Koch bodies in white cells.

Serologic Tests:

Agar gel precipitin test, complement-fixation, Coons test, and indirect fluorescent antibody.

Animal Inoculation:

Tick transmission is the most consistant means of reproducing infection. Animal inoculation with tissues of infected animals is only sporadically successful. The injection of infected tick tissues, or lymphoblast tissue cultures are more consistent in artificially inducing infections in susceptible cattle. Laboratory animals are refractory to infection.

APPENDIX 5

Description:

A highly pathogenic, tick transmitted, febrile disease of cattle affecting primarily the hemopoietic and lymphopoietic systems.

Cause and Distribution:

The protozoan - *Theileria parva*. East Africa.

Host:

All bovine species. African buffalo. Indian water buffalo.

Signs:

Swelling of the lymph nodes draining the area where infected ticks have fed. Sudden febrile response --105-106°F. Lachrymation, nasal discharge, corneal opacity. Diarrhea, lung-edema, emaciation. Course 8 to 25 days.

Pathology:

Lungs are generally edematous also subcutaneous and intramuscular edema, Highly variable areas of petechial hemorrhages, extending through the thoracic and abdominal cavities. Proliferation and enlargement of lymphoid tissue, including enlarged Peyer's patches and raised grey-white areas of proliferative foci of lymphocytes on the liver and kidneys resembling infarcts.

Diagnosis:

Field - A highly febrile and fatal disease associated with regional lymph node enlargement. Differential - Demonstration of Koch's bodies in lymphoid tissue and parasites in blood smears.

Incubation Period:

8 to 25 days with an average of 13 days.

Mode of Transmission:

Stage to stage transmission principally by the tick *Rhipicephalus*

APPENDIX 5 (Continued)

appendiculatus. Six other species of Rhipcephalus and three species of Hyalomma capable of transmission.

Period of Communicability:

R. appendiculatus may harbor infection as long as 34 weeks. Cattle and African buffalo may become carriers of infection for an undetermined period.

Control Measures:

Tick control by frequent dipping (7-10 day intervals), strict quarantine, restriction on all movement of cattle and in some instances slaughter.

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Review: Current Status of Control and Treatment With Drugs

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REVIEW: CURRENT STATUS OF CONTROL AND TREATMENT WITH DRUGS

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Anaplasmosis, an infectious disease of cattle, is recognized in both the acute and chronic form. The acute disease is most easily recognized, having a characteristic clinical syndrome which may lead to death in affected animals. For years treatment of anaplasmosis implied the use of drugs and supportive care of this phase of infection.[35] In recent years, however, greater attention has been given the chronic or carrier phase of infection. The disease in this phase does not usually pose a threat to the animal's well-being and is usually asymptomatic, but it does represent a reservoir of infection capable of transmission to non-infected animals. The elimination of carrier infection has assumed importance in those herds where preventive measures and disease eradication are feasible.[38]

A common pitfall in the evaluation of therapeutic agents used for anaplasmosis is the fairly large number of spontaneous recoveries which might mislead the observer to believe that a specific drug or therapeutic procedure was effective, when in reality recovery would have occurred without treatment. A second problem is that often the animal with anaplasmosis may go unrecognized until the terminal phases of infection, at which time even specific therapy is unsuccessful. Early treatment is recognized as being important to ensure success.[9,10]

This review will consider the treatment of anaplasmosis in the context of the acute infection and the chronic or carrier infection, the first being primarily concerned with the interruption of the clinical syndrome by reversal

of characteristic signs of infection, the second being the elimination of the causative organism, Anaplasma marginale, in the carrier animal. In recent years greater emphasis has been placed on the latter.

Treatment of Acute Anaplasmosis

Prior to the introduction of the tetracyclines and other chemotherapeutic compounds, treatment of acute anaplasmosis was limited largely to supportive therapy including a variety of hematinics, blood transfusions, good nursing, and care.[35] These procedures were aimed largely toward the elimination of stress and alleviation of the anemia until such time as the immune mechanism and hemopoietic system could adequately respond. It would appear that blood transfusions could and should play an important role in the treatment of acute anaplasmosis,[12,19,26] but there are conflicting reports[18] and some suggestion of caution in this procedure.[3,9] There is always a danger in treating weakened animals, particularly range cattle when the excitement and exertion associated with handling and restraining may do more harm than the treatment will do good.[9] Extensive treatment of the semi-wild range animal may be contraindicated; whereas, treatment of a docile dairy animal may be useful and successful.

Until the last few years, the only specific chemotherapeutic compounds have been the tetracyclines, principally oxytetracycline,* chlortetracycline,** and tetracycline hydrochloride.*** These compounds all appear similar in suppressing the reproduction of the Anaplasma organism. Foote[13] described this action in 1951, but noted that chlortetracycline was not effective when

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*Oxytetracycline: Terramycin - Chas. Pfizer & Co., Inc.

**Chlortetracycline: Aureomycin - Lederle Laboratories, American Cyanimide Co.

***Tetracycline Hydrochloride: Polyotic - Lederle Laboratories, American Cyanimide Co.

given late in the course of infection. The desirability of using the tetracyclines early in the course of infection was noted by others.[10,27,28] Carricaburu[9] indicated considerable success in treating naturally occurring field cases of anaplasmosis with 3 mg/lbs oxytetracycline given intramuscularly (I/M). Brock et al.[5] described the use of tetracycline hydrochloride. In their opinion, when used at the rate of 3 mg/lbs I/M it was essentially equal in effect to either oxytetracycline or chlortetracycline.

The chemoprophylactic effect of feeding low levels of the tetracycline drugs has been described.[7,11,34] It was found that as little as 0.5 mg/lbs fed daily would prevent acute anaplasmosis, even though exposure to A. marginale did occur.

Two new compounds, 356C61* and 4A65,** have recently been described as having a specific chemotherapeutic effect on Anaplasma.[2,20,21,22,23,25,30,31,32,33] In premunization experiments, Kuttler and Todorovic[24] showed 356C61 (5 mg/kg) and 4A65 (4 mg/kg) superior to oxytetracycline (12 mg/kg) in moderating the course of infection in adult cattle intentionally exposed to virulent A. marginale. Roby[30] has reported that the development of acute anaplasmosis in splenectomized calves has been inhibited with a single injection of 2.5 mg/kg 4A65. These drugs, 356C61 and 4A65, are not available commercially and, hence, have not been used extensively in cases of acute anaplasmosis but on limited experimental work show promise.

Treatment of Carrier Infections to Eliminate Anaplasma

Soon after the tetracyclines were observed to inhibit growth of Anaplasma,

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*356C61: Gloxazone - Alpha-Ethoxycthylglyoxal Dithiosemicarbazone; Burroughs Wellcome Company.

**4A65: Imidocarb - 3,3'-Bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride (or dipropionate); Burroughs Wellcome Company.

experiments were conducted to evaluate these drugs in relation to the removal of carrier infections.[4,6,14,16,29,31,36] These experiments indicated that such therapy would eliminate carrier infections, but only after the prolonged administration of fairly large amounts of these agents. Nevertheless, this significant breakthrough stimulated a great deal of research on this subject with the evolvement of numerous successful treatment regimes which will effectively eliminate carrier infections. A list of these procedures is given in Tables 1 and 2, which under varying conditions have proven successful.

Carrier status has been eliminated by the I/V or I/M injection of 5 mg/lbs tetracycline 10 times at daily intervals.[29] The injection of oxytetracycline and chlortetracycline is also effective at 12 to 16 daily injections.[36] These drugs are relatively non-toxic; however, they do produce considerable irritation at the site of I/M injections, and if given too rapidly I/V can produce a transient mild to moderate respiratory distress.

The smallest amount of chlortetracycline that has proven effective in eliminating infection was 0.5 mg/lbs orally for a 120-day period.[15] This treatment was administered during the winter season when re-exposure was not occurring. The negative status of treated animals was established by serologic means. A similar experiment, using 0.5 mg/lbs orally for 90 days, conducted during the vector season failed to eliminate the Anaplasma; however, containment and reduced infectivity level was detected.[11,34]

It is probable that oral treatment with low levels while effective may be influenced by factors such as continued exposure, lack of consumption by some animal, or differences in individual animals to the extent that 100% results may not always occur. For these reasons chlortetracycline levels higher than 0.5 mg/lbs are generally used. A treatment consisting of 5 mg/lbs daily for 30 to 60 days has generally been successful.[17,31,37] This treatment regime

gives room for animal variation and has provided more consistent removal of carrier infections.

The prolonged periods of treatment are frequently in conflict with various management practices. This, plus the expense of the drug and labor to supervise such a program, has led many workers to search for a better system. In 1965, Barrett et al.[2] described an alpha dithiosemicarbazone (356C61) which had specific activity against Anaplasma. This dithiosemicarbazone is an insoluble powder which is prepared as an aqueous suspension. If injected I/V, it may produce a respiratory distress. When diluted in PSS this can be minimized. Several workers have reported the apparent value of this drug.[8,20,21,22,23,25,32] Comparisons between 5 mg/kg 356C61 and 11 mg/kg oxytetracycline when given I/V to splenectomized calves showed 356C61 to be superior based on a significantly faster return of packed cell volumes to normal following treatment.[23] The injection of 356C61 10 times at the level of 5 mg/kg produced a fatal toxicosis in 6 of 7 animals.[1] Deaths occurred as early as 1 day and as late as 41 days after the last of 10 injections. Clinically, the animals showed chronic typanites, rumen atony, and depression before dying. Animals were treated symptomatically with ruminatorics and laxatives but failed to respond. Relief of bloat by trocharization or the passage of a stomach tube was only transitory.

Other attempts to eliminate the Anaplasma infection in carriers were made using 356C61. The injection of 5 mg/kg 5 times at 24-hour intervals was unsuccessful.[23] The injection of 356C61 at the rate of 5 mg/kg 3 times at 2-day intervals, 4 times over a 2-week period at 3- and 4-day intervals, 3 times at weekly intervals, and 3 times at 2-week intervals were all unsuccessful.

The addition of oxytetracycline to 356C61, giving both drugs simultaneously, was consistently successful in Anaplasma infected splenectomized

calves not previously treated.[20,21,22] Oxytetracycline (11 mg/kg) was combined with 356C61 (5 mg/kg), both drugs being diluted in 150 ml sterile saline and then injected I/V. Three treatments at either 24- or 48-hour intervals were successful. At 72-hour intervals, only 2 of 3 responded favorably.

Imidocarb (4A65), a white, readily soluble powder, originally recognized for its babesiacidal activities, has also shown promise for use in anaplasmosis.[21,30,33] Imidocarb given at the rate of 4, 5, and 6 mg/kg on 3 successive days has successfully eliminated the Anaplasma carrier status in splenectomized calves.[21] The addition of 356C61 to 4A65 appears compatible and was effective in eliminating Anaplasma with reduced dose rates of 4A65.[21] The use of 2 mg/kg 4A65 when given together with 5 mg/kg 356C61 3 times at 24-hour intervals was effective. In this instance, 4A65 was administered I/M and the 356C61 I/V. The reverse dosage consisting of 5 mg/kg 4A65 and 2 mg/kg 356C61 was also effective when administered as described. Unpublished evidence is available that the carrier status of splenectomized calves may be terminated with as little as 2 mg/kg of each 4A65 and 356C61 given 3 times at 24-hour intervals.

A total of 15 mg/kg 4A65 when given over a 3-day period was effective, but 15 mg/kg 4A65 given in 1 injection was ineffective in eliminating the carrier state.[21] A single injection of 4 and 6 mg/kg 4A65 combined with 15 mg/kg 356C61 was also ineffective.

The apparent synergistic or at least additive effect of oxytetracycline and 356C61 prompted the author to try combining oxytetracycline and 4A65. Every such trial was unsuccessful.

More recently Roby and Mazzola have described the use of 4A65 at the level of 5 mg/kg in adult carrier cattle.[33] A single injection was ineffective in eliminating the carrier status, but 2 injections of the same amount at 2-week intervals was successful in eliminating infection in all 5 cattle so

treated. They used both the dihydrochloride and the dipropionate salts successfully. Both salts had identical effects on the parasites; however, the dipropionate was less irritating at the site of inoculation.

Imidocarb at 5 mg/kg can be given safely either S/C or I/M. As little as 3 mg/kg given I/V may produce immediate respiratory collapse and death. A more likely response is labored breathing, excessive salivation, and lacrimation. These same signs often follow the S/C or I/M injection, but always much milder. In no instance has a fatal response occurred immediately after the S/C or I/M injection of 5 mg/kg. There appears to be considerable individual variation in tolerance to 4A65. On 1 occasion the S/C injection of calves with 15 mg/kg produced nothing more than transitory discomfort; whereas, on another occasion the S/C injection of 10 mg/kg was followed by death 2 days following the injection.

The toxicity of 4A65 while known to exist is still poorly understood. There have been unconfirmed reports of delayed signs of toxicity characterized by edema, lacrimation, depression, and death sometimes associated with elevated blood urea nitrogen and serum glutamic oxalacetic transaminase values. These signs have been seen 3-5 weeks after drug inoculation. These observations are limited and may or may not be drug related. Work is in progress to further examine this.

The discovery of these 2 new drugs represents a significant step forward in the treatment of anaplasmosis. When and if they become available commercially, they may provide a more practical approach for the elimination of carrier infection and eradication of anaplasmosis.

The possibility of combined drug therapy with reduced dose rates would further minimize the hazards of toxicity. More work is needed to explore other treatment programs.

There are several large-scale field trials using 4A65 presently underway.

Analysis of these results will undoubtedly contribute to our better understanding of the potential of drug therapy in an eradication program.

TABLE 1

Successful Treatment Procedures for the Elimination of *Anaplasma* Infection Using the Tetracycline Drugs

DRUG	RATE OF ADMIN.	ROUTE	NUMBER OF TREATMENTS	INTERVAL	REFERENCE
Tetracycline	5 mg/lbs	I/V or I/M	10	Daily	Pearson (29)
Oxytetracycline	5 mg/lbs	I/V or I/M	12-14	Daily	Splitter (36)
Chlortetracycline	15 mg/lbs	I/V	16	Daily	Splitter (36)
Chlortetracycline	1.0 mg/lbs	Orally	41	Daily	Franklin (16)
Chlortetracycline	2.5 mg/lbs	Orally	45	Daily	Franklin (16)
Chlortetracycline	0.5 mg/lbs	Orally	120	Daily	Franklin (15)*
Chlortetracycline	5 mg/lbs	Orally	30-60	Daily	Franklin (17)
Chlortetracycline	5 mg/lbs	Orally	60	Daily	Brock (6)
Chlortetracycline	2.5 mg/lbs	Orally	60	Daily	Brock (6)
Chlortetracycline	1.5 mg/lbs	Orally	60	Daily	Brock (6)
Chlortetracycline	5 mg/lbs	Orally	45-60	Daily	Roby (31)
Chlortetracycline	5 mg/lbs	Orally	30	Daily	Twiehaus (37)

*Negative Status Determined by Serologic Procedures

TABLE 2

Use of 4A65, Oxytetracycline, and 356C61
to Eliminate *Anaplasma* Infection

DRUG	RATE OF ADMIN.	ROUTE	NUMBER OF TREATMENTS	INTERVAL	REFERENCE
Oxytetracycline * 356C61	11 mg/kg 5 mg/kg	I/V	3	24 or 48 hours	Kuttler (20, 21, 22)
4A65*	5 mg/kg	I/M or S/C	3	Daily	Kuttler (21)
4A65 * 356C61	2 mg/kg 5 mg/kg	I/M or S/C	3	Daily	Kuttler (21)
4A65(1)	5 mg/kg	I/M or S/C	2	14 Days	Roby (33)

*Splenectomized calves used

(1) Adult cattle used

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The Effect of Imidocarb Treatment on *Babesia* in Calves and the *Boophilus microplus* Vector

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The Effect of Imidocarb Treatment on *Babesia* in Calves and the *Boophilus microplus* Vector

The efficacy of Imidocarb in the treatment and prophylaxis of cattle babesiosis has been reported. Beveridge (1969) found Imidocarb more effective than quinuronium, diamidine, and amicarbalide derivatives in preventing *Babesia rodhaini* infections in mice and rats. Imidocarb was also recognized as an active and effective compound for the treatment of *B. rodhaini* by Schmidt (1969). Callow and McGregor (1970) observed that 2.0 mg/kg Imidocarb was adequate to eliminate *Babesia argentina* infections in cattle, and that 0.6 mg/kg was sufficient to remove a *Babesia bigemina* infection in splenectomized steers. In a similar study, Brown and Berger (1970) reported that as little as 1 mg/kg injected intramuscularly (I/M) eliminated *B. bigemina* infections in cattle.

Both Hart *et al.* (1971), and Callow and McGregor (1970) have observed a prophylactic effect with Imidocarb. Working with mixed *Babesia* infections (*B. argentina* and *B. bigemina*), Todorovic *et al.* (1973) reported that 2 mg/kg Imidocarb prevented infection for 20 days, when the challenge consisted of *Babesia* infected blood, and for up to 15 weeks under natural field challenge.

In this study an attempt has been made to measure the duration of drug induced resistance in calves and the influence of this treatment on the infectivity and viability of *Boophilus microplus* ticks feeding on these calves.

MATERIALS AND METHODS

A total of 8 dairy type, male calves approximately 4 to 6 months of age were used in this study. To measure the effect of treatment checks for infectivity were made in 7 splenectomized calves. Calves 1, 2, 3, and 4 were injected I/M with 5 mg/kg Imidocarb dipropionate on day 0. Calves 5, 6, 7,

and 8 remained as untreated controls.

Infected Boophilus microplus larvae were obtained from a single pool of eggs obtained from several engorged females that had fed on a Babesia infected calf. From this pool the eggs were divided into 500 mg aliquots and allowed to hatch into larvae. Treatment of calf 1 was deferred until after infected ticks had been released onto calves 1 and 5. Calf 1 was given Imidocarb (5 mg/kg) 14 days later, after the development of diagnostic evidence of babesiosis. Infected larvae were placed on calves 2 and 6, 3 and 7, and 4 and 8, 14, 28, and 42 days after treatment of calves 2, 3, and 4. The Babesia infections observed resembled B. argentina morphologically, but the possibility exists that a mixed infection, including B. bigemina, was present.

Following the release of B. microplus larvae daily temperatures were taken. Blood samples in EDTA were taken at weekly intervals until evidence of a febrile response occurred, and were then taken daily. Giemsa stained thin blood smears were prepared and examined for evidence of hemoparasites. A packed red cell volume (PCV) was determined on each sample, using a micro-hematocrit centrifuge.

A total of 20 engorged females were collected from each calf (1 through 8). Each of the engorged females was weighed, the resulting egg mass was weighed, and the egg/female weight ratio determined to evaluate the influence of treatment on the Boophilus tick. After weighing the egg mass was pooled from each calf and divided into 500 mg aliquots for future use. When the larvae from a single calf were ready to feed they were then placed on a splenectomized calf to determine the influence of treatment on the presence or absence of infection in the larval ticks. This pattern was followed throughout with the exception of calf 8, a non-treated control. When in doubt as to a diagnosis of Babesia, blood inoculation (10-20 ml intravenously

[I/V]) into a splenectomized calf was made.

RESULTS

The response of Imidocarb treatment on Babesia infection in calves and ticks, and the response of treatment on Boophilus ticks is tabulated in Tables 1 and 2, respectively.

Calves 1 and 5 developed clinical signs of Babesia infection before treatment of calf 1. Both calves recovered, but a more persistent temperature response was noted in calf 5 and recovery was more protracted. Larval progeny from ticks recovered from calf 1 were not infective for Babesia when placed on a susceptible splenectomized calf. The larval progeny of ticks recovered from calf 5 transmitted Babesia infection to a splenectomized calf.

Calf 2, treated 14 days before, failed to develop evidence of Babesia infection after infective ticks were released. Larval progeny of ticks recovered from calf 2 were not infective for Babesia when placed on a susceptible splenectomized calf. Calf 6, a non-treated control, developed clinical signs of babesiosis, characterized by an elevated temperature and a drop in PCV, following the release of infected Boophilus larvae. The larval progeny of ticks recovered from calf 6 transmitted Babesia infection to a splenectomized calf.

Calf 3, treated 28 days before, failed to develop evidence of Babesia infection after infective ticks were released. The larval progeny of ticks recovered from calf 3, however, transmitted Babesia infection to a splenectomized calf. Calf 7, a non-treated control, developed clinical signs of babesiosis, characterized by an elevated temperature and a drop in PCV, following the release of infected larvae. The infection was confirmed by positive identification of a Babesia parasitemia. The larval progeny of ticks

recovered from calf 7 transmitted Babesia infection to a splenectomized calf.

Calf 4, treated 42 days prior to tick release, and calf 8, an untreated control, both developed clinical signs of babesiosis following the release of infected Boophilus larvae. The infection in calf 4 was confirmed by inoculating 10 ml of whole blood from calf 4 into a susceptible splenectomized calf, which died of acute babesiosis (B. argentina) 14 days after injection. Ticks recovered from calves 4 and 8 were assumed to be infective based on previous trials.

The average weights of 20 engorged females collected from all 8 test calves are recorded in Table 2. The average egg yield from each female plus the ratio of egg weight/female weight is also recorded. A clear pattern of influence attributable to either treatment or infection was not evident. No differences were detected in ticks or egg weights from calves 1 and 5. Ticks from calf 6, a non-treated control that developed acute babesiosis, were heavier than ticks from calf 2 which had been treated 14 days before tick infestation. Egg weights followed the same pattern and the egg/female weight ratio was unchanged. Comparisons of tick weights and egg weights on engorged females from calves 3 and 7, and 4 and 8 showed just the opposite trend. Ticks from calves 3 and 4, which had previously been treated, were heavier than ticks from the untreated controls. In one instance a significant difference in egg/female weight ratio occurred. Engorged female ticks from calf 3, in addition to being heavier, produced proportionally more eggs than did ticks from the untreated control calf 7.

DISCUSSION

Imidocarb administered I/M at the rate of 5 mg/kg was an effective treatment in one instance, and was sufficient to prevent clinical babesiosis

in calves exposed to known infected ticks when given 14 and 28 days before exposure. The Imidocarb blood level present when treatment was given 14 days after infected ticks were released, and at a time when clinical babesiosis was present, was sufficient to eliminate the infectivity of the larval progeny. The same observation was made when calves were treated 14 days before tick release. This calf was not only protected from Babesia but the engorged females from the animal failed to transmit the infection, if present, to the resulting larvae. It is not known if the drug sterilized the tick or if the drug merely prevented the development of the organism in the vertebrate host and hence interfered with reinfection of the tick. In any event Imidocarb was adequate to eliminate infection in both the vertebrate and invertebrate, thus, effectively preventing transmission of the organism.

Imidocarb when given 28 days before tick infestation prevented clinical babesiosis, but did not prevent infectivity of the tick. It was, regrettably, not determined if calf 3 actually developed a sub-clinical infection; hence, an explanation of this observation is difficult. It was obvious that injection of the drug 42 days prior to exposure did not prevent the development of Babesia.

The inconsistent pattern of female and egg weights of ticks recovered from treated and non-treated calves makes interpretation difficult and uncertain. The highly significant differences, however, suggest that this factor should not be ignored. It is possible that exposure of ticks as larvae to blood levels of Imidocarb (calf 2) present 14 days after treatment caused some retardation of tick growth which was reflected by lower average weights. The blood levels in calves 3 and 4, on the other hand, were so low that no influence was seen. The drug level in calves 3 and 4 may have been adequate to suppress Babesia growth to the extent that the ticks were not as

heavily exposed to the Babesia organism as they were in calves 7 and 8. The Babesia infection in the ticks per se may have been responsible for the reduced size of both the engorged females and their egg mass.

SUMMARY

Treatment of calves with 5 mg/kg Imidocarb dipropionate 2 weeks after exposure to Babesia infected larvae, and 2 weeks before exposure to infected larvae rendered the larval progeny from these calves incapable of transmitting Babesia infection. The drug administered 14 and 28 days before tick exposure prevented the development of clinical babesiosis. Larval progeny from a calf treated 28 days before infestation were, however, infective for Babesia. Treatment 42 days before exposure to infective larvae did not prevent the development of a Babesia parasitemia.

TABLE 1

Prophylactic Effect of Imidocarb Against *Babesia* Infection in Calves and Ticks

Calf	Time of Tick Release in Relation to Treatment	Febrile Response After Tick Release (in days)	Low PCV	High Febrile Response	*Babesia* Parasitemia	Infectivity of Tick Progeny for Splenectomized Calves
1 (Treated)	-14 days**	13	10	104.6	Neg.	Neg.
5 (Control)	same	13	12	105.4	Pos.	Pos. - developed babesiosis
2 (Treated)	14 days	None	25	102.4	Neg.	Neg.
6 (Control)	same	13	13	106.0	Neg.	Pos. - developed babesiosis
3 (Treated)	28 days	None	26	102.0	Neg.	Pos. - developed babesiosis
7 (Control)	same	13	16	104.3	Pos.	Pos. - developed babesiosis
4*(Treated)	42 days	14	30	104.8	Neg.	NT
8 (Control)	same	15	25	104.8	Neg.	NT

*Calf 4 was checked for *Babesia*, following the febrile response, by blood inoculation into a splenectomized calf. A *B. argentina* organism was recovered in the check calf.

**Treatment in this instance was given 14 days after tick release.

NT : No test.

TABLE 2

Influence of Imidocarb Treatment on *B. microplus* Ticks

	Avg. Wts. of 20 Engorged Females (mg.)	Avg. Egg Wts. From 20 Engorged Females (mg.)	Ratio Egg/Engorged Female Weights (mg.)
Calf 1	404 ± 32	228 ± 28	0.56 ± 0.06
5	409 ± 36	235 ± 29	0.57 ± 0.05
Significance	NS	NS	NS
Calf 2	348 ± 34	203 ± 21	0.58 ± 0.04
6	438 ± 58	248 ± 54	0.56 ± 0.12
Significance	P<0.01	P<0.01	NS
Calf 3	442 ± 41	263 ± 26	0.59 ± 0.04
7	385 ± 42	128 ± 30	0.34 ± 0.09
Significance	P<0.01	P<0.01	P<0.01
Calf 4	509 ± 43	304 ± 60	0.60 ± 0.12
8	461 ± 40	253 ± 40	0.55 ± 0.93
Significance	P<0.01	P<0.01	NS

NS : Not significant

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Arthropod Borne Protozoan Infections
(Affecting Domesticated, Food-Producing Animals)

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Introduction

The arthropod borne protozoan infections include a wide range of infectious agents which are generally hemotropic in nature and are transmitted by blood sucking insects such as ticks, flies, mosquitoes, etc. Transmission may occur mechanically by the transfer of infected blood from one animal to another by insects or improperly cleaned surgical instruments. More often, biological vectors are involved, in which the causative agent undergoes cyclical development in the invertebrate host, prior to producing infection in the susceptible vertebrate host.

These protozoan agents may be extra- or intra-erythrocytic in nature, but are generally characterized by febrile reactions in the acute phase, varying degrees of anemia, and carrier infections following recovery.

Anaplasmosis will be considered in this section because of the many similarities between this disease and the arthropod borne hemoprotozoan infections. Recent studies suggest that the causative agent, *Anaplasma marginale*, is more closely related to *Rickettsia* than *Protozoa*, although some question still exists as to its correct classification.

Included under this heading are diseases caused by *Theileria*, *Trypanosoma*, *Anaplasma*, *Babesia*, *Leucocytozoon*, and *Besnoitia*. A list of agents with the disease they produce, animal species affected, and the principal vectors is given in Table 1. East Coast Fever (*Theileria*) and trypanosomiasis (*Trypanosoma*) have been discussed in detail in separate chapters. Babesiosis and anaplasmosis, major animal disease problems throughout the world, are discussed in some detail in this chapter.

TABLE I

ORGANISM	ANIMALS AFFECTED	MORPHOLOGY OF ORGANISM	VECTORS
BABESIA (babesiosis)		Intraerythrocytic	Ticks
B. bigemina	Cattle	4.5μ X 2.5μ (Large, round and piriform; acute angle)	Boophilus annulatus, B. calcaratus, B. decoloratus, B. microplus, Haemaphysalis punctata, Rhipicephalus appendiculatus, R. bursa, R. evertsi.
B. bovis, B. berbera, B. argentina	Cattle	2.4μ X 1.5μ (Small and more rounded; obtuse angle)	B. annulatus, B. calcaratus, B. microplus, Ixodes persulcatus, I. ricinus, R. bursa.
B. divergens	Cattle	1.5μ X 0.4μ (Small, narrow and marginal location; obtuse angle)	Dermacentor reticulatus, Haemaphysalis punctata, I. ricinus.
B. major	Cattle	2.6μ X 1.5μ (Similar to B. bigemina but smaller)	B. calcaratus, Haemaphysalis punctata, I. ricinus, probably Boophilus ticks.
B. motasi	Sheep and Goats	3.0μ X 2.0μ (Large, piriform; acute angle)	D. silvarum, Haemaphysalis punctata, R. bursa.
B. ovis	Sheep and Goats	1.5μ X 1.0μ (Small and more rounded; obtuse angle)	I. persulcatus, R. bursa.

Table I Cont'd.

ORGANISM	ANIMALS AFFECTED	MORPHOLOGY OF ORGANISM		VECTORS
B. caballi	Horses	3.0μ X 2.0μ (Large, pear-shaped; acute angle)		D. marginatus, D. nitens, D. pictus D. silvarum, Hyalomma dromedarii, H. excavatum, H. marginatum, H. scupense, R. bursa, R. sanguineus.
B. equi	Horses	1.0 - 2.0μ (Small and rounded; Maltese Cross is characteristic)		Dermacentor spp., H. dromedarii, H. excavatum, H. marginatum, H. plumbeum, H. uralense, R. bursa, R. evertsi, R. sanguineus.
B. trautmanni	Swine	3.5μ X 2.0μ (Large, narrow and long; acute angle)		R. sanguineus, Dermacentor spp. and Hyalomma spp.
B. perroncitoi	Swine	0.7 - 2.0μ (Small and more rounded)		Vectors unknown.
		Erythrocytic Forms	Lymphocytic Forms	
THEILERIA (theileriosis)		Intraerythrocytic and Intralymphocytic		Ticks
T. parva	Cattle	1.8 X 0.75μ (Rod forms more common; very pleomorphic)	8.0μ in diameter	R. appendiculatus, R. ayrei, R. capensis, R. evertsi, R. jeanelli, R. neavei, R. simus, H. anatolicum, H. dromedarii, H. truncatum.
T. annulata (Egyptian Fever of Cattle, Tropical Theileriosis, Mediterranean Coast Fever)	Cattle	0.5 - 1.5μ (Oval forms more common)	Similar to T. parva	Hyalomma spp.

Table I Cont'd.

ORGANISM	ANIMALS AFFECTED	MORPHOLOGY OF ORGANISM		VECTORS
		Erythrocytic Forms	Lymphocytic Forms	
T. mutans (Tzaneen Disease)	Cattle (Generally non-pathogenic)	1.0 - 2.0μ diameter (Mostly round and oval)	Rarely seen but similar to *T. parva*	*B. annulatus*, *Haemaphysalis bispinosa*, *Rhipicephalus spp.*
T. lawrencei (Corridor Disease)	Cattle and African Buffalo	Similar to *T. parva*		Principally *R. appendiculatus*, *Rhipicephalus spp.*, probably the same as *T. parva*.
T. hirci	Sheep and Goats	0.6 - 2.0μ (Mostly round and oval)	4.0 - 10.0μ in diameter	*R. bursa*, *R. evertsi*.
T. ovis	Sheep and Goats (Generally non-pathogenic)	Similar to *T. hirci*		*Ornithodoros lahorensis*, others are probably involved.
TRYPANOSOMA (trypanosomiasis)		Extraerythrocytic		Flies
T. theileri	Cattle (Non-pathogenic)	25 - 120μ in length		*Tabanids*.

Table I Cont'd.

ORGANISM	ANIMALS AFFECTED	MORPHOLOGY OF ORGANISM	VECTORS
T. brucei	Horses, camels, and dogs; cattle are relatively resistant	15 - 35μ in length	Tsetse fly (*Glossina*).
T. gambiense	Man	Similar to *T. brucei*	Tsetse fly (*Glossina*).
T. rhodesiense	Man	Similar to *T. brucei*	Tsetse fly (*Glossina*).
T. congolense	Cattle	9 - 18μ in length	Tsetse fly (*Glossina*).
T. vivax	Cattle, sheep and goats	18 - 26μ in length	*Glossina*, *Tabanids* and other biting flies.
T. evansi (Surra)	Horses; but cattle are susceptible	15 - 34μ in length	*Tabanidae* (horseflies) and other biting flies, also vampire bats.
ANAPLASMA (anaplasmosis, Gall Sickness)		Intraerythrocytic	Ticks, mosquitoes and flies
A. marginale	Cattle	0.3 - 1.0μ in diameter (Coccoid)	20 species of ticks, 10 species of *Tabanus*; other flies and mosquitoes are potential vectors.

Table I Cont'd.

ORGANISM	ANIMALS AFFECTED	MORPHOLOGY OF ORGANISM	VECTORS
A. ovis	Sheep	0.3 - 1.0μ (Coccoid)	Unknown - probably similar to A. marginale.
A. centrale	Cattle (Benign infection)	In size similar to A. marginale - distinguished by its central location in the erythrocyte	Thought to be similar to A. marginale.
LEUCOCYTOZOON (leucocytozoonosis)		Intraerythrocytic	Flies or gnats
L. simondi	Domestic and wild ducks and geese	Gametocyte 14 - 15μ X 4.5 - 5.5μ	Simulium venustum, S. crosstoni, S. euryadminiculum, S. rugglesi.
L. smithi	Domestic and wild turkeys	Gametocyte 20 - 22μ X 6μ	S. occidentale, S. nigroparvum, and S slossonae.
L. caulleryi	Domestic chickens	Gametocyte Round and about 15μ in diameter	Culicoides arakawe.

BABESIOSIS
(Piroplasmosis, Texas Fever, Redwater, Tick Fever)

Definition: Babesiosis is a febrile, tick-borne disease of domestic and wild animals caused by protozoan parasites of the genus *Babesia* and characterized by extensive erythrocytic lysis leading to anemia, icterus, hemoglobinuria, and death.

Etiology: Babesiosis is associated with the appearance of the specific infecting *Protozoa* in the erythrocytes. Neitz (1956) recognized 17 distinct species of *Babesia* from various vertebrate hosts.

Babesia bigemina: This species causes one of the most important diseases of cattle in the tropics and subtropics. *Babesia bigemina* is a relatively large parasite; when ultrathin sections of infected erythrocytes were viewed by electronmicroscopy, the double membrane, nucleus, endoplasmic reticulum, and polar bodies were connected with a conoid structure which was located on the anterior part were found (Gonzalez *et al.*, 1971). The round forms are 2 to 3μ in diameter and the elongate ones 4 to 5μ long. In bovine erythrocytes, the parasites may assume various shapes, but the most characteristic is oval to pear-shaped. Paired *B. bigemina* usually diverge with an acute angle.

Babesia bovis: This species is the most important cause of European babesiosis. It is common in many regions of the world, but information on its true prevalence must await a decision as to whether *Babesia berbera* and *Babesia argentina* are synonymous. *Babesia bovis* is a small form measuring about 2.4 by 1.5μ (Davies *et al.*, 1958). The parasite usually appears to be on the periphery of the erythrocytes, with paired organisms diverging with an obtuse angle.

Babesia divergens: This species is smaller than *B. bovis*. The

organisms usually occur as paired, club-shaped organisms about 1.5 by 0.4μ; the angle of divergence of paired organisms is relatively large, so that they diverge more from each other than B. bovis; in addition, they tend to lie along the circumference of the host erythrocyte.

Babesia argentina: This species resembles, and may be synonymous with, B. bovis. Babesia argentina is more robust than B. bovis, the ring forms being commonly found. The paired forms are piriform with an obtuse angle, about 2.0 by 1.5μ, and usually lie slightly off center of the host erythrocyte.

Babesia major: This species also resembles B. bovis but is larger. The piriform, paired forms measure 2.6 by 1.5μ, and the round ones are 1.8μ in diameter. The parasites lie in the center of the host erythrocyte. It is distinguished morphologically from B. bovis by its elongated sub-piriform shape and by the fact that most paired forms meet at an angle less than 90°.

Babesia organisms are generally host-specific, and in addition to the bovine babesiosis the following parasites have been described in other species:

B. motasi, B. ovis, B. foliata, and B. taylori in sheep;

B. caballi and B. equi in horses;

B. trautmanni and B. perroncitoi in pigs;

B. canis and B. gibsoni in dogs;

B. felis in cats; and

B. rodhaini in mice and rats.

History: The classical investigations of Smith and Kilborne (1893) in the United States of America of the cause of Texas fever were the first to establish that a protozoan disease could be transmitted by an intermediate arthropod host; and their discovery that B. bigemina was carried by the

cattle tick, Boophilus annulatus, paved the way not only for the control of Texas fever, but also for the study of arthropod-borne diseases in general. Bovine babesiosis is of historical interest because it has now been virtually eliminated from the United States where it was once very prevalent. Its eradication was accomplished by eliminating the tick-vector, B. annulatus. Babesiosis is still present in many parts of the world.

Signs: Infections with B. bigemina and B. argentina are usually so similar that they can be distinguished only by microscopic examination of the blood. Moreover, mixed infections with these two parasites frequently occur. It is more convenient to deal with them together, emphasizing those differences characteristic of one or the other etiological agent. When bovine babesiosis is contracted from ticks, the incubation period is commonly two to three weeks; but when induced by the inoculation of infected blood, it is usually between five and 14 days, although it may be longer. There is a marked difference in susceptibility of individual cattle. Some cattle pass through an attack without showing any clinical signs; whereas, others become so severely affected that death may result. Calves may possess a natural resistance to the disease and show no clinical signs at all; yet, they may acquire an infection which protects them in later life. This resistance gradually diminishes with the age of the calf, so that by the time they are a year old they may be susceptible enough to suffer a severe attack. Well-bred bulls in good condition suffer severely, and the susceptibility of lactating cows and all pregnant and aged cows is high if they have never been previously exposed.

Development of high fever, up to 41.5° C. rectal temperature, is sudden in onset. There is anorexia and rumen atony. Often, the first

sign is that the animal isolates itself from the herd, becomes uneasy, seeks shade, and may lie down. Cattle may stand with an arched back, have a roughened hair coat, and show evidence of dyspnea, and tachycardia. The mucous membranes are first injected and reddened, but as erythrocytic lysis occurs, the color changes to the pallor of anemia. Anemia is a contributory factor to the weakness and loss of condition seen in cattle that survive the acute phase of the disease. The anemia may be very severe with up to 75% or more of the erythrocytes being destroyed.

When cattle are driven, they move with difficulty and they may stagger because of posterior weakness. Constipation is present in the early stages. Cattle suffering a severe reaction have hemoglobinuria and icterus. A decrease in milk production, which occurs with the initial rise of temperature, is generally the first sign noted in lactating cows. The febrile stage of the disease usually lasts about a week, and the course of the disease is about three weeks. Pregnant cows may abort. Calves may be less severely affected, pyrexia not being as severe, and hemoglobinuria being absent.

Under field conditions, mortality usually occurs in the third week. With B. argentina infections, there is usually more rapid development of clinical signs, such as higher fever, greater weakness, and earlier death, than with B. bigemina. The mortality rate may reach 90%, particularly in the hot weather, but in colder weather or when the disease is mild, the death rate is much lower. The average death rate is stated to be 60% in fully susceptible cattle. Death may be accelerated by forced exertion, and a sudden decrease in body temperature usually presages early death. Infection with B. argentina causes a higher mortality than with B. bigemina. In Australia, 70-80% of cattle infected with B. argentina, and only 30% with B. bigemina, develop a reaction severe enough to need chemotherapy. In cattle which survive, the temperature gradually decreases to normal and the appetite is

regained, but recovery is very slow and may take weeks or even months. Involvement of the central nervous system is not an unusual complication of babesiosis due to B. argentina. Among the clinical signs are depression, ataxia, convulsions, opisthotonus, mania, and finally coma.

Incubation Time: According to Riek (1968), the incubation period is 5 to 15 days but depends to some degree on the species of parasites involved.

Postmortem Lesions: Cattle dying from babesiosis show postmortem lesions which are somewhat characteristic and vary with the acuteness and severity of the clinical signs. In cattle that have died after a few days of high fever, the lungs may be edematous and congested. The pericardial sac contains some sero-sanguineous fluid and there are subepicardial and subendocardial petechial hemorrhages. The liver is enlarged and icteric, and the gall bladder, which may show hemorrhages on the mucous surface, is distended with thick, dark green bile. The spleen is markedly enlarged with a dark pulpy consistency. The abomasal and intestinal mucosa may be icteric with patches of subserosal hemorrhages. The blood is thin and watery; the plasma may be red tinged due to hemoglobinemia. The kidneys are congested, especially with B. argentina infection, and the urinary bladder distended with dark reddish-brown urine. Jaundice is commonly distributed in the connective tissue. The lymph nodes are edematous and often have petechiation.

In cattle which have suffered a more prolonged illness, acute lesions are much less conspicuous except that subepicardial petechial hemorrhages may be present. The carcass is usually emaciated and icteric; the blood is thin and watery; the intermuscular fascia edematous; the liver yellowish-brown in color and the bile contains flakes of semi-solid material.

The kidneys are pale and often edematous, and the bladder contains normal urine. Although the spleen is enlarged, the pulp is firmer than in the acute babesiosis.

Microscopic Pathology: The microscopic lesions are characteristic of an acute intravascular hemolytic anemia. Multicentric hematopoietic hyperplasia and metaplasia often occur in the spleen and bone marrow. Hepatic centrilobular necrosis may occur as a consequence of the anemia. The lymph nodes are hemorrhagic and edematous with reticulo-endothelial and lymphoid hyperplasia being prominent. The lymph nodes, tonsil, and spleen often have hemosiderosis, erythrophagocytosis, and hyaline degeneration of arterioles. The terminal hepatic veins of the liver may be engorged with leukocytes. In cases of central nervous system involvement, there may be perivascular lymphocytic infiltration of the cerebral and cerebellar capillaries which are engorged with parasitized erythrocytes, especially *B. argentina*. Cerebral and cerebellar congestion and edema are common lesions.

The erythrocytic count often decreases to less than 1 million at the time of death. Erythroid hyperplasia of the bone occurs in the disease as is manifested by the presence of immature erythrocytes. Extreme poikilocytosis generally occurs. The myeloid:erythroid ratio of the bone marrow decreases in the acute phases.

Diagnosis:

In the Field: Fever, anemia, jaundice, and hemoglobinuria are suggestive clinical signs in cattle from an area where babesiosis is enzootic. If these are coupled with splenomegaly and the usual postmortem lesions associated with erythrocytic destruction, the diagnosis of babesiosis is strengthened. However, a positive diagnosis of

babesiosis can be established by identification of the parasites in the blood, positive serological tests, and by transmission experiments.

Laboratory: Although the presence of B. bigemina can often be detected by the examination of thin blood films, B. argentina is often very difficult to find. However, Giemsa stained thick blood smears have been found to be a reliable and practical means for the detection of both species of Babesia. A wet film of blood, approximately 50μ thick, is made on slides, dried quickly at 65° C. for 45 minutes, and stained as soon as possible after drying with a 1:50 solution of Giemsa at pH 7.0-7.1.

Babesia argentina can be readily detected in capillaries of the brain by the brain-smear, biopsy technique, either at the abattoirs after death or from live cattle. Babesia appear as small, intraerythrocytic, paired, basophilic bodies. Clinically normal cattle with latent infections of B. argentina show the parasite in brain smears. The grey matter of the cerebral or cerebellar cortex is the tissue of choice when making smears. Babesia argentina was found to persist in the brain capillaries for 12 months in 50% of cattle tested. Babesia bigemina is found in brain tissue much less frequently than B. argentina. Biopsy specimens can be taken from the brains of cattle at three monthly intervals with a high degree of safety, although temporary nervous derangement may be observed.

The detection of the carrier state of bovine babesiosis has presented a particularly difficult problem because of the low parasitemias in a high percentage of carrier cattle. Fundamental knowledge concerning immunoserology of several Babesia spp. has led to the development of several diagnostic procedures for detection of Babesia antibodies. Todorovic et al. (1971) found that antigens originated from the infected parasites, as well as from plasma collected at the acute stages of infection, can be

employed in serological tests. The complement fixation reaction constituted one of the earliest tests, and in recent years considerable progress has been made in an improved complement fixation test which is an exceedingly valuable diagnostic tool, both in the field and in research.

Complement-fixing antibodies develop in the sera of cattle within seven to 21 days post-infection, and persist through all stages of infection from the commencement of a detectable parasitemia to a time when they are too few in number to be detected in thick blood smears. A positive result is a reliable indication of a current babesial infection, even though the parasites are too few to be found in thick films. On the other hand, a negative result cannot be reliably interpreted as freedom from infection.

Fluorescent microscopy is proving valuable in the study of babesiosis, and the staining of Babesia by fluorochrome acridine orange has been reported. This substance stains only nuclear material, thus, making it suitable for demonstrating parasites in red blood cells. Formalin preserved blood can be kept for three months, and smears from it stained with acridine orange and examined with the fluorescent microscope show Babesia as brilliant reddish objects in blue fluorescent erythrocytes, easily seen under low power.

In addition, the gel-precipitation, fluorescent antibody, direct and indirect hemagglutination techniques have been applied for detection of specific Babesia antibodies, utilizing antigens from the parasitized erythrocytes as well as plasma collected from animals with acute infection.

Differential: Other conditions which should be differentiated in cattle are: anaplasmosis, trypanosomiasis, theileriasis, leptospirosis, eperythrozoonosis, hemobartonellosis, post-parturient hemoglobinuria, cold water hemoglobinuria, onion toxicity, and bacillary hemoglobinuria. In cerebral babesiosis, the signs may be confused with those of rabies.

Prognosis: Babesiosis in susceptible cattle is frequently severe with mortality approaching 60 to 90%. The prognosis of cattle with hemoglobinuria is very poor. The prognosis may be more favorable in indigenous cattle where mortality may be much lower.

Epizootiology:

Geographic Distribution: Babesiosis has often been categorized as a tropical malady; however, reports have originated from temperate and frigid sections of the world. Due to a variety of species which are susceptible to infection, there is a constant danger that even unaffected areas may be invaded by these hemoprotozoan parasites.

Babesia bigemina has a worldwide distribution in cattle. The geographic distribution of this species is through Central America, South America, Europe, Africa, Australia, and Asia.

Babesia bovis: This is a European species, but it has been reported from Africa, Asia, and the East Indies. It occurs mainly outside the tropical zones. The geographic distribution of this species is in south and central Europe, Russia, Africa, and Mediterranean coasts. This is the most important case of European babesiosis. It is common in many regions, but information on its true prevalence must await new surveys in the light of the recent recognition that *B. divergens* is a separate species and also a decision as to whether *B. berbera* and *B. argentina* are synonymous.

Babesia argentina: This species is widely distributed throughout the tropics, mainly through South America, Central America, Mexico, and Australia. *B. argentina* may be synonymous with *B. bovis*.

Babesia divergens: The geographic distribution of this species is in Balkan states, Austria, and England.

Babesia major: This species has been reported from North, South, and West Africa, South America, and from the Baltic region, Russia, and England.

Babesia berbera: This species has been reported from North Africa, Russia, and northern Europe and may be synonymous with B. argentina.

Transmission: In North America, the major transmitting agent in the past was B. annulatus, known locally as the blue tick, the Texas fever tick, the fever tick, or the southern cattle tick. In a very limited area of southern Florida, the transmitting agent was Boophilus microplus, also known as the tropical fever tick. This species has a wider host relationship than B. annulatus.

In other parts of the world, other species of Boophilus are responsible for transmitting Babesia. In southern Europe, it is Boophilus calcaratus; in central Africa, Boophilus decoloratus; and in South and Central America, Mexico, Australia, Brazil, Venezuela, and some of the Caribbean Islands, the transmitting agent is B. microplus. Very little is known about the life history of B. bovis, although a number of vectors have been suggested, e.g. Ixodes, Boophilus, and Haemaphysalis. The main vector is probably Ixodes ricinus.

Early investigations in Australia showed that B. bigemina could be transmitted by nymphs and adult ticks and B. argentina by larvae of the female B. microplus which had engorged on infected cattle. Haemaphysalis bispinosa, Haemaphysalis bancrofti, Amblyomma triguttatum, or Ixodes holocyclus are found on cattle in Australia, but are not capable of transmitting babesiosis.

Babesia argentina: It is transmitted by species of B. microplus and possibly by Rhipicephalus bursa. Babesia major: The vector in Russia is B. calcaratus. Babesia berbera: The vectors are B. calcaratus and R. bursa. Transmission occurs through stage-to-stage. Babesia divergens: It is transmitted by species of I. ricinus and Haemaphysalis cinnabarina punctata by transstadial and transovarial stages.

Hosts: Babesia parasites are generally host-specific; however, Neitiz (1956) discussed the susceptibility of various animals to Babesia infections and concluded that Babesia parasites are by no means as host-specific as formerly believed. Babesia bigemina are found in cattle and water buffalo, B. argentina and B. berbera in cattle, and B. bovis and B. divergens in cattle.

Control and Eradication:

Preventive Measures: As Babesia are transmitted by ticks, prevention depends on the elimination of the tick vector. This is frequently exceedingly difficult depending upon the particular species of ticks involved, but it may be accomplished by regular and prolonged dipping at two-week intervals. This is possible in other areas only through the complete absence of the animal host for sufficient time to permit the ticks to die out. In the United States, the disease has been practically eliminated by systematic dipping of all cattle to destroy the principal vectors, B. annulatus and B. microplus.

Cattle which survive an attack of babesiosis become carriers, and as long as the infection persists, the animals are resistant to reinfection. In enzootic areas, the animals are constantly reinfected as long as they are subject to infestation with infected ticks. In the absence of reinfection, the Babesia gradually disappear, and the cattle again become susceptible to the disease. Immunity to babesiosis, in the absence of reinfection, persists for a varying time up to about one year. Young calves from immune dams are protected up to two months against babesiosis by antibodies received from their mothers through colostrum. The immunity which accompanies infection with babesiosis forms the basis of the programs of protective inoculation which are carried out in the countries where babesiosis is enzootic.

Treatment: Successful treatment of babesiosis depends on early diagnosis and the prompt administration of effective drugs. There is little likelihood of success if treatment is delayed until the animal has been weakened by fever and anemia. A number of drugs have been used to treat this disease in the different species of animals with varying results.

Trypan Blue - This was one of the first drugs used to control babesiosis. Trypan blue, the sodium salt of ditolyl diazo-bis-8-amino-1-naphthol-3,6-disulfonic acid, is best used as a 1% solution at the rate of 50 to 100 ml. injected intravenously. Even though the drug is inexpensive, there are disadvantages to its use, and more recently developed drugs are generally preferred.

Quinuronium Derivatives - This drug is sold under the proprietary names such as Acaprin, Babesan, Pirevan, Piroplasmin and is essentially 6,6'-di(N-methyl-quinolyl) urea dimethosulfate. It has been reported to be highly effective against the large *Babesia spp.* and of some value, also, against the smaller piroplasms, *B. bovis* and *B. argentina*. In cattle, the drug is injected subcutaneously at the rate of 1 ml. per 100 pound body weight, with a maximum of 6 ml.

Acridine Derivatives - The acridine derivatives, sold under such trade names as Acriflavin, Gonacrine, Flavin, and Euflain, are a mixture of 2,8-diamino-10-methylacridinium chloride with a small amount of the 2,8-diamino salt. An intravenous injection in cattle with 20 ml. of a 5% solution has given good results against both *B. bigemina* and *B. argentina*.

Diamidine Derivatives - Phenamidine, composed of 4,4'-diamidinodiphenyl ether, was first reported to be of value against canine babesiosis and subsequently against *B. bigemina*. It is tolerated in cattle up to a level of 22.5 mg. per kg. injected intravenously, but it has been found

toxic at 30 mg. per kg. when used as a 40% solution. Although some workers disagree concerning its efficiency, Berenil, also Ganaseg, (4,4'-diamidino diaxoaminobenzene diaceturate) has been recommended for use against a number of species in different animals. It is now used extensively in the treatment of B. bigemina and B. argentina at 2 to 3 mg. per kg. intramuscularly. First introduced in 1960, Diampron (3,3'-diamidinocarbanilide diesthionate) has been used successfully in the treatment of B. divergens, B. berbera, and B. bigemina infections in cattle when administered subcutaneously or intramuscularly and at dose rates of 5 to 10 mg. per kg.

Other Drugs - Quinine, ichthargan, neoarsphenamine, and aureomycin have been used to treat babesial infections with varying results; however, they do not appear to be of much value when compared with the more recent compounds. Imidocarb (4A65) (3,3'-bis-(2-imidazolin-2-YL)-carbanilide dihydrochloride or diproprionate-Burroughs Wellcome Lab.) has been demonstrated to have prolonged prophylactic value and curative action against B. bigemina and B. argentina at 2 to 5 mg. per kg. subcutaneously or intramuscularly.

Sanitation and Disinfection: Sanitation and disinfection, aside from that involved in control and elimination of tick-vector, do not contribute to an abatement of the disease incidence in enzootic areas.

Immunization: Immunization (co-infectious immunity or premunition) against babesiosis is performed by inoculating cattle with Babesia spp. with and without specific chemotherapy. Cattle having recovered from a premunizing parasitemia will have a high degree of immunity for the duration of the carrier infection which usually lasts 4 to 8 months in the absence of re-exposure. The continued presence of infected ticks serves to boost the immunity established by inoculation. The standard premunizing

inoculum usually consists of $1X10^6$ to $1X10^8$ Babesia parasites contained in 2 ml. of blood. Because of biological variation the premunizing reaction often cannot be predicted. More severe reactions will occur in cattle that have had little or no previous exposure to infected ticks. For practical purposes, the period of close supervision should be from 7 to 14 days after premunization. The number of cattle inoculated should be restricted to a number that can be supervised comfortably. On farms with small paddocks and good handling facilities, up to 300 can be inoculated. If fully susceptible cattle are inoculated in large numbers without aftercare, losses of 1 to 5% may occur. It is good practice to inoculate and dip at the same time. Pregnant cows may abort after severe premunizing reactions.

Treatment following premunization is indicated when sickness is apparent or when the morning temperature is 41.0° C. or more. Daily temperatures should be taken during the time when the reaction is expected. Losses are greatly diminished when treatment is promptly administered as soon as the temperature increases because the onset of fever usually precedes clinical signs of infection. Cattle treated promptly often suffer no ill-effects; whereas, cattle treated when they appear sick may take days or weeks to recover.

Cattle premunized with either B. argentina or B. bigemina are protected more thoroughly from a homologous infection. Some cross Babesia spp. protection does occur, but is not complete. While unlikely, there are instances where antigenic variations occur within a single Babesia spp.. so that the immunologic barrier may not be complete even against a homologous Babesia spp.

In the absence of a practical eradication program, premunization is a useful tool to prevent serious cattle losses but has the drawback of perpetuating infection.

ANAPLASMOSIS
(Gall Sickness)

Definition: Anaplasmosis is an acute or chronic, infectious, non-contagious disease of cattle. Acute infections are characterized by anemia, fever, and icterus. It is caused by the micro-organism, *Anaplasma marginale*, which is found in the erythrocyte and is transmitted by a variety of blood sucking arthropods. Chronic, or carrier infections, may persist for years without producing evidence of clinical disease, but during this time serve as a reservoir of infection from which arthropod vectors may transmit the causative organism to susceptible cattle.

Etiology: *Anaplasma marginale* was first thought to be a *Protozoa*, largely on the basis of its association with *Babesia*, and a disease syndrome similar to that produced by other hemoprotozoan agents. In the past, it has also been considered to be a viral agent, but currently the most acceptable theory is that it is more closely related to the *Rickettsia*, even though a degree of uncertainty still exists.

The intraerythrocytic *Anaplasma* body, measuring 0.3 to 1.0μ in diameter, is a small, dark blue or purplish coccoid body (using a Giemsa stain), generally located on the margin of the erythrocyte.

Larger *Anaplasma* bodies have been shown to be composed of several smaller units which are considered to be initial bodies. Reproduction of the organism is considered to be by binary fision. In the acute phase of infection, it is not uncommon to see parasitemias of 30-40% or higher with more than one body per erythrocyte. The parasitemia may drop rapidly after reaching a peak and is extremely difficult to demonstrate in carrier animals.

In addition to the typical coccoid forms including initial bodies, some workers have noted projections and ring-like forms. The projections are most

often seen with vital stains or by electron and phase microscopy. There is some uncertainty as to whether or not forms showing projections are A. marginale. Kreier and Ristic (1963) suggest that these atypical forms, while related to A. marginale, are antigenically different and have designated them Paranaplasma caudata.

History: Smith and Kilborne in 1893 described a small, deep staining, coccoid body on the erythrocyte margin which they considered a phase in the life cycle of Babesia bigemina. In 1910, Theiler recognized these marginal bodies as the causative agent of an entirely separate disease entity which he entitled anaplasmosis. He considered this organism to be a nucleus devoid of protoplasma, hence, the name Anaplasma. Theiler recognized a second disease entity caused by a centrally located, coccoid body, resembling A. marginale except for its central location in the erythrocyte, which he named A. marginale (variety centrale), now known and recognized as A. centrale. Anaplasma centrale is not an important animal pathogen due to the mild response associated with such infections, and has been widely used in South Africa as a premunizing vaccine against A. marginale.

Signs: The clinical characteristics of anaplasmosis are influenced by the host's age, virulence of the particular Anaplasma isolate, and to some extent, by the size and route of the infecting exposure. Calves less than a year of age are generally more resistant and in the absence of blood examinations, infections often go unnoticed. Recognizing considerable animal variation, increased age is generally associated with progressively more severe infection up to 3 or 4 years of age. Acute infections may occur in cattle 2 to 3 years of age, but severe infections are more common in older animals. Peracute, highly fatal infections commonly occur in splenectomized calves.

Infection is associated with a rise in body temperature, followed by progressive anemia associated with Anaplasma parasitemia, and may terminate as a fatal hypoxia. Most clinical signs are associated with the effects of anemia and erythrocytic destruction, and are usually characterized by pale mucous membranes, jaundice, weakness, and rapid breathing especially after exercise. Loss of weight and general condition is common.

Cattle with packed cell volumes (PCV) as low as 14-18% may show few, if any, signs of infection if viewed in a pasture under resting conditions. Frequently, when clinical signs are sufficiently severe to be noticed, the animal may be near death.

Incubation Time: This is extremely variable and depends largely on the size and route of the infecting inocula. Under field conditions, it will generally range from 16 to 40 days. Under laboratory conditions, using massive inoculations of infected blood, the incubation time can be shortened to as little as 2 to 3 days, and by using very small inoculums increased to as long as 60 to 80 days.

Pathologic Change:

Pathogenesis: Based on a recognizable parasitemia of 1% or over, the course of acute infection, while variable, may extend 4 to 14 days. Clinically, the course of infection may appear to be only a few days due to the lack of visible signs, which are dependent on a high parasitemia and a low PCV. When death does not occur, a rapid disappearance of the A. marginale parasitemia usually occurs, but recovery of condition and outward appearance may be more protracted. A persistent carrier infection usually occurs following recovery. Relapses may occur but they are generally less severe and usually will not cause signs of clinical illness.

The anemia of Anaplasma appears to be the result of an accelerated

removal of erythrocytes by macro-phages of the reticuloendothelial system, involving an auto immune response. The bile pigments resulting from this destruction are transported and excreted as urobilinogen by normal routes but at an accelerated rate. Hemoglobinuria does not occur.

The erythroid elements in the bone marrow of calves infected with anaplasmosis do not change significantly until the anemia becomes severe, at which time they become more prominent. The myeloid:erythroid ratios of the bone marrow decreases as the disease progresses. A rapid influx of immature erythrocytes in the circulating blood is generally favorable. Death may occur if the infection overwhelms the animal before the erythropoietic system can respond.

Postmortem Lesions: In acute anaplasmosis, there is an acute anemia which is characterized by pallor and icterus. The spleen is usually enlarged; the liver is enlarged with rounded edges, with evidence of icterus common. The gall bladder may contain dark and viscous bile. The epicardium may show evidence of petechial hemorrhages. Petechiation and edema of lymph nodes are common findings. There are no characteristic pathologic changes attributable to *A. marginale* in chronic carrier infections.

Microlesions: Depending on the length of infection before death, varying degrees of hyperplasia of the bone marrow is noted, along with extra medullary hemopoiesis in the spleen and liver. Marked reticuloendothelial and lymphoid hyperplasia occur in the lymph nodes, spleen, and tonsils. Perivascualr lymphatic infiltration occurs in many organs, e.g. liver, kidney, and lungs.

Diagnosis:

In the Field: Acute cases of anaplasmosis are characterized by pale mucous membranes, rapid breath, weakness, muscle tremor, a yellow tinge on

mucous membranes, and the availability of vectors. A firm diagnosis of anaplasmosis requires laboratory confirmation, based on finding Anaplasma bodies on Giemsa-stained blood smears or by a positive response to available serologic tests. There are usually no recognizable signs of chronic anaplasmosis, and diagnosis of this type infection is dependent on serologic means.

A number of serologic tests are available for the diagnosis of anaplasmosis. The oldest and still a standard is the complement-fixation test (CF). A capillary-tube agglutination test is available and in recent years a card agglutination test has been developed. Both of these procedures compare favorably with the CF test, all showing a high degree of correlation. The card agglutination test was developed primarily for field use and can be conducted on the farm within minutes of taking the blood sample. It can be conducted with serum or non-inactivated plasma and a stained antigen, which when combined shows specific agglutination when infection is present.

Differential: In tropical areas, anaplasmosis may be confused with babesiosis, leptospirosis, trypanosomiasis, theileriosis, or other hemotropic infections which may result in an anemic syndrome. It is not uncommon for cattle in tropical zones to be carrying two or more blood parasitic infections including anaplasmosis. Babesia parasitemias are generally more difficult to demonstrate than Anaplasma, but when present are distinctive. Hemoglobinuria occurs with babesiosis and leptospirosis, but is not seen with anaplasmosis. Elevated temperatures are a more consistent feature of babesiosis, even though a similar response is possible with anaplasmosis. The incubation time is considerably shorter in babesiosis. Anemia, while occurring in trypanosomiasis and theileriosis, is generally not as severe as in anaplasmosis and babesiosis. Young animals are more

resistant to anaplasmosis; whereas, age is not so much a factor with babesiosis, and calves are more severely affected with leptospirosis than are older animals. Identification of the hemo-parasite on Giemsa-stained blood smears or a positive serologic test response is essential for definite diagnosis.

Prognosis: In cattle over 3 years of age, the prognosis should be guarded; whereas, in calves and yearlings, recovery is more frequent. Non-apparent carrier infections may persist for the life of the animal following recovery.

Epizootiology:

Geographic Distribution: Anaplasmosis is present in most tropical and sub-tropical areas of the world and is also found in many temperate zones of North America. The distribution is generally limited to those areas having adequate vectors, but the wide variety of transmitting agents results in extensive distribution.

A recent survey in Colombia in South America showed a marked correlation between infection and altitude. Altitude influenced mean temperatures, hence, the presence or absence of vectors, to the extent that at elevations of 2600 meters or more anaplasmosis was not encountered in mature cattle even in tropical latitudes.

Transmission: A large variety of arthropods may serve as transmitting agents. These vectors in some instances are true biological carriers, and in others transmission occurs by mechanical means. Any transfer of infected blood to susceptible cattle, even in minute amounts, can produce infection.

There are at least 20 species of ticks that have been shown to transmit infection, including *Boophilus*, *Hyalomma*, *Rhipicephalus*, *Dermacentor*, *Ixodes*, and *Haemaphysalis* ticks; and at least 10 species of biting flies

(mostly Tabanids) have been shown capable of transmitting anaplasmosis. It is possible that other biting insects may be involved in transmission. It has been suggested by Roberts (1968) that Hippelates flies (eye gnats) may play a role in anaplasmosis transmission.

In the United States, the biting insects are thought to be the principle vectors in the Gulf coast and southeastern states; Dermacentor andersoni is probably the most important vector in the intermountain areas; Dermacentor occidentalis is a major vector in the Pacific coast areas of California. In tropical South America, the Boophilus ticks apparently play a major role in transmission. In Africa and Asia, the Boophilus and Rhipicephalus ticks are important vectors.

It is reported that transmission of anaplasmosis by biting flies will occur only within a few minutes after a blood meal has been obtained from an infected animal. The situation is much different with ticks, as the organism can maintain itself in this host for an extended period of time. Transstadial and transovarial transmission occur with tick vectors.

Hosts: The principle host for anaplasmosis is cattle. Sheep, goats, and white-tailed deer (Odocoileus virginianus) can be infected artificially, but it is doubtful if these animals play a major role in the maintenance of infection in a given area. Black-tailed deer (Odocoileus hemionus columbianus) are carriers of anaplasmosis in nature and act as a reservoir of infection to cattle in California. The mule deer is also naturally infected, but its role in the epidemiology of anaplasmosis is not fully known.

Anaplasma marginale has been isolated from elk, buffalo, and several African antelope. It is not recognized as an economically important disease in animals other than the bovine.

Control and Eradication:

Preventive Measures: Insect control and the segregation or elimination of infected animals will prevent transmission and effectively limit the infectious agent, thus, reducing animal losses. Serologic tests are useful in identifying carrier animals. These measures are not successful in those areas of tick vectors and where reservoirs of infection are present in wild animals. In these areas a practical plan for eradication has yet to be developed.

Treatment: In acute anaplasmosis, success is frequently dependent on early recognition of infection and prompt treatment. The tetracycline compounds given early in the course of infection have been shown to be effective when administered intravenously or intramuscularly at a rate of 3 to 5 mg. per pound of body weight. Good nursing and supportive treatment, including blood transfusions, are helpful. In recent years, two additional chemotherapeutic agents have been recognized as being effective against anaplasmosis, Gloxazone* (alpha-ethoxyethylglyoxal dithiosemicarbazone) and Imidocarb* (3,3'-Bis(2-imidazolin-2-YL)-carbanilide dihydrochloride). Gloxazone is effective at 2 to 5 mg. per kg. intravenously, and Imidocarb at 5 mg. per kg. intramuscularly or subcutaneously.

In recent years, the use of treatment to eliminate carrier infections has become an integral part of control and eradication programs. Tetracyclines, administered orally at the rate of 5 mg. per pound daily for 60 days, has been reported effective in eliminating carrier infections. Carrier infections are also reportedly eliminated by 10 to 15 daily I.M. or I.V. injections of 5 mg. per pound tetracycline. If carriers in a herd are recognized by serologic means and treated, infection can, theoretically, be

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*Burroughs Wellcome and Co., Inc., Raleigh, North Carolina.

eradicated with no further hazard of transmission within the herd. This test and treatment approach has been successfully employed in a few instances.

Experimental evidence is available to suggest that Gloxazone given with oxytetracycline is more effective than either drug alone in eliminating carrier infections. Imidocarb in combination with Gloxazone, or administered alone, has been effective in eliminating carrier infections. Roby (1972) has reported the successful elimination of carrier infections by the subcutaneous or intramuscular injection of 5 mg. per kg. Imidocarb given 2 times at a 14-day interval.

Control measures based on diagnosis and treatment are particularly effective in areas where the principal vectors are biting flies and where transmission is primarily mechanical. Where biological vectors occur, such as the Dermacentor, Boophilus, and Rhipicephalus ticks, and where there are known wildlife reservoirs, the problems of control and/or eradication are greatly compounded.

Immunization: Soon after the identification of A. marginale by Theiler in 1910, he discovered a second agent, now referred to as A. centrale, which produces a mild infection and a high degree of protection against virulent A. marginale. This organism is still being used successfully in Africa and Asia as an immunizing agent against anaplasmosis. It has not been used in North America for this purpose.

A partial immunity has been produced, using killed vaccines which consist primarily of Anaplasma bodies derived from infected erythrocytes added to oil adjuvants. A commercially available vaccine, using two subcutaneous injections 4 to 6 weeks apart, claims to reduce the severity of infection and prevent death loss due to anaplasmosis. There is recent

evidence to suggest that the use of this vaccine is occasionally associated with a hemolytic anemia affecting calves born to dams previously vaccinated.

An attenuated live vaccine has been developed which, like A. centrale, produces a mild infection, but has the advantage of greater antigenic similarity to A. marginale from which it was derived. Cattle immunized with this agent are protected from challenge with a virulent A. marginale isolate of common origin.

Experimental evidence is available which suggests the presence of antigenic differences in various Anaplasma isolates. During experiments in South America, both the killed adjuvant vaccine and the live attenuated vaccine were overwhelmed by natural exposure to the local Anaplasma. This may have been due to antigenic variants, to the size of the challenge infection, or factors presently unknown.

Both A. centrale and the attenuated A. marginale depend on premunition for their protective potential. Premunition can also be induced by virulent A. marginale. Calves in enzootic zones exposed to vectors carrying Anaplasma frequently become infected early in life and because of their natural resistance may recover without apparent illness. Such infections may persist for life, resulting in an Anaplasma resistant animal. Older cattle may become premunized with virulent A. marginale, but in the absence of treatment to reduce the severity of infection, this procedure is hazardous. Cattle premunized with virulent Anaplasma show the greatest resistance to later field challenge, and for this reason therapeutic procedures aimed at reducing the severity of infection without eliminating infection are useful.

LEUCOCYTOZOONOSIS

Protozoan parasites belonging to the genus Leucocytozoon (L. smithi, L. simondi, L. caullery) are members of the hemosporidia and occur in the blood of many species of wild and domestic birds. Heavy losses of wild ducks, turkeys, and grouse have been attributed to these organisms.

The causative organism undergoes schizogony in the endothelial and parenchymatous cells of the liver, heart, and kidney of the infected host. Gametocytes occur in the circulating blood and the infected erythrocytes become grossly distorted, being elongated with pointed ends and may assume a spherical form. No pigment is produced. The vectors are gnats or black flies (Simulium). A sexual cycle occurs in the vectors.

Young birds more often show acute signs of infection. These consist of anorexia, emaciation, debility, incoordination, and sometimes evidence of central nervous system involvement. Death may occur in 2 or 3 days. Infections are generally less acute in older birds. Birds that recover generally remain as carriers.

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BESNOITIOSIS
(Globidiosis)

Besnoitia besnoiti is usually transmitted by ingestion, but it has been reported to have been mechanically transmitted by *Glossina palpalis*. The parasite is known to occur in South Africa, Congo, Sudan, Angola, southern France, and Portugal. Cattle are primarily infected. The parasites measuring 5μ - 9μ X 2μ - 5μ are found in the dermis, subcutaneous tissues, and fascia and in the laryngeal, nasal, and other mucosae. Cysts may develop measuring up to 600μ in size in cattle.

As the disease progresses, hair falls out, the skin thickens and may crack with exudation of blood. Mortality is usually less than 10%, but the animals lose condition, pregnant cows may abort, bulls may become sterile, and hides become valueless. Premunition follows infection. At present there appears to be no specific treatment.

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APPENDIX 2
(Babesiosis)

Microbiological Procedure:

Babesia parasites from blood or internal organs (spleen, liver, brain capillaries, lymph nodes). Giemsa staining technique.

Histological Examination:

Multicentric hematopoietic hyperplasia and metaplasia, hepatic centrilobular necrosis. Lesions of intravascular hemolytic anemia. Lymphoid hyperplasia, hemosiderosis, erythrophagocytosis, hyaline degeneration, decrease of myeloid:erythroid ratio, perivascular lymphocytic infiltration, cerebral and cerebellar congestion and edema. Erythroid hyperplasia and parasites in erythrocytes.

Serologic Tests:

Complement fixation, agar gel precipitation, capillary agglutination, direct and indirect hemagglutination, direct and indirect fluorescent antibody and agglutination tests.

Animal Inoculation:

Laboratory animals are refractory to infection with bovine *Babesia spp*. Cattle inoculation with blood, tissue of infected animal is successful for inducing artificial infection.

APPENDIX 5
(Babesiosis)

Description:

Febrile, tick-borne disease characterized by anemia, icterus, and hemoglobinuria.

Cause and Distribution:

Babesiosis has a worldwide distribution related to the presence of *Boophilus spp.* of ticks in warm climates and to *Ixodes spp.* in temperate climates.

Host:

Domestic and wild animals naturally susceptible to *Babesia spp.*

Signs:

Fever, severe hemolytic anemia, icterus, hemoglobinuria, weakness, weight loss, and poor condition followed by death.

Pathology:

Severe emaciation. Blood thin and watery. Spleen, liver, lymph nodes enlarged and edematous. Multicentric hematopoietic hyperplasia and metaplasia of bone marrow. Perivascular lymphocytic infiltration of the cerebral capillaries.

Diagnosis:

Clinical signs and pathology. Presence of *Babesia spp.* in blood smears or demonstration of complement fixing, precipitating, or agglutinating antibodies in sera of tested animals. Transfer of blood to a susceptible splenectomized animal.

Incubation Time:

From 5 to 15 days, but depends on the *Babesia spp*. involved.

Mode of Transmission:

Under natural conditions transmission is carried out by ticks of the *Genera Boophilus*, *Ixodes*, and *Rhipicephalus*.

Period of Communicability:

Cyclical in tick to tick; transstadial in larvae, nymph, and adults. Transovarial or hereditary from ova to larvae of ticks. Cattle may remain carriers for a period of years.

Control Measures:

Control of ticks. Premunition - coinfectious immunity with *Babesia* infective blood. Vaccination - sterile immunity with killed *Babesia* parasites. Chemotherapy with premunition - *Babesia spp*. infective blood and drug therapy a week later. Chemoprophylaxis.

APPENDIX 2
(Anaplasmosis)

Microbiological:

Anaplasma marginale bodies in Giemsa-stained blood smears.

Histological Examination:

Hyperplasia of bone marrow, evidence of extra medullary hemopoiesis in spleen and liver, and reticulocytosis. *Anaplasma* parasitemias in acute cases.

Serologic Tests:

Complement fixation, capillary-tube agglutination, and card agglutination tests commonly used for diagnosis. Other tests have been described.

Animal Inoculation:

The inoculation of susceptible cattle with blood from acute or chronic infections readily produces infection. Sheep and deer can also be infected experimentally. Laboratory animals are refractory to infection.

APPENDIX 5
(Anaplasmosis)

Description:

An acute or chronic infectious blood disease of cattle characterized by a severe hemolytic anemia in acute cases, and transmitted in nature by a variety of blood sucking arthropods.

Cause and Distribution:

Anaplasma marginale. North and South America, Asia, Africa, Australia, and southern Europe. Presence of the disease is largely dependent on the availability of insect vectors capable of transmission.

Host:

Primarily cattle. Deer, elk, buffalo, sheep, and goats can be infected.

Signs:

A severe hemolytic anemia with the resultant shortness of breath, weakness, pale mucous membranes, icterus, and fever. Signs of infection are more severe in older animals. When death does not occur, weight loss and poor condition follow infection.

Pathology:

Low erythrocytic counts, low hemoglobin, and low packed cell volumes. Pallor, icterus, enlarged spleen and liver and other lesions related to the anemia. Evidence of bone marrow hyperplasia.

Diagnosis:

A febrile disease with evidence of acute anemia in an area of arthropod vectors is suggestive of anaplasmosis. Presence of *Anaplasma* bodies in blood smears or a positive serologic test for anaplasmosis is diagnostic.

Incubation Time:

Highly variable but under natural conditions it usually is 21-49 days.

Mode of Transmission:

A wide variety of arthropods. Biting flies and ticks are the most important vectors. Flies are mechanical vectors; ticks may be mechanical or biological vectors with transmission being transstadial or transovarial.

Period of Communicability:

Following recovery of acute anaplasmosis, cattle may remain carriers for a period of years and possibly the life of the animal.

Control Measures:

Vector control, identification of carrier animals with their removal from the herd, or by treatment to eliminate infection. Vaccines are present to reduce the severity of acute infection, but they allow the development of chronic carriers.

Techniques of Premunization for the Control of Anaplasmosis

K. L. Kuttler and R. A. Todorovic

TECHNIQUES OF PREMUNIZATION FOR THE CONTROL OF ANAPLASMOSIS

K. L. Kuttler and R. A. Todorovic

Anaplasmosis premunition, or co-infectious immunity, represents an immune or resistant state in the absence of clinical evidence of disease brought about by the presence of a chronic or carrier infection. This immunity persists as long as the infectious organism remains present. The use of premunization for the prevention of acute episodes of anaplasmosis has been known for almost as long as the disease has been recognized. Theiler[11] in 1911, only a year after his first publication concerning *Anaplasma marginale*,[10] described the use of *Anaplasma centrale* to induce premunition and a resistance to *A. marginale*. The subsequent use of *A. centrale* as a premunizing vaccine was widespread throughout much of Africa and Asia. Ristic[7] suggests that premunition is the only effective means for producing a practical and effective immunity to anaplasmosis.

In 1937, Schmidt[8] described the use of field isolates of *Anaplasma* in the U.S. for premunizing cattle against anaplasmosis, which consisted of injecting blood from known carriers into susceptible cattle. He reported a 1.3% mortality from this procedure in over a 1,000 cattle. These infections were induced during the cool season and in cattle less than 15 months of age.

Franklin and Huff[3] proposed a method of premunization with small inocula in which only moderate response occurred but protection was induced. Protection from subsequent exposure is generally good against antigenically similar anaplasms. The existence of antigenically different organisms in South America has been suggested by Kuttler and Zaraza.[5] In these instances, however, premunition does appear to produce a degree of cross protection.

Premunition is contraindicated in areas where the disease is sporadic and treatment or management programs offer the hope of control and/or eradication. In endemic areas of the tropics, premunization is often considered the only effective means for control. Field premunition by natural vectors is known to occur at an early age in many areas.[4] Because of the innate resistance of young animals, recovery from such infection is usual, with the resultant premunition preventing future acute infections. The introduction of Anaplasma-free adult cattle into such areas is often associated with severe losses, unless some form of immunization or treatment is practiced.

During the past few years, experimental data has been accumulated from Africa, South America, and the U.S. on the premunization of cattle of various age groups, using several different techniques. Generally these practices have been successful, but experience shows the continued need for care in this procedure. The intent of this paper is to review and report data involving premunization of both calves and adult cattle and the response of these animals to challenge with virulent Anaplasma.

MATERIALS AND METHODS

Premunization response using varying methods was measured in 129 cattle. A total of 78 adult cattle 18 months of age and older of varying breed, predominantly European type (Bos Taurus), were premunized, using 4 different Anaplasma isolates in selected groups (Tables 1 and 2). Two of these anaplasms represent attenuated organisms and 2 are fully virulent field isolates. The fully virulent organisms were used with and without treatment. Treatment

consisted of 4A65* (Imidocarb), 356C61** (Gloxazone), and oxytetracycline*** (Liquamycin). Premunization consisted of injecting subcutaneously (S/C) 2-5 ml fresh blood from carrier animals, except where the attenuated A. marginale (AAM)[+ 7,12] was used and in most instances this organism was obtained from frozen inocula which was thawed and injected in 5 ml amounts S/C.

Nine cattle averaging 7.3 years of age were inoculated with virulent A. marginale isolated in Texas (TAM). This organism has been repeatedly passaged in experimental cattle. These 9 cattle were not treated in any way to moderate infection. Six young adults averaging 1.5 years of age were premunized with TAM and then treated with 4A65 at the level of 4 mg/kg administered S/C 31 days after exposure. Six cattle averaging 3.8 years of age were premunized with TAM and then treated with 356C61 at the level of 5 mg/kg given I/V 25 days after exposure (Table 1).

A group of 23 cattle from Anaplasma-free areas, averaging 1.5 years of age were each premunized with a virulent A. marginale isolate from the north coast of Colombia (Table 1). Thirteen of the 23 were treated with 12 mg/kg of oxytetracycline 9 times beginning 21 days after exposure and being repeated on days 41, 45, 46, 47, 48, 49, 50, and 51. Five were treated with 5 mg/kg 356C61 given 7 days after exposure. An additional 5 were treated with 5 mg/kg 356C61 given 7, 28, 29, and 30 days after exposure.

A regression correlation analysis was made of 20 adult cattle listed in Table 1 on which exact ages were available to determine the possible influence

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*4A65: Imidocarb - 3,3'-Bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride (or dipropionate) - Burroughs Wellcome Company, Inc.

**356C61: Gloxazone - Alpha-Ethoxyethylglyoxal Dithiosemicarbazone - Burroughs Wellcome Company, Inc.

***oxytetracycline: Liquamycin - Chas. Pfizer & Company, Inc.

+Diamond Laboratories

of age on the severity of infection in these particular animals. Even though a range in age from 1 year to 13 was observed, no statistically significant correlation between the percent of normal PCV at the height of infection and age was detected.

In Table 2 the effects and efficacy of premunizing 34 cattle with attenuated anaplasms is compared with the group of 9 TAM infected cattle previously listed in Table 1. Fourteen cattle averaging 2 years of age were premunized with A. centrale (AC). No treatment was given to moderate the resulting infection. All cattle were subsequently exposed within 3 months of premunization by the inoculation of blood containing a virulent A. marginale. Fifteen cattle averaging 5.6 years of age were premunized with AAM. No treatment was given after premunization. All cattle were subsequently challenged within 3 months of premunition by the inoculation of 5 ml blood containing a virulent A. marginale. An additional 5 cattle averaging 1.5 years of age were premunized with AAM and exposed to a field challenge on the north coast of Colombia.

A group of 29 young intact calves was premunized with 3 different Anaplasma isolates (Table 3). No treatment was used in these premunization attempts. Twelve calves averaging 3.5 months of age were premunized with TAM, and 5 were later subjected to field challenge on the north coast of Colombia. Six calves averaging 2.3 months of age were premunized with AC, and all were later challenged by the inoculation of blood containing virulent A. marginale. Eleven calves averaging 5.2 months of age were premunized with AAM, and 2 were later exposed to field challenge, and 3 were inoculated with blood containing virulent A. marginale. A total of 17 calves were actually injected with the AAM infected blood which had been frozen, but only 11 or 65% showed evidence of a premunizing infection. Six or 35% failed to develop evidence of infection as characterized

by a complement fixation response and/or an Anaplasma parasitemia. These calves were not included in our tables because in effect no premunization occurred.

Schmidt[8] recommended premunization in the winter and early spring, during cool weather. The suggestion was made that Anaplasma reactions were less severe at this time. Franklin[2] observed greater parasitemias in antigen production cows when the temperature was increased. During the course of our studies we have had occasion to premunize adult cattle and intact calves at both Texas and Colombia when there was considerable variation in mean ambient temperature. Table 4 presents a comparison of the reactions produced by AAM in adult cattle in Texas and those in Colombia. The source of the AAM in both instances was Diamond Laboratories. Seven cattle with an average age of 3.6 ±2 years were premunized on the Bogota savannah where the mean temperature was 13° C. Eight cattle with an average age of 7.2 ±2 years were premunized at College Station in August when the mean temperature was 29.4° C. In addition, 4 intact calves were premunized in Colombia and 7 in Texas (Table 4).

In an effort to further characterize the apparent temperature response on the course of anaplasmosis, the course of infection was carefully followed in 14 splenectomized calves, one-half of which were exposed to summer heat and the other half maintained in an air-conditioned room. The mean temperature of those exposed to summer conditions was 30.0° C.; whereas, the mean temperature in the air-conditioned stall was 18.5° C. All calves were inoculated with the TAM organism and the course of infection observed for a period of over 60 days (Table 5).

A group of 12 intact calves averaging 5.2 months of age were premunized with TAM and used to monitor the effect of oxytetracycline treatments in moderating the premunizing infection. Four calves were treated with oxytetracycline

at the rate of 5 mg/lbs I/M 14 days after TAM exposure. Four calves were treated in the same manner 21 days after exposure, and 4 remained as untreated controls (Table 6).

The comparative response to virulent A. marginale challenge imposed on 11 splenectomized calves previously premunized with an A. marginale isolate of reduced virulence (not AAM)* and A. centrale was measured and recorded in Table 7. Seven splenectomized calves premunized with A. centrale and 4 splenectomized calves premunized with a mild A. marginale were challenged with virulent A. marginale by the inoculation of 5 ml infected blood. Response to this challenge is recorded in Table 7.

Packed cell volume (PCV), parasitemia, complement fixation (CF) response, and the duration of immunity were the parameters recorded in tables to measure the severity of infection. The PCV is expressed as the low percent of normal occurring during the primary infection. If the pre-infection PCV normal was 32% and the low PCV during the primary Anaplasma infection was 12% then the percent of normal would be 12/32 or 37.5%. Persistence of anemia represents the time in days that the PCV remains lower than 75% of the pre-infection normal. The response to challenge was expressed as a severe response (SR) where the low PCV was 50% or less of normal, a moderate response (MR) where the low PCV was 50-75% of normal, or no response (NR) where the low PCV was 75-100% of normal.

Serum samples were tested for evidence of specific complement-fixing antibodies, using modification of the USDA (1958) technique.[1] Average CF titers were obtained using a logrithmic transformation of the dilution factor. An analysis of variance was conducted for the establishment of significance.[9]

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*Obtained in 1964 from T. O. Roby, Beltsville, Maryland.

RESULTS

Results tabulated in Table 1 show relatively severe reactions occurred in adult cattle premunized with TAM, with 1 of 9 cattle dying. Treatment with 4A65 and 356C61 were generally successful in moderating the severity of infection. The PCV, parasitemia, and persistence of anemia were significantly different reflecting the value of treatment. Premunization of young adult cattle with NCAM produced a severe response when attempts were made to moderate infection with oxytetracycline. The other 2 groups in which 356C61 was used showed significantly more moderate infections.

The attenuated anaplasms tabulated in Table 2 resulted in moderate infections which were significantly milder than the control TAM infections. No significant difference could be detected between AC and AAM infections, except for the lower CF titers with AC infections. Challenge infection induced on the AC carrier animals resulted in 3 moderate reactions, but 11 showed no response. A TAM challenge imposed on 15 AAM premunized cattle failed to produce any evidence of renewed Anaplasma reaction. Animals with AAM infections exposed to a field challenge on the north coast of Colombia developed 3 severe reactions, 1 moderate response, and 1 showing no response.

A similar comparison of TAM, AC, and AAM infections induced in intact calves is recorded in Table 3. A trend toward more moderate reactions with AC and AAM was noted but the only significance was in the persistence of anemia. The anemia persisted significantly longer following TAM premunization than was observed in AC infections. An A. marginale challenge given 6 AC premunized animals resulted in 4 showing no response, 1 a moderate response, and 1 a severe response. A similar challenge administered to 3 calves having an AAM infection failed to produce any signs of a challenge response. Five TAM premunized calves when exposed to a north coast (Colombia) field challenge showed a moderate

response in 3 and no response in 2. A similar challenge of 2AAM premunized calves resulted in 2 moderate reactions.

Significant differences in the reaction of adult cattle premunized with AAM at Bogota, Colombia and College Station, Texas was detected. There was a time lapse of about 2 years between the time this work was done in Colombia and when it was done in Texas, but the organism used had the same origin. The cattle in Colombia were mostly Angus while in Texas they were predominantly grade Hereford cattle. The cattle used in Texas were somewhat older but correlation regression analysis plotting the low PCV response against age failed to show a significant correlation. The 2 most outstanding differences are the altitude and temperature. Premunization was carried out in Texas in August at about sea level when the mean temperature was 29.4° C. In Bogota, with an altitude of 2,600 M., the mean temperature was only 13° C. A significantly more severe response occurred in Texas even though the average reactions were considered moderate. All 15 cattle as seen in Table 3 were solidly immune to TAM challenge. Similar differences in response were noted among intact calves but they were not so marked and failed to reach significance.

The attempt to duplicate this finding using 14 splenectomized calves was generally unsuccessful as indicated in Table 5. There were no significant differences between PCV response to TAM infection of calves kept at 18.5° C and those held at approximately 30° C. A trend of more severe response was noted in those calves held at 30° C but the differences were not significant.

The administration to intact calves of 11 mg/kg oxytetracycline 14 and 21 days after exposure to virulent Anaplasma failed to significantly alter the course of infection (Table 6), which was extremely mild in the controls.

A virulent A. marginale challenge of 7 splenectomized calves premunized with A. centrale resulted in 5 showing a severe response, 1 a moderate response,

and 1 no response. Similar challenge of 4 calves premunized with a mild A. marginale resulted in 2 showing a moderate response, with 2 showing no response to challenge (Table 7).

CONCLUSION & DISCUSSION

Premunization of adult cattle with fully virulent A. marginale is feasible when the infection is moderated by appropriate drug therapy. Both 4A65 (Imidocarb) and 356C61 (Gloxazone) have proven effective. Under the conditions of these trials oxytetracycline was relatively less effective than Imidocarb and Gloxazone for this purpose; however, only 2 trials with oxytetracycline were attempted.

From a time and economy point of view, the attenuated anaplasms offer more promise as premunizing agents. Needle challenge with a virulent Texas isolate (TAM) showed complete protection in cattle previously premunized with the AAM strain. Protection against field challenge was not complete, even though a degree of protection was apparent. It is recommended that controlled studies be made to characterize possible antigenic differences between the organism being used for premunization and the locally occurring A. marginale isolate, whenever possible.

Premunization of calves less than 6 months of age can probably best be accomplished using a virulent A. marginale which has first been characterized as to its virulence. An isolate such as is present at Texas (TAM) produced only a moderate response in intact calves. It is probable that some field isolates might be excessively virulent for use even in young animals. The AAM organism that has been frozen is often too mild or too low in titer to produce consistent premunization in intact calves. This observation was prompted by a failure of 6 of 17 calves (35%) to develop evidence of premunizing infection

following their inoculation with this material. The injection of freshly collected blood from carrier animals infected with either AC or AAM appeared more satisfactory for premunizing purposes.

The observations of Schmidt[8] and Franklin[3] relative to the influence of ambient temperature on the severity of infection appeared to be confirmed when reactions produced by AAM in Colombia were compared to reactions in Texas. Our attempts to reproduce this effect in splenectomized calves was unsuccessful but an insufficient range of temperature differences may not have been present. Three factors which could contribute to the differences noted between Colombia and Texas are altitude, temperature, and possible strain (AAM) differences occurring during the time interval between trials.

The greater antigenic similarity of a mild A. marginale (MAM) to virulent A. marginale than A. centrale is quite obvious in the results of Table 6. For this reason attenuated or mild A. marginale isolates would have distinct advantages over A. centrale as premunizing vaccines.

SUMMARY

Attempts at Anaplasma premunization in varying age groups have been reported using a virulent A. marginale of Texas origin, a virulent A. marginale of Colombian origin, an attenuated A. marginale, and A. centrale. Results of premunization response and the response to field and artificial challenge have been reported.

Premunization is a practical approach for the prevention of clinical losses associated with anaplasmosis, but a series of variables must be considered if success is to be achieved. Some of these factors are: (1) age of the animals being premunized, (2) virulence, potency, and size of the premunizing inoculum, and (3) the strain, or size, of the expected challenge

exposure. In some instances, the use of a highly virulent A. marginale in adult cattle resulted in overly severe reactions even with treatment. Gloxazone (356C61) and Imidocarb (4A65) were superior to oxytetracycline in moderating the premunizing infection. Attenuated strains of A. marginale when used in young intact calves failed to produce the desired premunizing effect; however, they were very effective in adult cattle. Premunization is dependent on establishing an active infection, and in the absence of such infection, susceptibility to field or experimental challenge occurs.

Successful premunization resulted in almost complete protection following challenge with antigenically similar A. marginale isolates. Protection was only partial, however, in instances where heterologous challenge was encountered.

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TABLE 1

Premunization of Adult Cattle With 2 Virulent *Anaplasma* Isolates Modified by Treatment

Anaplasma Isolate Injected	No. of Animals	Est. Avg. Age	Low PCV % of Normal	Avg. High Paras.	Average High CF Response	Persistence of Anemia	Treatment
		years				days	
TAM	9*	7.3	42 ±10	14 ±8	1:508	33 ±16	No Treatment
TAM	6	1.5	63 ±5	2 ±1	1:202	10 ±4	4A65(1)
TAM	6	3.8	64 ±15	8 ±6	1:452	11 ±8	356C61(2)
Significance			P<0.01	P<0.01	NS	P<0.05	
DRS			14.6	8.6		18.2	
NCAM	13	1.5	43 ±11	6 ±5	1:465	30 ±15	Oxytet.(3)
NCAM	5	1.5	71 ±7	.4 ±.2	1:1280	6 ±6	356C61(4)
NCAM	5	1.5	72 ±6	.4 ±.1	1:139	13 ±14	356C61(5)
Significance			P<0.01	P<0.05	P<0.05	P<0.01	
DRS			13.4	5.2	1:139 lower than 1:1280 only	19.0	

TAM - virulent *Anaplasma*, Texas isolate

NCAM - virulent *Anaplasma*, north coast of Colombia isolate

(1) 4A65: 4 mg/kg given I/M 31 days after exposure

(2) 356C61: 5 mg/kg given I/V 25 days after exposure

(3) Oxytetracycline: 12 mg/kg given 9 times on days 21, 41, 45, 46, 47, 48, 49, 50, 51

(4) 356C61: 5 mg/kg given I/V 7 days after exposure

(5) 356C61: 5 mg/kg given I/V 7, 28, 29, and 30 days after exposure

DRS - Difference Required for Significance

NS - Not Significant

*1 cow premunized without treatment died

±: Standard Deviation

TABLE 2

Premunization of Adult Cattle With a
Virulent and 2 Attenuated Anaplasms Not Modified
By Treatment, But Exposed to Heterologous Challenge

Anaplasma Isolate	No. of Animals	Est. Avg. Age	Low PCV % of Normal	Avg. High Paras.	Average High CF Response	Persistence of Anemia	Response to Challenge		
							NR	MR	SR
		years				days			
TAM	9*	7.3	42 ±10	14 ±8	1:508	33 ±16	-	-	-
AC	14	2.0	72 ±14	4 ±4	1:131	8 ±9	11	3	0
AAM	15	5.6	74 ±16	3 ±4	1:305	8 ±12	15	0	0
AAM	5	1.5	76 ±4	1.5±.8	1:907	2 ±3	1**	1**	3**
Significance			P<0.01	P<0.01	P<0.01	P<0.01			
DRS			16.0	6.0	1:131 lower than 1:508 1:907	14.5			

TAM - virulent *Anaplasma*, Texas isolate

AC - *Anaplasma centrale*

AAM - attenuated *Anaplasma marginale* (Diamond Laboratories)

NR: no response to challenge - the low PCV percent of normal being >75%

MR: moderate response to challenge - the low PCV percent of normal being 50-75%

SR: severe response to challenge - the low PCV percent of normal being <50%

* 1 of the 9 animals died of acute anaplasmosis

** subjected to field challenge on the north coast of Colombia

±: Standard Deviation

TABLE 3

Premunization of Intact Calves With a
Virulent and 2 Attenuated *Anaplasma* Isolates Followed By Challenge

Anaplasma Isolate	No. of Animals	Est. Avg. Age	Low PCV % of Normal	Avg. High Paras.	Average High CF Response	Persistence of Anemia	Response to Challenge		
							NR	MR	SR
		mos.				days			
TAM	12	3.5	63 ±11	1.0±.5	1:214	28 ±30	2*	3*	0
AC	6	2.3	67 ±21	6 ±8	1:320	5 ±8	4	1	1
AAM	11	5.2	72 ±8	1.4±2.0	1:220	6 ±6	3	2*	0
Significance			NS	NS	NS	P<0.05			
DRS						22			

TAM - virulent *Anaplasma*, Texas isolate

AC - *Anaplasma centrale*

AAM - attenuated *Anaplasma marginale* (Diamond Laboratories)

NR: no response to challenge - the low PCV percent of normal being >75%

MR: moderate response to challenge - the low PCV percent of normal being 50-75%

SR: severe response to challenge - the low PCV percent of normal being <50%

NS - Not Significant

* field challenge on north coast of Colombia
all others are needle challenges with virulent *A. marginale*

± : Standard Deviation

TABLE 4

Comparative Response to AAM Premunition
of Adult Cattle and Calves Located in Colombia and in Texas A&M

ADULT CATTLE	No. of Cattle	Avg. Age (yrs.)	Avg. Ambient Temp.	PCV Response (% of Normal)	Duration of Anemia
					days
Colombia	7	3.6±2	13 C	83.3 ±7	0
Texas A&M	8	7.2±2.0	29.4 C	66.1 ±18	15.2 ±13
		P<0.01		P<0.05	
INTACT CALVES		(mos.)			
Colombia	4	3.0±2	13 C	77.8 ±7.9	4.5 ±3.0
Texas A&M	7	6.4±3	29.4 C	68.6 ±6.0	7.7 ±6.1
		P<0.01		NS	NS

NS - Not Significant

±: Standard Deviation

TABLE 5

Influence of Temperature on PCV Following Infection of Splenectomized Calves With *A. marginale* (TAM)

	No. of Animals	Avg. Temp.	Low PCV % of Normal
Outside	7	30.0 C	30.2%
Air-Conditioned Room	7	18.5 C	33.5%

No Significant Different

TABLE 6

Moderation of Premunizing *A. marginale* (TAM) Infection In Calves By the Use of Oxytetracycline

No. of Calves	Avg. Age	PCV % of Normal	Time Required After Exposure to Reach Low PCV	Paras.	CF Response	Treatment
	mos.		days			
4	5.2	56.8 ±8	24.2 ±3	0.65 ±.4	1:190	Oxytetracycline 11 mg/kg I/M on day 14
4	5.5	60.5 ±7	21.8 ±7	0.42±0.05	1:270	Oxytetracycline 11 mg/kg I/M on day 21
4	5.0	54.8 ±3	23.5 ±7	0.58 ±.30	1:270	No Treatment
		NS	NS	NS	NS	

NS - Not Significant

±: Standard Deviation

TABLE 7

Comparative Response to Virulent *A. marginale* Challenge Imposed on Splenectomized Calves Premunized With a Non-Virulent *A. marginale* and *A. centrale*

Premunizing Infection	No. of Animals	Low PCV % of Normal Associated With Premunition	Challenge With Virulent *A. marginale*		
			NR	MR	SR
AC	7	44 ±8	1	1	5
MAM	4	40 ±11	2	2	0
		NS			

AC - *Anaplasma centrale*

MAM - a mild *A. marginale* isolate (obtained in 1964 from Dr. T. O. Roby, Beltsville, Maryland)

NR: No Response

MR: Moderate Response

SR: Severe Response

NS - Not Significant

±: Standard Deviation

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A Comparison of the Antigenic Properties of Erythrocytic *Babesia bigemina* in Acute and Chronic Blood Borne and Tick Borne Infections in Cattle

K. C. Thompson

A COMPARISON OF THE ANTIGENIC PROPERTIES OF ERYTHROCYTIC *BABESIA BIGEMINA* IN ACUTE AND CHRONIC BLOOD BORNE AND TICK BORNE INFECTIONS IN CATTLE

Evidence is accumulating as to the possible existence of variants occurring with *Babesia* species. This is analogous to trypanosome and plasmodium species in which variants do occur.

Immunological studies on *Babesia*, investigations into the epidemiology of babesiasis and the development and application of a *Babesia* vaccine must take antigenic variation into account.

This report adds more evidence to those confirming the existence of antigenic variation in *Babesia* species. A total of 71 Holstein-Friesian calves, 12 guinea pigs and 15 New Zealand White rabbits were utilized in the present study.

Experiment 1. Purification of *Babesia bigemina*.

Rapid serial passage of blood containing *Babesia argentina*, *B. bigemina*, *B. major*, and *Anaplasma marginale* through 5 splenectomized calves resulted in the purification of *B. bigemina* and elimination of the other species present. The blood containing the purified *B. bigemina* was then frozen and formed the stabilate used in the remaining experiments. The stabilate was checked for viability in an intact calf and monitored 2 months in the calf to ensure its purity.

Experiment 2. Isolation, Purification and Maintenance of *Boophilus microplus*.

Engorged adult female *B. microplus* were collected from adult Holstein-Friesian dairy cattle and allowed to deposit eggs. The eggs from each female were maintained separately so as to identify progeny of clean versus progeny from *Babesia* infected ticks. The larvae resulting from these eggs were placed on splenectomized calves known free of hemotropic diseases. Hence, the progeny were screened and labeled clean or infected. The clean larvae were allowed to

develop to adults and lay eggs and these progeny again tested. The splenectomized calves used for the third progeny testing were monitored for 3 months and after this period the third batch of larvae were designated clean (free of hemotropic diseases) and used in subsequent experiments.

Maintenance of B. microplus larvae was attempted on disease free guinea pigs with only moderate success. Immature stages and adults were also kept in desiccator jars, with ammonium chloride to regulate the percent relative humidity, in an attempt to see of it were possible to maintain them for long periods without feeding. Moderate success was attained as the external environment proved to be more economical and more efficient.

Experiment 3. Infection of B. microplus free of B. bigemina.

Approximately 20,000 uninfected B. microplus larvae were placed on each of 2 splenectomized calves. The calves were inoculated intravenously (IV) at nymphal molt with an infection dose (1×10^9 organisms) of purified B. bigemina stabilate. The incubation period of the stabilate was 5 to 7 days which coincided with the period of rapid engorgement of the adults (the last 24 hours of adult repletion).

The infected adults resulting from these calves were collected and maintained for use in subsequent experiments.

Experiment 4. Acute and Chronic Blood Borne Infection.

Five intact calves known free of hemotropic diseases were inoculated IV with an infection dose of 1×10^9 organisms of purified B. bigemina stabilate. At the time of highest parasitemia, 500 ml of blood pooled from the 5 calves was subinoculated IV into a splenectomized calf. At maximal parasitemia the calf was bled and 100 ml of infected blood was subinoculated into another

splenectomized calf. When the second splenectomized calf had a peak parasitemia, it was exsanguinated and an infective blood stabilate prepared along with an acute blood antigen, designated AB antigen.

Three months after infection these intact calves were again bled, the blood pooled and subinoculated into a splenectomized calf. At maximal parasitemia, this calf was bled and 100 ml subinoculated into a second splenectomized calf. When this calf reached peak parasitemia it was exsanguinated and another infective blood stabilate prepared along with a chronic blood antigen, designated CB antigen.

Experiment 5. Acute and Chronic Tick Borne Infection.

Twenty thousand to 40,000 infected B. microplus larvae were allowed to feed on the bodies of 5 intact and 1 splenectomized calves known free of hemotropic diseases. Acute tick (AT) antigen and chronic tick (CT) antigen and infective blood stabilates were prepared similar to that described in experiment 4 for the AB antigen and CB antigen.

Results of Experiments 4 and 5.

Tick borne infected calves showed less number of recrudescenses, greater weight loss, higher temperatures, lower packed cell volume (PCV), lower % parasitemia and lower complement fixation (CF) titers.

Experiment 6. Isolation and Purification of the Babesia Erythrocytic Antigens.

Carotid artery canulation of the second splenectomized, highly parasitized calves, experiments 4 and 5 resulted in satisfactory antigen production by the method of Mahoney (1962, 1967). Purification by means of molecular sieving (Sibinovic et al., 1968) was also attempted. The Babesia erythrocytic antigen mixtures were passed through a 2.5 by 65 cm Sephadex G-100 column and a 2.5 by

48 cm Sephadex G-200 column from which 3 ml samples were collected. These samples were then further characterized by the slide agar gel precipitation test and the slide agar gel electrophoresis (Davis, 1964; Ornstein, 1964; Todorovic, 1967).

Results.

AB antigen showed more lines of identity with rabbit anti-AT antigen (RAT) than with either rabbit anti-CB antigen (RCB) or rabbit anti-CT antigen (RCT). AT antigen showed lines of identity with RCB but no lines of identity with RCT and CT antigen showed lines of identity with RCB. No other antigen-rabbit antiserum (specific) showed lines of identity.

The passive (indirect) hemagglutination test (Stavitsky, 1954; Butler, 1963; Curnow, 1968; Campbell *et al.*, 1970) aided in further characterizing the *Babesia* erythrocytic antigens.

Results.

The highest homologous titer was AT X AT while heterologously the highest titer was AT X CT and the lowest heterologous titer was CT X AB.

The final test which was used to characterize the *Babesia* erythrocytic antigens was the CF test (Mahoney, 1962) and a microtiter procedure similar to that described by Hidalgo and Dimopoullus in 1967.

Result.

The highest titers with known positive serum were obtained with the AT antigen and the lowest with the CB antigen.

Experiment 7. Preparation of Specific Antiserum.

Three New Zealand White rabbits were used for each antigen produced for

the production of specific antiserum. A normal bovine blood antigen (NB) and rabbit antiserum were also prepared and used as controls. These antiserums were those described in experiment 6.

Experiment 8. Host Response to the Babesia Erythrocytic Blood Stabilates (Infective).

Thirty-six Holstein-Friesian calves were divided into groups of 2 or 3 and inoculated with 1 X 10^9 organisms IV and 28 days later were homologously and heterologously challenged IV with 1 X 10^{10} organisms.

Results.

The group with the highest % parasitemia up to day 28 was AB and after day 28 till day 48 was group AT that was challenged with CT. The highest CF titer was attained in group CB challenged with CB, the highest temperature recorded in group AT challenged with CT, the lowest PCV in group AT challenged with CB and the greatest weight loss in group AT challenged with CB.

Bovine Babesiosis: Its Diagnosis and Control

By

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SUMMARY

The investigation was conducted to develop new systems and to evaluate existing ones for the diagnosis and control of bovine babesiosis in Colombia, South America. Antigens of Babesia bigemina and Babesia argentina were isolated and used in the complement fixation and rapid agglutination tests for the diagnosis of babesiosis in cattle. Three systems were evaluated for the control of bovine babeiosis: (1) vaccination of susceptible cattle with killed Babesia spp. vaccine to produce resistance based on sterile immunity; (2) premunition of cattle with virulent Babesia spp., followed by chemotherapy to produce resistance based on co-infectious immunity; and (3) chemoprophylaxis based on the activity of babesiacidal compounds with prolonged residual action. All these systems were found effective in controlling bovine babesiosis under the conditions of these experiments. The epizootiological conditions of babesiosis enzootic areas will indicate which system is applicable. In zones with high incidence of Boophilus microplus, the premunition is indicated; in areas where tick population is controlled, or in areas at constant risk of tick exposure, the system of inducing resistance with killed Babesia spp. or chemoprophylaxis is indicated.

INTRODUCTION

Although bovine babesiosis is recognized as a serious economic disease of cattle for more than 80 years,[2] the practical method for diagnosis and effective control programs have not yet been achieved.[31] This hemoprotozoan malady causes a considerable production and death loss of cattle, especially in lowlands of tropical zones of the world where there are ticks.[30,33,38] The identification of the acutely infected cattle has not presented a problem because Babesia spp. can be easily detected in Giemsa stained blood films.[15,27] However, if cattle are latently infected, the diagnosis presents a difficult

problem since the blood from these animals does not contain sufficient number of Babesia spp. parasites upon which to base diagnosis.[27,30,31] Therefore, a great deal of the past interest has been directed toward development of serologic techniques for the diagnosis of bovine babesiosis.

In the last decade several serologic tests have been developed for the detection of specific Babesia spp. antibodies in the infected cattle. Complement fixation,[21,23,45] hemagglutination,[10,14] capillary agglutination,[20] fluorescent antibody,[12,17,34] precipitation in gel[45] have been reported. These serologic tests showed a high degree of specificity for each Babesia spp. tested, and due to the sensitivity differences of each technique, antibodies can be detected for varying periods after infection depending on the test used. The complement fixation (CF) test which has the lowest sensitivity may be reliable to detect antibodies to B. argentina for several months and to B. bigemina for 4 months after a single infection.[26] With indirect fluorescent antibody and passive hemagglutination techniques periods in excess of a year have been reported.[14,16,34] Because of reinfection under actual natural conditions, the loss of detectable antibodies should rarely occur after the first infection has been established.[26] All these techniques are laboratory tests; they are tedious to perform and considerable skill and equipment are required. Therefore, there is an evident need for a rapid, simple serologic test which can be applied under field and laboratory conditions for the diagnosis of bovine babesiosis.

Control of bovine babesiosis is still a problem particularly in the tropical areas of the world where this blood disease is most prevalent.[9,13,26,28,29,30,33,38] Since the natural transmission of Babesia spp. is dependent on certain species of ticks, infection can be prevented by an adequate tick control program to keep cattle free from tick infestation.[30,32] This can be accomplished by regular dipping of cattle, and this method has resulted in the eradication of bovine

babesiosis in the United States.[28] However, in the lowlands of subtropical and tropical areas of the world, a vector control program as a measure of Babesia spp. control, while appealing, is virtually impossible for various reasons.[42] Since ticks occur abundantly in permanent grasslands of the tropics and feed regularly on both wild and domestic animals, it is difficult to eradicate them. At the present time, in the countries where a tick control program is not possible, the only measure which can be used to prevent production and death losses and to control the disease is an effective vaccination or chemoprophylactic program.[4,8,22,25,30,40,41,43,44] In order to achieve this goal a series of investigations were undertaken to test and evaluate different control systems for bovine babesiosis in tropical zones of Colombia, South America, where babesiosis is most prevalent. These systems included: (1) vaccination of susceptible cattle with killed Babesia spp. vaccine to produce a resistance to disease based on the phenomenon of sterile immunity,[5,20,24,25] and (2) premunition of susceptible cattle with virulent Babesia spp. isolated from cattle acutely infected with these parasites. The post premunition reactions were controlled by specific chemotherapy. After recovery from the premunition reactions, cattle developed a resistance based on the phenomenon of co-infectious immunity.[36] In addition to these trials, attempts were made to control bovine babesiosis with (3) chemoprophylaxis. This system was based on a resistance to natural infection as a result of the action of a babesiacidal compound with prolonged residual property.[7] The protective period lasts for a few weeks, and after this time the drug level gradually decreases to the point when Babesia spp. can establish a mild infection. As a result of the interaction between drug and Babesia spp. parasites, chemoprophylactically treated cattle develop a resistance to babesiosis based on co-infectious immunity known as premunition.[44]

This work is mainly concerned with development of a more practical serologic

test for identification of Babesia spp. infected cattle and secondly with evaluation of three different systems for control of bovine babesiosis under tropical conditions in Colombia, South America.

MATERIALS AND METHODS

I. Serologic Diagnosis of Bovine Babesiosis.

A. Complement Fixation (CF) Technique: Experiments were carried out to evaluate the various serologic techniques for detection of Babesia spp. antibodies in cattle and to develop a new serologic test for the diagnosis of babesiosis. The complement fixation test used to detect Babesia spp. antibodies in the serum of naturally and experimentally infected cattle was modified from the original test reported by Mahoney.[21,23] The techniques consisting of (1) the isolation, purification and titration of the B. bigemina and B. argentina antigens, (2) preparation of the hemolytic system; (3) complement titration; and (4) performance of the test in tubes and in the microtiter system, are described in detail by Todorovic, et al.[37, 45]

B. Rapid Agglutination (RA) Technique: Babesia bigemina and B. argentina were used for infecting calves and for producing B. bigemina and B. argentina antigens. Babesia spp. antigens were prepared from blood in which at least 20% of the erythrocytes were infected. High parasitemias were developed by serial passage of the parasites through 2 to 4 splenectomized calves. In order to enhance development of parasitemia, 9-alpha-fluoro-16-alpha-methylprednisolone* at 0.5 mg./kg. were injected intramuscularly (IM) for 3 days prior to infection. During the course of each clinical attack following blood passage, thin and

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*Dexametazone - Schering Corp., Bloomfield, N. J.

thick[27] blood films of peripheral blood were taken every 3 to 4 hours to determine the portion of parasitized erythrocytes. When the parasitemia remained constant, as indicated by little or no change of the successive examinations, the organisms were passed to the next animal. After the second or third passage with B. bigemina and B. argentina, up to 20% of the erythrocytes were parasitized. Blood for the preparation of antigen was drawn into flasks containing ethylene diamine tetraacetic acid (EDTA), 1.2 g/liter, from calves with 20% or more parasitemia. Plasma was removed by centrifugation at 1085 x g for 30 minutes at 4° C. The infected erythrocytes were washed twice in 0.85% NaCl solution. The last wash was in antibiotic saline solution containing 100 units of penicillin and 11 mg. of streptomycin.* Then, 5 volumes of sterile 0.35% NaCl was used to lyse the infected erythrocytes for 1 hour at 4° C.[23] The lysate was centrifuged at 1085 x g for 30 minutes and resuspended in antibiotic saline solution. Thirty-five ml. of Babesia spp. suspensions were passed through the French Pressure Cell** at 1200 P.S.I. Then, the material was centrifuged at 10.000 x g at 3° C. for 30 minutes. The supernatant fluid was discarded and the concentrated Babesia spp. parasites were resuspended in equal amounts of sterile antibiotic solution. The suspension was honogenized in tissue Ten-Brock grinders. One percent of fast green dye*** was added at the rate of 0.1 ml./25 ml. of parasite suspension, mixed by shaking, and the mixture passed again through the French Pressure Cell at 1200 P.S.I. Excess dye was removed from the antigen by decanting the supernatant fluid after centrifugation at 10.000 x g for 30 minutes. The antigen pelet was resuspended in equal amounts of antibiotic solution and homogenized in a Ten-Brock grinder, sonified in a Cell Disruptor**** for 3 minutes at a 70 cycle

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*Gibco Biological Co., Grand Island, N. Y.

**American Instrument Co., Silver Springs, Md.

***Allied Chemical Co., N. J.

****Heath Systems Ultrasonic, N. Y.

power source, and tested for antigenic activity in a plate system. Serial dilutions of antigen and antisera were made and an approximate 0.025 ml. of antigen was mixed with the equal amount of serially diluted serum samples. Reference samples of positive and negative serums for evaluating Babesia spp. antigen reactivity and stability were obtained from 10 calves in a carrier phase of Babesia spp. infection and from several normal calves originating from an area free from babesiosis. The test was performed in a manner similar to the technique used in the plate test for the diagnosis of brucellosis. Disposable plastic trays to perform the test were used.

II. Vaccination of Cattle With Killed Babesia spp. Vaccine.

The Palmira experimental station of the Colombian Institute of Agricultural Research (ICA) located in the Cauca River Valley was used to carry on vaccination trials. The elevation was approximately 1000 meters above sea level and the average annual temperature was 26° C. The average rainfall measured 850 mm. per year and the heaviest rainfall occurred from March to May. Blood was collected from a splenectomized calf infected with 20% Babesia spp. for preparation of killed Babesia spp. vaccine. Blood was collected in EDTA as an anticoagulant by canulation of the carotid artery. Blood was lyophilized in a Virtis*-dry-freeze appartus in 20 ml. aliquots and stored at -20° C. Before use, the vaccine was restored to 1/3 of the original volume with sterile distilled water, and 5 ml. of this solution was injected IM into each calf. Preparation of the other types of killed Babesia spp. vaccine was described in detail by Todorovic, et al.[40] The degree of protection in 10 vaccinated animals was assessed by comparison of the following parameters: packed cell volume, parasitemia, antibody titer, body weight, and mortality with those of a comparable group of 5 non-vaccinated control

*The Virtis Co., Inc., Gardiner, N. Y.

calves. The field challenge was possible because all these calves were not included in any tick control program and the B. microplus tick load permitted sufficient numbers to build up on these animals.

III. Premunition of Cattle With Virulent Babesia spp.

Babesia bigemina and B. argentina were isolated from naturally infected cattle located in the Cauca River Valley.[41] These isolates were frozen using an emperical technique based on that described by Barnett.[3] On resuscitation, the frozen material was thawed rapidly in a 37° C. water bath and passaged once through a splenectomized calf before being used for premunition experiments. Standard infection of recently splenectomized calves gave rise to a 1% Babesia spp. parasitemia in a mean prepatent period of 4.5 days. Control of post premunition reaction, and determination of immune responses to premunition and B. microplus challenge were described in detail by Todorovic, et al.[39,41,43]

Five ml. of blood was inoculated IM, approximately 10^7 Babesia spp. parasites per ml. A new experimental compound, Imidocarb* (3,3'-bis-(2-imidazolin-2-YL)-carbanilide dihydrochloride), at a dosage of 0.75 mg. of salt/kg. was injected IM to control post premunition reaction in this experiment. Following all inoculations, the cattle were examined daily and 2 times per week for the presence of B. bigemina and B. argentina parasitemia by the use of thin and thick blood films technique,[27] packed cell volume,[35] and bi-weekly complement fixing technique.[45] Rectal temperature and body weight changes were also recorded. All cattle, 5 controls and 10 principals, were examined in the period of 8 months for immune responses during the experimentation. Ticks were collected from all animals and were preserved in absolute alcohol for identification

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*Imidocarb - Burroughs Wellcome & Co., Tuckahoe, N. Y.

purposes.

IV. Chemoprophylaxis.

The Burroughs Wellcome Company's experimental compound, Imidocarb, was used in this experiment. The drug, received as a water soluble salt, was dissolved in sterile distilled water as a 10% solution and used within 24 hours after preparation. This solution was administered IM at a dose of 3 mg./kg. in this experiment. Four weeks after inoculation, all 5 calves including the non-treated controls were injected with 100 ml. of blood containing 2% *B. bigemina* and 0.1% *B. argentina*. Additional experiments carried out to determine therapeutic and prophylactic effects of Imidocarb were described in detail by Adams and Todorovic[1] and Todorovic, *et al.*[44] The prophylactic efficacy of Imidocarb was determined by resistance to artificial and natural[44] challenge with *Babesia spp*. The therapeutic efficacy of Imidocarb was determined by recovery from acute infection after drug administration.[1] Inoculation of 500 ml. of blood into susceptible splenectomized calves was carried out to determine the presence or absence of *Babesia spp*. infection before and after treatment. All treated calves were observed for drug toxicity symptoms within 24 hours after treatment.

RESULTS

I. Serologic Diagnosis of Bovine Babesiosis.

A. Complement Fixation (CF) Test: The sera from the babesiosis experimentally and naturally infected cattle were subjected to the CF test for a period of more than 3 years. These samples were obtained from cattle located in babesiosis enzootic areas in the Cauca River Valley, the Magdalena River Valley, the Llanos, and the north coast of Monteria. In addition to these samples, serum was tested from cattle with known histories of experimental

babesiosis located at our laboratory. It was possible to obtain samples of serum from cattle before and after tick-borne or blood-borne infections. By means of the CF test the specific Babesia spp. antibodies were detected on the 8th day post infection. Babesia bigemina and B. argentina in the blood films stained by Giemsa were observed microscopically 3 to 8 days after experimental infection (Fig. 1). The specific Babesia spp. antibodies were actually detected after observable parasitemia an average of 3 to 5 days following the peak of parasitemia. The peak of parasitemia with Babesia spp. usually coincided with anemia and acute signs of acute babesiosis. Approximately 8 to 10 days after infection, the first CF titer was detected and they persisted in a variable manner with the death of the animal. On day 10, a 2 plus reaction was observed in the micro system with a titer of 1:10. The following 2 days the sample usually gave a 4 plus reaction with the titer 1:40 (Fig. 2). There has been a good agreement between the CF reaction and the known histories of animals with artificial and natural infections. Approximately 96% of serum samples with known babesiosis infections gave CF reactions; whereas, approximately 4% gave discordant reactions. The latter were especially noted in serum from several of the carrier animals.

B. Rapid Agglutination (RA) Test: Lysis of B. bigemina and B. argentina infected erythrocytes with sterile 0.35% NaCl yielded an antigen composed of concentrated Babesia spp. parasites. After passage through the French Pressure Cell, Babesia spp. parasites were ruptured and the unstained Babesia spp. antigen was grayish white and free of visible hemoglobin. Fine small particles of Babesia spp. parasites were stained with Fast Green dye; this mixture was stable and uniform. Preliminary studies with this antigen revealed that degree of specific agglutination reaction occurred with the serum from Babesia spp. infected cattle (Fig. 3). The reaction was visible during a period from 3 to 10 minutes of plate aggitation. However, non-specific agglutination occurred with

sera collected from cattle inoculated with a large volume of bovine blood. Experiments are in progress to prepare and test an antigen without using the French Pressure Cell for its isolation.

II. Sterile Immunity.

The results of hematologic and serologic responses of cattle injected with killed Babesia spp. vaccine and exposed to B. microplus infected ticks are shown in Figure 4. The first injection of killed Babesia spp. vaccine is indicated as week -8 and the second injection as week -6. The tick-borne challenge is indicated by an arrow as week 0. Prior to vaccination, the non-vaccinated control calves had a PCV of 33%, and the vaccinated ones 32%. After vaccination, PCV values remained in the normal range. The CF titers of 1:5 were detected in all vaccinated calves 4 days following vaccination. At the time of B. microplus field exposure all calves had approximately the same PCV (33%) values. Calves injected with killed Babesia spp. vaccine developed resistance to clinical babesiosis, which was evidenced with negative blood examinations for B. bigemina and B. argentina. In comparison, non-vaccinated control calves had 0.5% Babesia spp. parasitemia at 3 to 4 weeks after field exposure. At the time of Babesia spp. parasitemia, all calves developed anemia which was characterized by low PCV values of 18%, when the calves required a specific treatment against babesiosis to prevent production and possible death losses. Eleven to 12 weeks after field exposure all calves, vaccinated and non-vaccinated, developed signs of clinical anaplasmosis, evidenced by an A. marginale parasitemia and the PCV value decreasing to 20%; at this time all calves were treated with specific chemotherapy against anaplasmosis to prevent death losses. Complement fixing titers for Babesia spp. were not statistically significant between vaccinated and non-vaccinated calves. All calves responded with rising CF titers of 1:5 to 1:80, and the titers

persisted during the period of observations.

III. Co-infectious Immunity.

The results of hematologic and serologic responses of calves simultaneously injected with blood acutely infected with B. bigemina and B. argentina and subsequently treated with 0.75 mg./kg. of Imidocarb to moderate post premunition reaction are shown in Figure 5. All premunized calves responded severely to blood inoculation. Babesia bigemina and B. argentina were evident on day 4 and reached a maximum parasitemia of 0.3% on day 9 post premunition. After administration of Imidocarb, Babesia spp. parasitemia disappeared the day following treatment. During the Babesia spp. parasitemia, all inoculated calves developed anemia which was characterized by a low PCV of 23%; after drug treatment, all calves recovered from anemia by returning to the normal PCV values of 30%. At the time of field exposure to B. microplus ticks all calves, controls and premunized, had PCV values of 32%.

After tick-borne challenge with B. microplus ticks naturally infected with Babesia spp., all premunized calves had a high degree of resistance to field exposure. Babesia bigemina and B. argentina were not detected in thick and thin blood films stained by Giemsa. Packed cell volumes were in the normal range of 28-30%, while the non-premunized control calves developed a severe clinical babesiosis at 4 to 8 weeks following field exposure, which was evidenced with a 0.5% Babesia spp. parasitemia and low PCV values of 17%. All infected calves required a specific treatment against babesiosis to prevent production and death losses. Twelve weeks following field exposure all calves, premunized and controls, suffered a clinical anaplasmosis, characterized with a 1% A. marginale parasitemia, and they required a specific chemotherapy against anaplasmosis. Following treatment all calves recovered from the anemia and A. marginale parasitemia. The CF Babesia spp. titers were first detected on

Table 1. Serologic Diagnosis of Bovine Babesiosis

AUTHOR(S)	SEROLOGIC TECHNIQUE	SPECIES
Mahoney (1962)	C.F.	*B. bigemina*; *B. argentina*
Mahoney (1967)	C.F.	*B. bigemina*; *B. argentina*
Currnow and Currnow (1967)	I.H.	*B. argentina*
Ross and Lohr (1968)	C.A.	*B. argentina*
Lohr and Ross (1969)	I.F.A.	*B. bigemina*
Todorovic, *et al.* (1971)	C.F.	*B. bigemina*; *B. argentina*
Goodger (1971)	I.H.	*B. bigemina*; *B. argentina*
Goldman, *et al.* (1972)	F.A.	*B. bigemina*; *B. berbera*
Joyner, et al. (1972)	I.F.A.	*B. divergens*: *B. major*

C.F. - Complement Fixation

I.H. - Indirect Hemagglutination

C.A. - Capillary Agglutination

I.F.A. - Indirect Fluorescent Antibody

F.A. - Fluorescent Antibody

13 days post premunition, and they persisted with increasing levels of 1:20 to 1:80 during the period of experimentation.

IV. Chemotherapy.

The results of chemoprophylactic treatment against bovine babesiosis with Imidocarb and subsequent blood-borne challenge are shown in Figure 6. Immediately following an IM injection of Imidocarb all calves had salivation, lacrimation, coughing, and difficulty in breathing. These toxic signs were observed within 3 hours after drug administration. At day 0, or 28 days following drug inoculation, all calves were injected IV with *Babesia spp.* infected blood. At this time all calves had PCV values of 32%. Three days after blood-borne challenge, the calf not treated with Imidocarb had 0.1% *B. bigemina* and on day 5, 0.01% *B. argentina* parasitemia. *Babesia spp.* parasitemia persisted until day 13 when the calf died with signs of acute babesiosis, characterized by low PCV values of 12%. Three calves treated with Imidocarb 28 days before blood-borne challenge did not have any detectable *Babesia spp.* and their PCV values remained at a 31% level during the period of observations. The CF antibody response was detected only in the control calf with *Babesia spp.* parasitemia on day 8 following challenge with a titer of 1:5.

DISCUSSION

I. Serologic Diagnosis of Bovine Babesiosis.

A characteristic feature of *Babesia spp.* infection is that cattle which recover from an acute infection become carriers of the respective hemoparasites. These cattle cannot be diagnosed by blood films with any of the contemporary staining methods.[13,15,21,23,45] Thus, in order to identify a carrier animal it is necessary to develop serologic methods to detect specific antibodies rather

than the Babesia spp. parasites themselves (Table 1). The CF technique constituted one of the earliest tests for the diagnosis of bovine babesiosis.[21] A specific reaction for Babesia spp. parasites has been found useful in detecting CF antibodies in the serum of cattle with latent babesiosis; however, the practical application of the CF test was not conclusively demonstrated. The CF antigen used in our laboratory was isolated from the infected erythrocytes of cattle with acute babesiosis. Because of the impurities of these antigens they can be used only in the CF test; under these circumstances, the variables and difficulties of the CF technique become more pronounced and the dependability of the CF test lowered.[23] Obviously, similar technical and mechanical errors which are inherent of other CF procedures also apply to our technique. However, when the test is carefully conducted using an antigen prepared from high B. bigemina or B. argentina parasitemias, a minimum of error in accuracy and specificity should occur.

The preliminary results of the rapid agglutination test for detection of specific agglutinins in sera of cattle infected with Babesia spp. are encouraging and methods of eliminating some inherited problems of specificity of the agglutination reaction are being studied. In adjusting the agglutination reaction to detect a low level of Babesia spp. antibodies, care has to be taken so that the agglutinating antigen is not overly sensitive causing it to react with serum samples from uninfected cattle. Certain sera collected from cattle previously injected with large volumes of bovine blood were found to have a false positive agglutination reaction with Babesia spp. antigen stored at 3-5° C. for 10 days. The problem of preservation of Babesia spp. agglutination antigen is apparent.

Methods devised to date for serologic diagnosis of bovine babesiosis are still obviously imperfect. Improvements must be sought and serologic procedures standardized especially for practical use. Nevertheless, the admittedly imperfect techniques reported by several investigators (Table 1) now available are already

capable of diagnosing bovine babesiosis. As a result of the investigations and observations by others,[26] it is apparent that more research is needed for the perfection of a serologic technique for the diagnosis of bovine babesiosis.

II. Sterile Immunity.

The theoretical concept for the experiments was based on the possibility that sterile immunity might be induced in bovine babesiosis. Although sterile immunity is not usually encountered in blood protozoan infections, it does, however, occur naturally in *Theileria parva* infections.[28] Mahoney[24] reported that the species-specific *Babesia* antigen required for cross-protection immunity between different strains of *Babesia spp.* parasites was found in the infective plasma during the acute phase of infection. Todorovic, *et al.*[40] found that different *Babesia spp.* preparations had different immunogenic properties. The protection was more evident in cattle injected with a vaccine prepared from plasma rather than from the infected erythrocytes. The results of experiments reported here indicate that cattle inoculated with blood containing killed *Babesia spp.* developed a resistance to field exposure with *B. microplus* ticks, while non-vaccinated cattle suffered a clinical babesiosis kept under the same field conditions. Following field challenge, difficulties were encountered with *A. marginale* infection resulting in a severe anemia of experimental cattle. This fact indicates that the unilateral control of babesiosis alone is not economical under most tropical conditions.[41,43] The freeze-dried inactivated *B. bigemina* and *B. argentina* as described here are capable of imparting protection against babesiosis. The sterile immunity reported in the present study appears more effective than the type of immunity reported by Australian workers.[22,24,25] This difference can be attributed to the fact that intact calves were used in our work and the killed *Babesia spp.* vaccine was prepared from blood. Mahoney and Goodger[25] studied the immugenic reactions of splen-

ectomized calves inoculated with infected plasma and they found protection, characterized by an abrupt removal of B. argentina from peripheral blood, only in the experiments in which the challenged parasites were antigenically identical with those in the infective plasma used for immunization. Similar results were reported by Todorovic, et al.[40] who studied the degree of protection produced by 2 preparations, (1) plasma and (2) infected erythrocytes. It appears that the immunogenic material contained was present mostly in the plasma collected during the acute stage of infection especially at the peak of Babesia spp. parasitemia, rather than in the infected erythrocytes. The complete protection observed in cattle vaccinated with both preparations can be explained by differences reported by other investigators.[22,24] The results of experiments designed to ascertain the mechanism of sterile immunity to bovine babesiosis strongly suggest that this type of immunity in B. bigemina and B. argentina exists and plays an important role in the development of acquired immunity to bovine babesiosis.

III. Co-infectious Immunity.

The co-infectious immunity in bovine babesiosis is referred to as a premunition,[36] labile infection, latent infection, or immunitas non sterilizans.[28] The immunity persists as long as the animal remains a carrier of the infection. Several variables must be solved before premunition as a method of immunizing cattle can be recommended on a large scale for the control of babesiosis in cattle.[4,9,16,39,41] These variables include: (1) volume of blood used as inoculum; (2) number, infectivity, and history of Babesia spp. parasites; (3) phase of infection when blood is collected from cattle either recovered from or reacting to Babesia spp.; (4) storage of infected blood from time of collection to time of inoculation; (5) age and breed of animals used for premunition; and (6) drugs used for moderating the post premunition reaction.

The recent reports have been designed to investigate these factors associated with premunition of Colombian cattle and to limit the number of variables to as few as possible.[39,40,41,43] In the present work, the source of infected blood used as premunition inoculum was always from a splenectomized calf acutely infected with Babesia spp. This method gave us an advantage because the infective inoculum always contained approximately the same number of Babesia spp. parasites (10^7) per ml. Callow and Tammemagi[9] reported that low infectivity for B. argentina was the cause of vaccination failures in the field. According to these authors, 1/3 of cattle inoculated with 5 ml. of blood from 6 different carriers of B. argentina between 1 and 6 months after primary infection failed to become infected.[9] In our work a splenectomized calf was used to produce adequate infection, so that the blood inoculum taken from this calf contained approximately the sufficient number of Babesia spp. parasites to accomplish premunization. The second obstacle to an effective premunition program is severity of the post premunition reaction. In view of the effectiveness of Imidocarb[1,7,44] against B. bigemina and B. argentina, this problem would not be serious except that cattle are made susceptible if the compound is used in excess and sterilizes the infection.

Plata[29] reported on the common distribution of bovine babesiosis and anaplasmosis in Colombia and the high frequency of concurrent Babesia spp. and A. marginale infections in cattle. Our findings confirmed his observations and suggest development of control programs for simultaneous control of babesiosis and anaplasmosis in tropical zones of Colombia.[41]

The mechanism of acquired immunity to bovine babesiosis is complex in nature and is not quite elucidated. In a study of immunity to babesiosis it became apparent that the state of equilibrium which develops between the bovine host and the hemoprotozoan parasite is not permanent.[26] The parasite will ultimately be eliminated in the absence of continued infection by ticks, and

the animal may become susceptible to a clinical attack if reinfection occurs after a certain lapse of time.[28] In enzootic areas in Colombia, due to the repeated reinfection from infected ticks, it is not unusual for cattle to retain immunity for several years.

IV. Chemoprophylaxis.

The search for a new drug which can be administered to Babesia spp. susceptible cattle as a prophylactic measure in shorter or longer terms before exposure is obviously needed. In Australia, Callow and McGregor[7] recently reported that cattle treated with 2 mg./kg. of body weight of Imidocarb were refractory to infection with B. bigemina and B. argentina. Todorovic, et al.[44] confirmed their observations on the prophylactic effect of Imidocarb against Babesia spp. of Colombian origin. Cattle were protected up to several weeks after blood- or tick-borne babesiosis. In addition, Imidocarb was found highly effective against acute infection with B. bigemina and B. argentina.[1,7,15,18,44] The mechanism of Imidocarb action is not clear. Severe toxic reactions of cattle injected with Imidocarb were reported.[44] Varying degrees of salivation, lacrimation, labored breathing, coughing, and eventual death were observed if the drug was administered intravenously at a dose of 3 mg./kg. These side effects appeared less severe when Imidocarb was administered intramuscularly or subcutaneously. The potential use of Imidocarb as a prophylactic drug is indicated in a number of circumstances.[7,15,44] To protect susceptible cattle moving from a tick- and Babesia-free country through a tick infested area, Imidocarb can be used to replace hazardous premunition procedures with virulent Babesia spp. Furthermore, application of Imidocarb and exposure of cattle to infected ticks with Babesia spp. might help in the development of natural co-infectious immunity without severe post premunition reactions. In addition, the prophylactic effect of Imidocarb was effective for a longer period of time than any babesiacidal drug

tested. However, severe toxic effects were found which might limit its application, but need to be determined.[44]

The efficacy of 3 systems with which the spread of bovine babesiosis can be controlled depends upon the actual epizootiological conditions. In an area where the tick population is not effectively controlled the system of premunition is indicated. However, in tick-free zones or areas at a constant risk of tick exposure and babesiosis, cattle could be vaccinated with killed *Babesia spp.* vaccine, or chemoprophylactically treated to be protected against the introduction and sudden outbreaks of bovine babesiosis.

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Bovine Babesiosis: Co-Infectious Immunity to

Babesia bigemina and *Babesia argentina* Infections

by

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SUMMARY

A total of 48 intact and 8 splenectomized calves were used to evaluate different systems of immunization against *Babesia bigemina* and *Babesia argentina* infections. These systems were based on premunition or co-infectious immunity in which the continued presence of a relatively small number of *Babesia* parasites protects the bovine host against otherwise lethal infections. *Babesia spp.* parasites were isolated from naturally infected cattle in the Cauca Valley and Monteria and pure isolates of *B. bigemina* and *B. argentina* were maintained in calves at the Laboratory. The infectivity [illegible] athogenicity of *B. bigemina* and *B. argentina* were established in splenectomized calves.

Co-infectious immunity was produced by two methods: (1) acutely infected blood with *B. bigemina* and *B. argentina* was used as the source of inoculum and the post injection reactions were controlled with Imidocarb or Ganaseg; and (2) by artificially inducing babesiosis and anaplasmosis with the blood of patent carriers of *B. bigemina* and *B. argentina* without chemotherapy. Since babesiosis and anaplasmosis occur as concurrent infections under tropical conditions, attempts were made to produce co-infectious immunity against both diseases.

Immune responses of calves to premunization and challenge were assessed by hematocrit values (H), parasitemia (P), temperature (T), body weight (BW), complement fixing antibody titers (AT), and mortality. The degree of resistance was determined by blood-borne and tick-borne challenges. Ticks were collected from all animals and identified as *Boophilus microplus* and *Dermacentor nitens*. Premunized cattle demonstrated a high degree of resistance to babesiosis; however, non-premunized suffered severe attacks of babesiosis and were treated with specific therapy to prevent death losses.

INTRODUCTION

The knowledge of mechanism of immunity to bovine babesiosis is far from complete (Rick, 1968). Nevertheless, some basic findings concerning immunopathology of hemotropic infections have been made during the last 20 years (Ristic, 1968). The problem of bovine babesiosis still exists, particularly in the subtropical and tropical areas of the world where there are ticks. In Colombia, South America, babesiosis causes great losses in susceptible cattle and the incidence of infection appears to be related to the occurrence and activities of tick-vectors in various altitudes (Velasquez, 1938; Roman, 1945; Plata, 1956; Todorovic *et al.*, 1969; Gonzalez *et al.*, 1971). The disease can be prevented by an adequate tick control program to keep animals free from tick infectations. This method has resulted in the eradication of bovine babesiosis in the United States. At the present time in Colombia, a vector control program as a measure of *Babesia* control, while appealing, is impossible for various reasons (Todorovic *et al.*, 1971). Therefore, the only measure which can be used to prevent losses and control disease is an effective vaccination program (Todorovic *et al.*, 1970). In order to achieve this goal a series of investigations were undertaken to study the mechanism of co-infectious immunity to babesiosis so that cause ontoward results of premunization and vaccination against babesiosis may be elucidated and better methods of producing vaccines devised. The experiments were carried out to obtain (1) information on the infectivity and virulence of the blood from cattle naturally infected, (2) information from cattle undergoing a primary reaction with *Babesia spp.*, (3) responses after premunition and chemotherapy, and (4) resistance against tick-borne (*B. microplus*) challenge. The results of these experiments are reported in this paper.

MATERIALS AND METHODS

Experimental Design.

Systems of Premunition and Blood-Borne Challenge: Experiment 1. Ten male Holstein-Friesian calves 8 months of age located at Tibaitata (alt. 2,600 meters) were intramuscularly inoculated with 5 ml of infected blood containing 1% *B. bigemina*, 0.1% *B. argentina*, and 2% *Anaplasma marginale*. Blood was collected from splenectomized calves acutely infected with these parasites. *Anaplasma marginale*, *B. bigemina*, and *B. argentina* were isolated from cattle naturally infected in the north coast of Colombia (Monteria). All premunized calves were simultaneously treated against babesiosis with Imidocarb* at a dosage of 0.75 mg/kg of body weight given intramuscularly, and against anaplasmosis with Gloxazone** at a dosage of 4 mg/kg of body weight given intravenously 7 days after premunition. Five calves (Group B) were additionally treated with Gloxazone at a dosage of 4 mg/kg on days 28, 30, and 32 after premunition against anaplasmosis. Nine weeks after premunition all calves were intramuscularly injected with 5 ml of infected blood collected from acutely infected calf with homologous parasites used for premunition. The premunized calves were transported to Putumayo, a tropical zone of Colombia, 12 and 14 weeks after premunition.

Experiment 2. One Brown Swiss and 3 Holstein-Friesian bulls 2 years of age located at Bogota (alt. 2,600 meters) were used for this experiment. All bulls were subcutaneously injected with 5 ml of infected blood containing 2% *B. bigemina*, 0.01% *B. argentina*, and 5% *A. marginale* parasitemias. Infected blood for this experiment was collected from naturally infected cattle located in the zone of Rio Sumapaz. Blood from a field case of anaplasmosis and babesiosis was collected in EDTA as anticoagulant, inoculated into a splenectomized calf at LIMV, and used as a source

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*Imidocarb: 3,3'-Bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride. Burroughs Wellcome & Co., Tuckahoe, New York.

**Gloxazone: (alpha-ethoxyethylglyoxal dithiosemicarbazone) Burroughs Wellcome & Co., Tuckahoe, New York.

of infection with B. bigemina, B. argentina, and A. marginale for premunization trials. After premunition, all bulls were treated against Babesia spp. with Ganaseg* at a dosage of 1 mg/kg of body weight on days 6 and 8, and against anaplasmosis with Gloxazone at a dosage of 4 mg/kg of body weight given intravenously on days 22 and 24 and with Liquamycin** at a dosage of 12 mg/kg of body weight on day 28. On day 36 all animals were injected with the original inoculum used for premunition.

Systems of Premunition and Tick-Borne (B. microplus) Challenge.

Experiment 3. Two groups of Holstein-Friesian male calves 4 months of age were used for this experiment; these animals were located at Palmira in the Cauca Valley (alt. 1,000 meters). The first group, consisting of 15 calves, was inoculated with 5 ml of blood infected with B. bigemina and B. argentina isolated from the naturally infected calves on this farm. Blood was maintained in intact calves at Bogota and used as a source for premunition trials. At the time of injection, calves were patent carriers without clinical symptoms or detectable parasitemias. Blood from these calves was pooled and injected into a splenectomized calf; at the time of 0.5% B. bigemina and 0.01% B. argentina parasitemia, this calf was bled and his blood was used for premunition experiments in Palmira. After 75 days of premunition, the calves were exposed to tick-borne challenge. Five calves of the same age and breed were not premunized and were used as controls (Group B).

Experiment 4. Two groups of Holstein-Friesian male calves 8 months of age located at Palmira in the Cauca Valley were used for this experiment. The first group (Group A), consisting of 5 calves, was inoculated with 5 ml blood from a splenectomized calf used as a donor of infected blood for calves in experiment 3. Ten weeks after premunition, all calves were exposed to tick-borne challenge. The second group of

*Ganaseg - Squibb, Cali, Colombia.

**Liquamycin - Chas. Pfizer & Co., New York.

calves (Group B), consisting of 5 calves, was injected with the same blood as Group A but this group of calves was premunized 3 months previously against anaplasmosis. All calves were exposed to tick-borne challenge at 10 weeks after premunition against babesiosis.

Controls.

Two groups of control calves were used to evaluate the efficacy of the premunition techniques used in this study. All control non-premunized calves were raised on the same farm and they were of the same breed and age as the prinicpal animals. The first group of 2 control intact calves was exposed to ticks on the same day as the premunized animals. After exposure they were not treated with specific therapy against babesiosis and anaplasmosis. The second group of control calves (Group B) consisted of 2 calves of the same age and breed as the premunized calves. After tick-borne challenge, they were treated with specific chemotherapy against babesiosis and anaplasmosis.

Control of Infectivity of Blood Used for Premunition.

Eight splenectomized calves were used to evaluate the pathogenicity of the *B. bigemina* and *B. argentina* parasites used for the premunition trials. Calves were bought from a babesiosis- and tick-free area in Bogota. These calves were splenectomized and maintained at LIMV for these trials. The calves were used for isolation of *Babesia spp.* and for the evaluation of the pathogenicity of *Babesia spp.* parasites from the patent carriers; blood from these calves was used for preparation of premunition inocula.

Preparation of Blood Inocula for Premunition.

Two intact Holstein-Friesian male calves 6 months of age were injected with *B. bigemina* and *B. argentina* infected blood which originated from the Los Llanos Orientales and Cauca Valley, respectively. When they recovered from primary infection, approximately 2 months thereafter, they were reinoculated 2 times with

the same organisms. These calves were used as a source of carrier blood to inoculate a splenectomized calf for production of infected blood for experiments in Palmira.

Three splenectomized calves were used as a source of acute infected blood with B. bigemina, B. argentina, and A. marginale, respectively, for premunition trials in Bogota. These calves were inoculated with blood of naturally infected cases in the northern coast of Colombia (Monteria). At the time of primary reaction, blood was collected in EDTA (1.2 g/l) as anticoagulant, stored at 5° C, and used for premunization on the same day of collection. The infective dose was 5×10^7 of Babesia spp. or A. marginale parasites.

Determination of Immune Responses to Premunition and Challenges.

Following all inoculations, the calves were examined daily and 2 times per week for the presence of parasitized erythrocytes with B. bigemina, B. argentina, or A. marginale parasitemias (P) by the use of thin and thick blood smears described by Mahoney (1962). Packed cell volumes were determined by the micro hematocrit method, and bi-weekly complement fixation tests (AT) were performed on collected samples according to the method described by Todorovic et al. (1971). Rectal temperature (T) and weekly body weight (BW) changes were also recorded. All animals, control and principals, were examined in the period of 1 year for immune responses during the experimentation. Ticks were collected from all the animals and were preserved in absolute alcohol for identification purposes.

RESULTS

Immune Responses of Calves to Premunition With Blood Collected From Acute Infections.

The results of hematologic and serologic responses of calves simultaneously injected with blood acutely infected with B. bigemina, B. argentina, and A. marginale

and subsequently treated against babesiosis with Imidocarb and against anaplasmosis with Gloxazone are shown in Figure 1. Five calves (Group A) were treated once on day 7 post injection. All calves in this group responded severely to premunition. The B. bigemina and B. argentina parasitemias were evident on day 3 and reached a maximum on day 8 post premunition. After treatment, the degree of parasitemia in a given calf varied from 0 to 0.1% until completely disappearing on day 13. The infected calves became anemic on day 8 as a result of Babesia spp. infection. After treatment against babesiosis, hematocrit values increased temporarily but started to decrease again due to an A. marginale parasitemia which was evident at 3 weeks post premunization. Four weeks after premunization, all calves except 1 recovered from anemia which was manifested with a return to normal hematocrit values. Only 1 calf suffered a prolonged anemic state and finally recovered after 11 weeks of premunition.

The second group of calves (Group B) was treated against anaplasmosis on days 28, 30, and 32 and responded more uniformly to the A. marginale premunition. At 9 weeks post premunition all calves in Groups A and B were inoculated with the original inoculum of Babesia spp. and A. marginale. They did not show any reactions; all had resistance to this challenge. All calves in Groups A and B reacted with complement fixing antibody titers to Babesia spp. and A. marginale with titers gradually increasing during the premunition period. All calves (Group A and B) were exposed to tick-borne challenge at 12 and 14 weeks post premunition. They were transported to Putumayo and did not require treatment against babesiosis or anaplasmosis during the 3 months of observations.

The results of hematologic and serologic responses of cattle simultaneously injected with blood acutely infected with B. bigemina, B. argentina, and A. marginale, and subsequently treated against babesiosis with Ganaseg on days 6 and 7 and against anaplasmosis with Gloxazone on days 22 and 24 and Lyquamicin on day 26 are shown in Figure 2. The parasitemia with B. bigemina and B. argentina became evident on day

5 post injection and reached a maximum on day 6 when the first injection of Ganaseg was given. After the second treatment with Ganaseg, Babesia spp. parasitemia decreased and completely disappeared on day 11 of premunition. Anaplasma marginale parasitemia became evident on day 17 and reached a maximum on day 19 post premunition. After treatment, the degree of parasitemia decreased and disappeared from peripheral circulation on day 27 post premunition. At the time of B. bigemina, B. argentina, and A. marginale parasitemias all animals had a fever and a loss of body weight, with an anemic state which was characterized by low hematocrit values. The lowest hematocrit values of 18% were recorded after appearance of an A. marginale parasitemia. All animals recovered from the premunition reaction and had normal parameters after 30 days post premunition. The first appearance of detectable complement fixing antibodies was on day 12 for Babesia spp. and on day 16 for A. marginale. At 36 days post premunition all animals were injected with the original inoculum and were found resistant to this challenge. All animals were exposed to tick-borne B. microplus challenge on day 90 post premunition; they were found resistant to babesiosis and anaplasmosis and in good health after field exposure.

Immune Responses of Cattle to Premunition With Blood Collected From Patent Carriers.

The results of hematologic and serologic responses of cattle injected with blood collected from patent carriers of B. bigemina and B. argentina are shown in Figures 3 and 4A. The parasitemia with B. bigemina and B. argentina became evident on day 8 post premunition, reached a maximum on day 12, gradually decreased on day 16, and thereafter was no longer detectable in the peripheral circulation. All calves developed an anemia which was characterized with low hematocrit values averaging 25% on day 14 after premunition. At the time of field exposure with naturally infected B. microplus ticks with Babesia spp. all calves had hematocrit values averaging 33%. After tick-borne challenge, B. bigemina and B. argentina

were not detected in the peripheral blood films as a result of resistance to babesiosis; however, calves suffered clinical anaplasmosis and A. marginale parasitemias were demonstrated in blood films 4 and 6 weeks after challenge. After specific chemotherapy against anaplasmosis, calves recovered from anemia and the A. marginale parasitemia. Complement fixing antibody titers were first detected on day 10 after premunition and they persisted with increasing titers during the period of challenge. Control calves not premunized developed clinical babesiosis which was manifested with B. bigemina and B. argentina parasitemias and they needed treatment to prevent death losses. Two calves not treated died with acute babesiosis. These control calves also suffered clinical anaplasmosis and they were treated to prevent death losses.

The results of hematologic and serologic responses of calves previously premunized against anaplasmosis and 3 months later injected with blood from patent carriers of B. bigemina and B. argentina are shown in Figure 4B. The parasitemia with B. bigemina and B. argentina became evident 5 days after premunization, reached a maximum on day 10, and gradually decreased and disappeared 18 days after premunition. At the time of B. bigemina and B. argentina parasitemias all calves became anemic, but all recovered from a post premunization reaction without treatment on day 24 with a return to normal hematocrit values. After tick-borne challenge with naturally infected B. microplus ticks, all premunized calves had a high degree of resistance to field challenge. Babesia bigemina, B. argentina, and A. marginale parasites were not detected in blood films. Temperatures and hematocrit values were in normal range. Complement fixing antibody titers were first detected on day 21 after premunition. They persisted with increased titers during the period of 13 weeks of field exposure to B. microplus infected ticks with B. bigemina and B. argentina. All calves had a fever at the time of B. bigemina and B. argentina post premunition reactions.

Immune Responses of Non-Premunized Control Calves to Field Challenge.

The results of hematologic and serologic immune responses of intact and splenectomized control calves used to determine the degree of resistance of premunized calves to babesiosis and pathogenicity of B. bigemina and B. argentina parasites are shown in Figures 3B, 5A, 5B, and 6A. All splenectomized calves inoculated with B. bigemina and B. argentina blood collected from acute infections were severely infected. All intact calves inoculated with blood collected from patent carriers developed a slight infection. Non-premunized calves exposed to field challenge with naturally infected B. microplus ticks suffered clinical babesiosis and they were treated to prevent death losses; non-treated calves died with signs of acute babesiosis.

Responses of Premunized Calves in Body Weight.

The effect of premunition against bovine babesiosis and anaplasmosis in body weight of vaccinated cattle is shown in Figure 7. At the time of premunition against babesiosis, all calves temporarily lost body weight in comparison with non-premunized control calves; however, after field exposure to naturally infected B. microplus infected ticks with B. bigemina and B. argentina, significant differences in body weight were found between premunized and non-premunized calves. As a result of resistance to B. bigemina and B. argentina infections, premunized calves gained 53 kg more in body weight during the 11 months of observations than the non-premunized control calves.

DISCUSSION

The classical concept of premunition or co-infectious immunity was first introduced by Sergent et al. (1924) and the phenomenon was based on the observation that the immunity to babesiosis persists as long as the animal remains a latent carrier of Babesia spp. parasites. If parasites disappeared as a result of

autosterilization, effective chemotherapy, the animal became fully susceptible. The immunity against babesiosis can be achieved by inoculating cattle with small doses of blood containing Babesia spp. parasites. This method will usually accomplish the purpose of co-infectious immunity although the method is, of course, somewhat hazardous in itself (Todorovic, 1970). According to Australian investigators (Callow and Tammemagi, 1967; Legg, 1939) who practiced this type of vaccination, losses can be considerable. If fully susceptible cattle are premunized in large numbers without aftercare, losses of approximately 1 to 5% may occur. The inoculated cattle should be observed very closely in case severe reactions develop. Legg (1939) reported two instances in which two-thirds and one-third, respectively, of the premunized cattle in 2 groups died from severe B. argentina post premunition reactions. Several variables must be solved before premunition as a method can be recommended on a large scale for the control of bovine babesiosis.

In early reports on the post premunition reactions, it was evident that many uncontrolled variables were responsible for the inconsistent results (Callow and Tammemagi, 1967; Johnston and Tammemagi, 1969; Kemron et al., 1964, 1962; Legg, 1939; and Riek, 1968). These variables included: (1) volume of blood used as inoculum; (2) number of Babesia spp. parasites; (3) infectivity and virulence of Babesia spp. parasites; (4) history of parasites; (5) infectivity and virulence of blood collected from cattle either recovered from or reacting to Babesia spp. parasites; (6) storage of infected blood from time of collection to time of inoculation; (7) age and breed of animals used for premunition; and (8) chemicals (type and dosages) used to control post premunition reactions. The present study was designed to investigate the factors associated with the premunition of Colombian cattle and to limit the number of variables to as few as possible.

This work was undertaken to determine immune responses of Colombian cattle premunized against B. bigemina and B. argentina, their relationships to chemotherapy, the infectivity of blood from acute and patent infections, and in

addition, simultaneous premunization against bovine babesiosis and anaplasmosis in comparison with unilateral control of either one of these tropical maladies. The volume of inoculum, including the challenged doses when applicable, were standardized at 5 ml of blood (10^7 parasites per ml). The blood samples that were used as inoculum were obtained during the initial acute phase of B. bigemina and B. argentina infections. The blood to be used as premunition inoculum was obtained when the B. bigemina parasitemia was 1% and the B. argentina between 0.1 to 1%. The source of blood used as premunition inoculum was always from a splenectomized calf either injected with blood from patent carriers or from acutely infected cattle with Babesia spp. This method gave us an advantage because the infective inoculum always contained approximately the same number of Babesia parasites. The criterion for infectivity and virulence of B. bigemina and B. argentina parasites was determined as a percent of the parasitemia of splenectomized calves, degree of anemia, and the mortality rate. It was established that the demonstration of B. bigemina or B. argentina parasites in the erythrocytes is the necessary criterion of infections.

Callow and Tammemagi (1967) reported that low infectivity for B. argentina was the cause of vaccination failures in the field. One-third of cattle inoculated with 5 ml of blood from 6 different carriers of B. argentina, between 1 and 6 months after primary infection, failed to become infected. In order to avoid this obstacle, a splenectomized calf was injected with carrier blood to be sure the inoculum would contain an adequate number of B. bigemina and B. argentina parasites to accomplish premunition. The present work also clearly demonstrated that when a splenectomized calf was inoculated with a large amount of patent carrier blood, the post inoculation reactions were mild, which indicate the attenuated nature of the Babesia spp. parasites in carrier blood. This was confirmed by the fact premunized calves at Palmira did not require treatment.

The results of our work in Palmira, Colombia, and also in Australia indicated

that the problem of effective premunition against B. bigemina or B. argentina infections was associated with variability in the infectivity of the blood of carrier animals, and that obstacle can be overcome by using a splenectomized calf to standardize inoculum (Callow and Mellors, 1967). The second obstacle to an effective premunition program is the severity of the post premunition reaction in cases where blood was collected from animals naturally infected during the acute stage of disease. In view of the effectiveness of various chemicals against B. bigemina and B. argentina this problem would not be serious, except that animals are made susceptible again if drugs are used in excess and sterilize the infection (Callow and McGregor, 1970). In our work, and in works reported by Callow and McGregor (1970) and Roby et al. (1968), new and commercially available compounds were found satisfactory to control post premunition reactions in calves inoculated with virulent blood collected from animals acutely infected with B. bigemina, B. argentina, and A. marginale.

Zaraza et al. (1969) reported on the common distribution of babesiosis and anaplasmosis in Colombia, and the high frequency of concurrent Babesia spp. and A. marginale infections in cattle. Our work confirmed their observation and demonstrated that unilateral control of hemotropic diseases is not practical. Our data indicate that premunition against B. bigemina, B. argentina, and A. marginale can be accomplished simultaneously without any severe post premunition reactions if animals are properly treated. The second approach might also be practical, as our data indicate, to premunize against babesiosis and after the animals completely recover to premunize against anaplasmosis. It appears that under Colombian conditions control of babesiosis and anaplasmosis is necessary due to concurrent incidence (Roman, 1945). We found that clinical and pathological manifestations of concurrent infection with B. bigemina, B. argentina, and A. marginale were more severe than those observed during infection with either of the hemotropic parasites alone, and were attributed to the concurrent infection being additive in nature (Todorovic et al., 1971). Simultaneous premunition against babesiosis and anaplasmosis will be more practical, but in some cases when it is not possible premunition of an individual disease is

also shown satisfactory.

On the basis of the results obtained in Palmira, it appears that calves premunized with blood from patent carriers maintained shortly into a splenectomized calf had mild post premunition reactions, manifested with a transient parasitemia, without clinical signs; treatment was not necessary to control such reactions. The complete protection of these calves was observed upon field exposure to ticks naturally infected with B. bigemina and B. argentina. The mechanism of immunity was based on the fact that calves were resistant to superinfection and they never show any signs of infection.

In this work, the acquired immunity of cattle to B. bigemina and B. argentina was investigated by challenging cattle either by blood- or tick-borne homologous challenges. Our results indicate that complete immunity was produced in cattle against homologous challenges. These results are in agreement with data reported by Callow (1967) that homologous immunity develops quickly, being sufficient to prevent parasitemias in cattle exposed to field challenge, in comparison with heterologous challenge where a slight degree of parasitemia is noted without severe clinical diseases. In all calves premunized at Palmira, the homologous immunity was developed in all instances and was apparently sufficiently solid to prevent any signs of diseases at a time when controls died from the same challenge.

The exact mechanism of immunity to babesiosis is complex in nature and is not quite elucidated. In a study of immunity to babesiosis, it becomes apparent that the state of equilibrium which develops between the host animal and the protozoan parasite is not permanent. The parasite will ultimately be eliminated in the absence of continued infection by ticks, and the animal may become susceptible to a clinical attack if reinfection occurs after a certain lapse of time (Mahoney, 1962). In endemic areas of Colombia, due to the repeated reinfection from infected ticks, it is not unusual for animals to retain their immunity for a considerable period of time. It has been shown that B. bigemina and B. argentina in the absence of reinfection

usually persist for 10 to 12 months and that severe relapses may occur at varying intervals within this period. Cattle which recover from clinically evident babesiosis develop a co-infectious immunity which may last for 1 to 12 months (Mahoney, 1962). However, it should be noted that the duration of this resistance varies considerably both with the species of Babesia and with individual animals (Rick, 1968).

The results obtained from our work on development of co-infectious immunity in Colombian cattle indicate that the lasting immunity to babesiosis can be established by providing for the constant maintenance of the immunopathologic process associated with subclinical infection controlled by chemotherapy. This process would activate the host's anti-Babesia humoral and cellular defenses, and finally produce resistance to natural infection. Furthermore, it was found that methods of premunizing cattle by injecting them with blood collected at the acute stage of B. bigemina and B. argentina infections and subsequently controlled reactions with chemotherapy was most effective.

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Bovine Babesiosis: Sterile Immunity to

Babesia bigemina and *Babesia argentina* Infections

by

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SUMMARY

Killed *Babesia bigemina* and *Babesia argentina* vaccine was prepared from the infected erythrocytes (AG-E) and from the infected plasma (AG-S) collected from acutely infected calves with *Babesia bigemina* and *Babesia argentina*. The vaccine was tested in Colombian cattle under field conditions in the Cauca Valley. A total of 40 calves two and one half months of age received killed-*Babesia* vaccine, and 5 calves were not vaccinated; they served as controls. Vaccinated and non-vaccinated control calves were exposed to field-borne challenge with *Boophilus microplus* infected ticks. On the basis of the data obtained in these experiments, it was found that a high degree of sterile immunity to *Babesia bigemina* and *Babesia argentina* can be produced in calves injected with killed-*Babesia* vaccine. It appears that sterile immunity plays an important role in the mechanism of acquired immunity to babesiosis other than well-known co-infectious immunity known as premunition.

INTRODUCTION

Since the discovery of Babesia bovis (Babes, 1888) much work has been done on various aspects of immunity to bovine babesiosis, however, the pathogenic and immunogenic mechanisms in this and other hemoptropic infections have not been eluciadated. Among these processes is mechanism of aquired resistance to Babesia bigemina and Babesia argentina infections. It is thought, that resistance to clinical infections is generally observed in cattle harboring the parasites or being in state of immunity known as "premunition" (Sergent et al. (1926). Recent evidence observed by the Australian investigators suggested that a sterile immunity exists and plays an important role in the development of aquired immunity to bovine babesiosis.

Sterile immunity was reported in cattle which had recovered from an infection with Babesia bigemina, however, this type of immunity was directed only against homologous strain of Babesia bigemina (Callow, 1964). Attempts to produce a sterile immunity with injecting cattle with k .lled-Babesia argentina parasites had resulted in only partial protection (Mahoney, 1967). Recently, Mahoney and Goodger (1972) found that complete protection characterized by abrupt removal of Babesia argentina from the periferal blood circulation was observed in the experiments in which the parasites used to challenge immunity were antigenically identical with those that produced the infected plasma used for immunization.

The following is a report on the immunizing properties of killed-Babesia bigemina and Babesia argentina of Colombian origin, produced by a freeze-dried technique to inactivate Babesia spp. parasites either in infected erythrocytes or plasma collected at an acute stage of infection.

MATERIALS AND METHODS

Location of Experiments:

The Palmira experimental farm of the Colombian Institute of Agricultural Research (ICA) located in the Cauca Valley was used to carry on vaccination trials. This farm is located in a subtropical zone of South America. The elevation was approximately 1,000 meters (3,195 ft.) and the average annual temperature was 26° C. (78.3° F.). The average annual rainfall measured 849 mm (33.8 in.) per year and the heaviest rainfall occurred in March, April, and May.

Experimental Animals:

Forty-five male, Holstein-friesian calves weighing from 69 to 97 kg, born and raised on this farm, were used for evaluation of the vaccines against babesiosis. Two days after birth, the calves were taken from the dams and placed in individual concrete stalls under conditions that precluded accidental *Babesia spp*. infections. The calves were fed milk and calf pellet concentrate. All calves were determined to be negative to *Babesia* infection by the complement-fixation test blood transmission and blood examination of Giemsa stained films; calves were in good health and free of tick infestation prior to vaccination. At the age of 2-½ months, they were used in vaccination experiments. Calves were drenched with Thiabendazole* against intestinal parasites and they were bled weekly for samples for serologic and hematologic studies. Ten weeks after vaccination, the calves were kept on pasture.

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*Thiabenzole, Merck and Company, Rahway, N.J.

The Parasites:

Blood was collected from infected cattle located on this farm and injected into splenectomized calves at the Veterinary Laboratory of the Colombian Institute of Agricultural Research in Bogota. *Babesia bigemina* and *B. argentina* were isolated from the acutely infected cattle. *Babesia bigemina* and *B. argentina* were maintained in splenectomized calves and used for vaccine preparations. Disodium ethylenediamine tetraacetate (EDTA) 1.2/1 of blood was used as anticoagulant. Blood was frozen using an emperical technique based on that described by Barnett (1964). Ten ml aliquots of infected blood in bijou bottles with 7.5 percent glycerol as the cryoprotectant were slowly frozen to -79° C. at the rate of approximately 1° C. a minute. The aliquots were stored at a temperature of -60° C. for periods of up to 6 months. On resuscitation, the frozen material was thawed rapidly in a 37° C. water bath and passaged once through a splenectomized calf before being used for vaccine preparation. Standard infection of recently splenectomized calves gave rise to a 1 percent parasitemia in a mean prepatent period of 4.5 days.

Preparation of AG-E Vaccine:

Blood was collected from a splenectomized calf infected with *B. bigemina* (25%) and *B. argentina* (1%) for preparation of the AG-E vaccine. Blood was collected in EDTA as anticoagulant by canualation of the carotid artery. Plasma was separated from the infected erythrocytes by centrifugation (2,500 rpm/20 minutes) and saved for further study. The infected erythrocytes were pooled and washed 3 times in 0.85% physiologic solution. These erythrocytes were lysed by freezing and thawing and lyophilized in Virtis*

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*Virtis Co., Inc., Gardiner, New York.

dry-freeze apparatus in 10 ml aliquots. The lyophilized lysate of infected erythrocytes with B. bigemina and B. argentina was termed as vaccine AG-E This vaccine was stored at -20° C.

Preparation of AG-S Vaccine:

Plasma separated from the infected erythrocytes with centrifugation was frozen at -20° C. and recentrifuged for removal of fibrinogen at 3,000 rpm/30 minutes. Ten ml aliquots of this material was distributed in bijou bottles and lyophilized in Virtis apparatus. This material was termed AG-S vaccine, and stored at -20° C. until use for vaccination trials.

Vaccination Procedure:

Vaccine prepared from the infected erythrocytes (AG-E) and from plasma (AG-S) was removed from the refrigerator and restored to one-third of the original volume with sterile distilled water and admixed with Freunds* complete adjuvant in equal amounts before injection. Vaccines with adjuvant or without were injected intramuscularly in the hip of calves at 14-day intervals. Four groups of calves (10 calves each, 2½ months of age) randomly distributed were divided in 4 groups. The experimental calves designated as Group A were intramuscularly inoculated with 5 ml of AG-S vaccine. The calves in Group B were inoculated with 5 ml of AS-S vaccine with equal amount of Freunds complete adjuvant. Ten calves in Group C were injected with 5 ml of AG-E vaccine. Ten calves in Group C were injected with ml of AG-E vaccine with equal amount of Freunds complete adjuvant. Five calves of the same breed and origin kept under the same condition as principal calves were not vaccinated but used as controls.

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*Freund's Complete Adjuvant, Difco Company, Detroit, Michigan.

Determination Assessment of Immune Responses of Vaccinated Calves:

Ten weeks after vaccination, all calves were exposed to natural field challenge with Boophilus microplus ticks naturally infected with B. bigemina and B. argentina. Blood samples from each animal were collected weekly in tubes under aseptic conditions. The blood tubes were refrigerated as soon as possible after collection by placing them in an ice chest and refrigeration was maintained until the blood samples were processed. Thin blood smears were made using the Giemsa technique described by Vizcaino et al. (1969). The films were fixed in absolute methanol and stained with Giemsa* for 20 minutes using phosphate buffered water pH 7.1 to prepate the stain solution. Each prepared smear was examined under the oil immersion lens of a light microscope at 1,000 X magnification. Twenty fields of slides were examined for parasitized erythrocytes and the percentage of parasitemias calculated on the basis of the number of infected erythrocytes in relation to the total number of cells in each field.

Hematocrit values were determined by the microhematocrit methods as outlined by Schalm (1965). Plain capillary tubes were filled to approximately 1 cm of the end with unclotted blood and the empty end of each tube was fused in the flame of a gas burner. The filled tubes were then centrifuged for 5 minutes at 11,500 rpm and the packed cell volume read directly from a calibrated tube reader. The complement-fixation test for Babesia spp. antibody detection was performed according to the technique described by Todorovic et al. (1971). Serum samples collected from each calf were screened for the presence of complement-fixing antibodies of B. bigemina and B. argentina infections.

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*Giemsa Stain, Gradwohl Laboratories, St. Louis, Missouri.

The weight of each calf was determined by a Nasco* weight tape. Each animal was restrained in a normal standing position and the tape placed around the calf at the heart girth. The weight in kilograms was read directly from the tape.

Ticks were collected from each calf at weekly intervals and preserved by immersing them in tubes with absolute alcohol. Clinical observations and necropsies of the dead animals were performed by veterinarians working in Pathology section of the Colombian Institute of Agriculture in Palmira.

The degree of protection in all animals vaccinated was assessed by comparison of the parameters measured with those of a comparable group of control non-vaccinated calves. The field-challenge was possible because all the calves were not included in any tick control program and the Boophilus microplus tick load permitted sufficient numbers to build up on animals.

RESULTS

Immune Responses of Calves to Vaccination.

The results of hematologic and serologic responses of calves injected with killed- Babesia spp. AG-S vaccine (Groups A & B) with or without complete Freund adjuvant are shown in Figure 1. The first injection of killed - Babesia spp. vaccine is indicated as week - 10, and the second injection as week - 8. The time of tick-borne (Boophilus microplus) challenge is indicated by an arrow as week 0. Prior to vaccination, the hematocrit values of 34% were essentially the same for all Groups A & B. Two weeks after

*Metric tape, Nasco, Fort Artkinson, Wisconsin.

vaccination, calves in Groups A & B developed a slight temporary transient anemia, which was evidenced by drop of hematocrit values to 29%. This anemic state lasted for a week and all calves in Groups A & B recovered from anemia by returning to the normals hematocrit values of 32 to 35% per Group, at the time of tick exposure. The complement-fixing antibody titer response of calves in Groups A & B were not statistically significant. The daily weight gains of the cattle in Groups A & B at time of vaccination were essentially the same as in non-vaccinated control calves.

The results of hematologic and serologic immune responses of calves injected with killed - Babesia spp. vaccine/AG-E injected with or without Freund's complete adjuvant is shown in Figure 1 (Group C & D). The calves in Group C injected with killed - Babesia spp. AG-E vaccine developed a transient anemia which was evident by drop of hematocrit values to 26% at two weeks after vaccination. However, calves in Group D had delay in showing anemia for one week. All calves recovered from this anemic state at time of field exposure. The complement-fixing antibody titers and the daily average body weight gains were not statistically significant between the two Groups. All calves in Groups (A, B, C, D) were 5 months old at the time when they were exposed to Boophilus microplus ticks naturally infected with B. bigemina and B. argentina. Significant hematologic serologic immune responses were not observed among different Groups of calves injected with various types of killed - Babesia spp. vaccines.

Immune Responses of Vaccinated Calves to Field-Challenge:

The results of hematologic and serologic immune responses of calves to tick-borne Boophilus microplus naturally infected ticks with Babesia bigemina

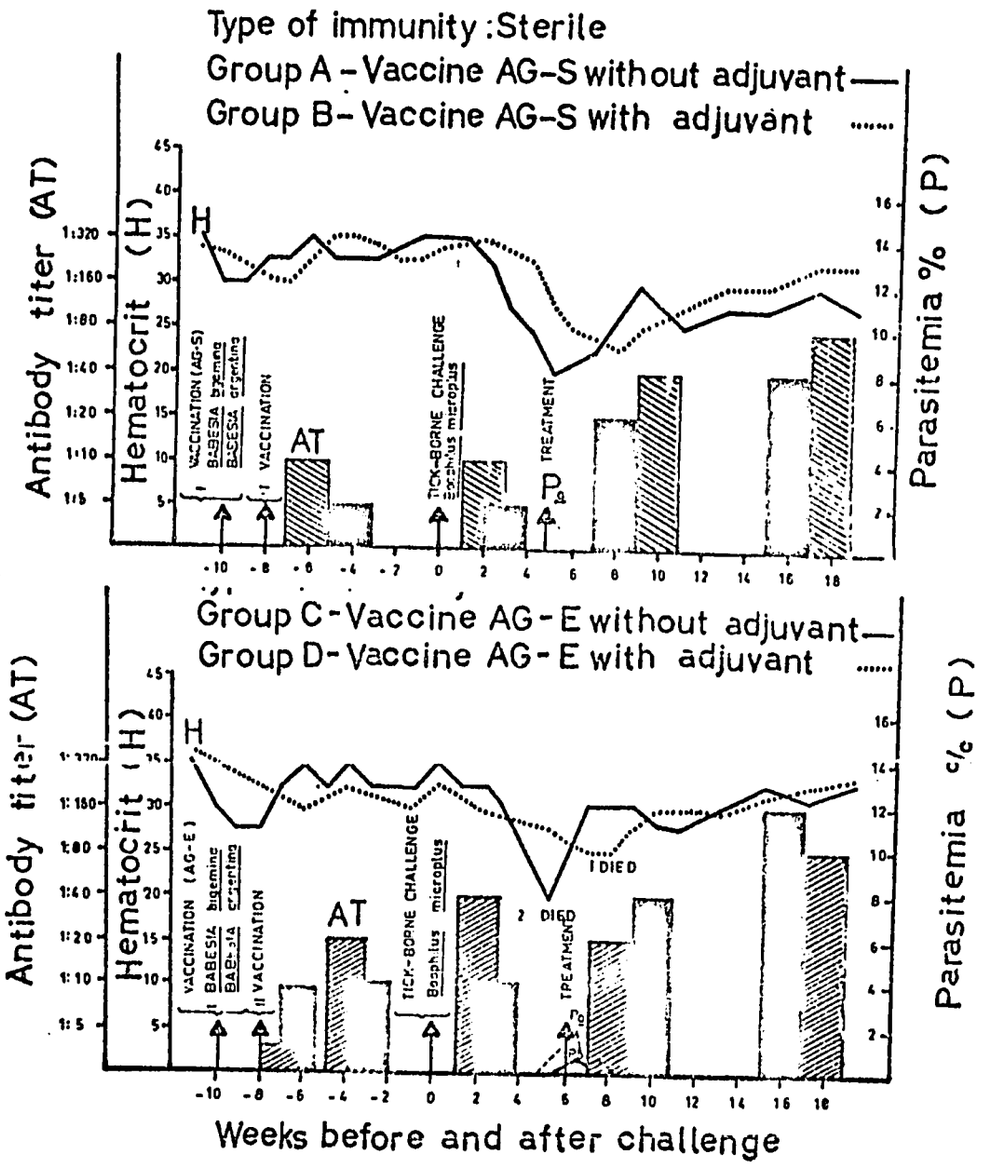

Fig. 1. Hematologic and serologic responses of cattle injected with killed Babesia spp. vaccine AG-S (infected plasma) Groups A & B; and AG-E (infected erythrocytes) Groups C & D. Graphic representation of mean values for anemia (H), parasitemia (P), and antibody titer (AT) and their relationships to vaccination and tick-borne (Boophilus microplus) challenge.

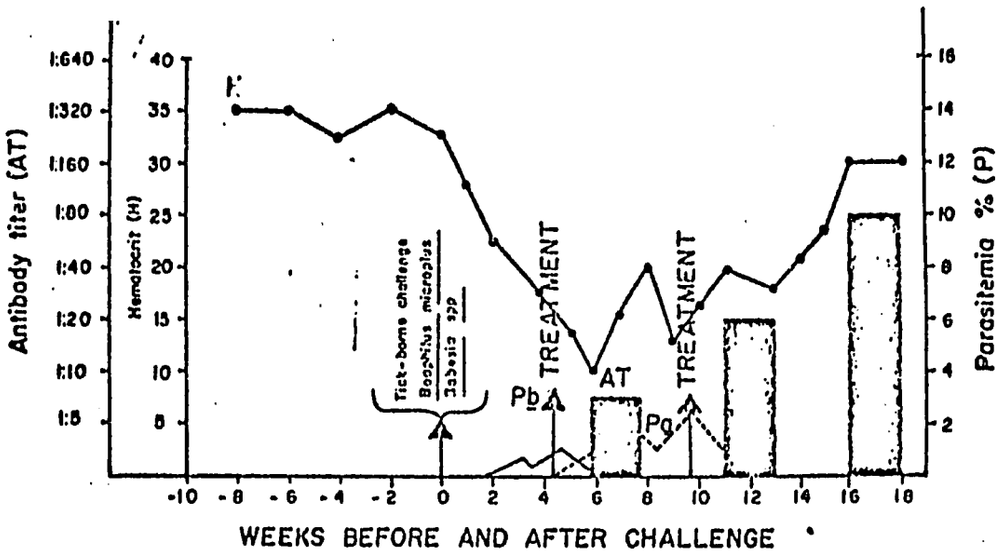

Fig. 2. Hematologic and serologic responses of five control calves exposed to Boophilus microplus ticks naturally infected with Babesia bigemina and Babesia argentina. Graphic representation of mean values for anemia (H), parasitemia (P), and antibody titer (AT) and their relationships to challenge and treatment.

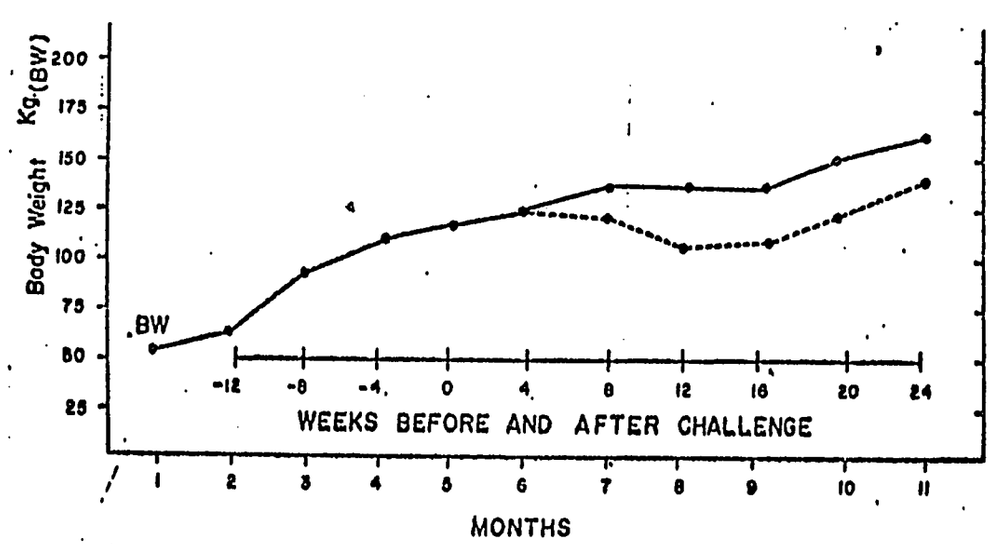

Fig. 3. Average body weight of vaccinated calves with killed - Babesia AG-S vaccine (continued line) in comparison with non-vaccinated control calves (dotted line). Time of tick-borne Boophilus microplus challenge is indicated as week 0. The vaccine was injected 10 weeks before tick-borne challenge. Age of calves is indicated by months on the abscissa.

and B. argentina at Palmira, Cauca Valley, are shown in Figure 1.

The average of hematocrit values at the time of field exposure was essentially the same in all groups. Calves in Group A & B vaccinated with killed Babesia spp. AG-S vaccine with or without adjuvant developed resistance to clinical babesiosis, which was evidenced with negative blood examinations for B. bigemina and B. argentina parasites. In comparison with these groups, non-vaccinated control calves had parasitemias with Babesia spp. at 2 to 3 weeks after field exposure. Four to 5 weeks after field exposure, calves in control as well as in vaccinated groups developed signs of anaplasmosis, which was evidenced by an Anaplasma marginale parasitemia, drop of hematocrit values. In order to prevent further complications, all calves were treated with oxytetracycline against anaplasmosis infection. During 18 weeks of observation not a single calf had any evidence of babesiosis infection. No significant differences were observed in hematologic or serologic responses in calves vaccinated with AG-S adjuvant and non-adjuvant vaccines. Twenty to 60 ticks were found on each calf during field exposure, and these ticks were idenified as B. microplus and Dermacentor nitens.

Five to 6 weeks after field challenge with B. mircoplus infected ticks, all calves in Group C and D also had signs of acute anaplasmosis infection which was evidenced by 2% A. marginale parasitemia, and only 1 calf (Group D) and 2 calves (Group C) had signs of babesiosis infection, which was evidenced with B. bigemina and B. argentina parasitemia. All calves (Group C and D) were treated against anaplasmosis with oxytetracycline to prevent losses; however, in spite of treatment there, calves died from acute anaplasmosis which was confirmed by necropsy findings.

Complement-fixing antibody titer were not statistically significant among the four groups of vaccinated calves, in comparison with non-vaccinated controled group of calves. All animals responded with rising complement-fixing antibodies titers of 1 : to 1:160 and the titers persisted during the time of experimentation. Average daily weight gains of calves were only statistically significant from control non-vaccinated calves for Groups A & B. All other Groups of calves did not have significant differences in daily weight gain from control calves at 10 months of age, when the experiments were terminated.

Immune Responses of Non-vaccinated Control Calves to Tick-Borne Challenge:

The results of hematologic and serologic immune responses of non-vaccinated control calves to tick-borne *Boophilus microplus* field challenge are shown in Figure 2. At the time of tick-borne challenge all 5 calves were in good physical condition which was evident by normal hematocrit values of 33%. However, after 2 to 4 weeks after exposure to naturally infected ticks with *B. bigemina* and *B. argentina* all calves were found infected with these organisms. Two calves died due to acute babesiosis and the further death losses were prevented by specific treatment with Ganaseg (Berenil). At the time of acute infection, the average hematocrit values were approximately 12%. Two weeks after the babesiosis attack clinical signs of anaplasmosis were evident. *Anaplasma marginale* parasites were detected in blood smears stained by Giemsa, and the low hematocrit values were noted. Parasitemia with *A. marginale* was 0.2 to 2% average during 4 to 8 weeks after field exposure. All animals were specifically treated

with Oxytetracycline 12 mg/kg to prevent death losses due to anaplasmosis. Complement-fixing antibody responses were not statistically significant between control and 4 groups of vaccinated calves.

Responses of Vaccinated Calves in Body Weight.

The effect of vaccination against bovine babesiosis with killed *Babesia spp.* vaccines (AG-S) in body weight of vaccinated cattle is shown in Figure 3. The first difference in body weight was evident after 4 weeks at field challenge between control and vaccinated calves. Thereafter, the average daily weight gains were significantly different as a result of resistance to *B. bigemina* and *B. argentina* infections. Vaccinated animals with AG-S vaccine gained 25 kg of body weight more than non-vaccinated control calves during the 11 months of observations.

DISCUSSION

Control of bovine babesiosis involves control of the tick vectors by dipping or premunition of cattle by injecting them with infected blood followed by specific chemotherapy to prevent post-vaccination reactions. The premunition is somewhat hazardous in itself due to perpetual infection, and losses without aftercare might be considerable (Riek, 1968). This work introduced a new approach to control bovine babesiosis in susceptible cattle with production of sterile immunity. This procedure might be of practical application in countries where babesiosis is enzootic or in the tropical zones of the world where cattle are at the permanent risk of exposure to babesiosis (Todorovic *et al.*, 1969).

This type of immunity will protect them until they develop solid acquired resistance.

The theoretical concept for these experiments was based on the possibility that sterile immunity might be induced against bovine babesiosis (Callow, 1967; Mahoney, 1967; and Mahoney and Goodger, 1972). Although sterile immunity is not usually encountered in blood protozoan infections according to Neitz (1957), it does, however, occur naturally in Theileria parva infections. Sterile immunity was reported in malaria in rats which had recovered from an infection with Plasmodium berghei (Corradetti, 1950); however, attempts to produce a sterile immunity with killed Plasmodium parasites had resulted in only partial protection. Following immunization of rats with crude P. berghei antigens, Zuckerman et al. (1965) obtained a moderate degree of resistance; whereas, Corradetti et al. (1966) did not. The inconclusive results obtained with killed vaccines have been attributed to the loss or denaturation of a soluble antigenic fraction during the preparation of the vaccines. Furthermore, the killed parasites may have been too rapidly destroyed in the host to elicit a discernible protective effect. Sinton (1939) postulated that amount and the rate of development of immunity to malaria may depend upon the amount and duration of antigenic stimulation. It has also been demonstrated following experimental infection and cure in a number of mammalian and avian malaria (Corradetti, 1950). Some evidence shows that P. berghei infected mice cured by drug therapy become resistant to a challenge infection (Box and Gingrich, 1958).

Todorovic et al. (1967) reported sterile immunity in avian malaria after inoculating birds with ex-plasmodial antigens isolated from the serum

collected from acutely infected birds with Plasmodium gallinaceum. Vaccinated birds developed a high degree of immunity and survived a high challenge dose of parasite, although these birds developed parasitemias but not other pathologic signs of disease. A disease comparable in some aspects with malaria is bovine babesiosis, so the experience obtained in developing a resistance against Plasmodium could be exploited in developing a resistance against Babesia infection since the mechanism of immunity in both blood infections is similar.

The principal facts of resistance immunity which are encountered in infection with Babesia spp. are: (1) natural resistance, (2) innate species resistance, (3) idiopatic resistance and (4) acquired resistance or immunity. The acquired immunity in bovine babesiosis can be defined as the immune state of an animal resulting from exposure to Babesia spp. parasites. If Babesia parasites are used to infect an animal and specific chemotherapy is administered shortly thereafter to destroy the infectious agent, the residual immunity which persists after the hemoparasite have been eliminated was called sterile immunity. The second type of sterile immunity results from exposing a susceptible Babesia-free animal to killed Babesia organisms or their antigens.

Not many Babesia spp. preparations have been investigated as vaccine materials in the past. In general, two sources of antigenic materials were reported: (1) the infected erythrocytes and (2) plasma collected from acute infection Ferris et al. (1968), Ristic et al. (1971). The preparations were obtained by treatment of infected blood by a variety of physical and chemical including among these irradiation, lysed by saponin, freezing and thawing, heat

inactivation, and from infected plasma by ammonium salts precipitation, column chromatography and differential centrifugation. The freeze-dried technique, one of the simplest procedures, appears to have been the least investigated.

Studies of the production of killed Babesia spp. vaccines have been complicated by the intracellular habitat of the B. bigemina and B. argentina parasites and the lack of an in vitro system for propagation and cultivation Gonzalez et al. (1971). The mechanism of immunity to Babesia infection is not clear, and it has not been definitely ascertained whether the host recognized the parasitized erythrocytes or the parasite alone. Although Babesia antigens have been detected free in the plasma of acutely infected animals and in the infected erythrocytes, the role of these antigens in production of sterile immunity has not been investigated.

The results of vaccination with killed Babesia spp. vaccine at Palmira, Colombia suggest the possibility of using it in zones where bovine babesiosis occur; however, conditions must be such that the introduction of infected ticks must be controled so that the exposed animals with sterile immunity develop consequently coinfectious immunity. Results of experiments in Palmira indicated that calves vaccinated with killed Babesia spp. vaccine (AG-S) developed a resistance to field challenge with B. microplus ticks infected with B. bigemina and B. argentina, while non-vaccinated calves suffered a clinical babesiosis under the same field conditions. Vaccine AG-S has proven to stimulate better protection than killed Babesia spp. vaccine (AG-E) produced from the infected erythrocytes. Vaccination with the killed Babesia AG-S vaccine, or without adjuvant, produced a characteristic complement-fixing antibody response with no parasitemina, and a slight reduction in hematocrit values.

Following tick-borne challenge with B. microplus naturally infected with Babesia spp., no losses occurred in the vaccinated calves; however, non-vaccinated control calves suffered acute babesiosis and needed specific treatment to survive. Following field challenge, difficulties were encountered with A. marginale infection resulting in some death losses. Although some vaccinated calves with AG-E vaccine suffered clinical anaplasmosis and 3 died in these groups, the results of vaccination are somewhat inconclusive; however, evidence of resistance observed in calves vaccinated with killed Babesia spp. vaccines clearly indicates the existence of sterile immunity. The degree of resistance was different between calves which received the AG-S vaccine and those the AG-E vaccine. In calves injected with the AG-S vaccine, the degree of resistance was more pronounced. This was evidenced by the lack of detectable parasitemias in all these animals.

The protection of cattle against Babesia infection produced in a response to killed Babesia vaccines demonstrated the importance of humoral factors in the mechanism of acquired immunity. Although bovine babesiosis is a disease in which immunity almost certainly depends on the presence of infection, the demonstration of the presence of protective antibodies to B. bigemina and B. argentina in the serum of infected cattle was regarded as evidence that the humoral mechanism was also an important factor in acquired immunity to babesiosis (Mahoney, 1967). The possibility of stimulating the production of protective antibody by vaccination is supported by this investigation. A suspension of infected erythrocytic lysate and plasma were freeze-dried and used as the vaccine preparations. Freund's complete adjuvant was used as a vehicle for the intramuscular administration of killed Babesia spp. vaccines to cattle, because in similar experiments with B. argentina a greater degree of protection had been stimulated (Mahoney, 1967). However, our results indicate that killed Babesia AG-S vaccine alone is highly immunogenic.

The freeze-dried inactivated B. bigemina and B. argentina as described here is noninfective and capable of imparting a level of protection against tick-borne babesiosis. These findings are similar to those obtained by Mahoney (1967) and Mahoney and Goodger (1972) involving immunization by killed B. argentina vaccine. The type and degree of protection against B. bigemina and B. argentina in cattle was essentially the same as that reported for calves following vaccination with killed B. argentina vaccine (Mahoney and Goodger, 1972).

In all experiments with intact, 5-month-old calves, the animals injected with killed Babesia spp. vaccine showed a higher degree of resistance to subsequent field challenge than the controls. This was particularly evident in the first (Group A) and second (Group B) experiments in which calves showed little or no general clinical signs after challenge. In contrast, the controls were obviously sick during the acute phase of the disease and 3 deaths occurred. The difference in the amount of erythrocytic destruction as indicated by reduction of hematocrit values between the immunizing and control groups was significant and this was the most reliable measure of protection.

None of the non-injected cattle were protected against field challenge. All unprotected animals developed progressive severe diseases characterized by high parasitemias and severe anemias. The vaccine protection appears to be caused by specific Babesia antigens; however, many of the factors involved in immunization against the blood stage of Babesia vaccination are still in their earliest stages of research. The studies described here promise to contribute much to these facets of Babesia vaccination technology and mechanism of immunity.

Degree of resistance produced by killed Babesia spp. vaccines is not the same mechanism as in co-infectious immunity, but the sterile immunity is sufficient to protect cattle from acute diseases until the animal's body develops solid immunity and maintains it by regular superinfection with infected ticks. The importance of frequent superinfection to promote adequate protective anti-

body levels is even necessary in cattle in artificially induced co-infectious immunity (Mahoney, 1967).

The protection afforded to cattle by injection of killed Babesia AG-S vaccine alone was the same as with the adjuvant vaccine. The successful immunization of intact calves (Group B) without adjuvant indicated that the killed Babesia spp. vaccine may be immunogenic alone when given by the intramuscular route. Mahoney and Goodger (1972) studied the immunological reactions of calves inoculated with plasma collected from acutely infected calves with B. argentina. Their study was directed to ascertain the general nature of the antigen contained and its possible role in the development of acquired immunity. The authors found that complete protection, which was characterized by an abrupt removal of parasites from peripheral blood, was found only in experiments in which the parasites used to artificially challenge immunity were antigenically identical with those that provided for immunization in the infected plasma. This finding was supported with data from our observation.

The results of experiments designed to ascertain the mechanism of sterile immunity to bovine babesiosis strongly suggest that the immunity to B. bigemina and B. argentina in cattle can be produced by injecting cattle with killed Babesia spp. vaccines. The protection was more evident in calves injected with AG-S vaccine prepared from plasma collected during the acute stage of infection than from AG-E vaccine prepared from the infected erythrocytes with B. bigemina and B. argentina.

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DEVELOPMENT OF RAPID AGGLUTINATION TEST FOR DIAGNOSIS OF BOVINE BABESIOSIS

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SUMMARY

Splenectomized calves were treated with an immunosuppressive drug (Azium) before they were infected with Babesia bigemina or Babesia argentina. These calves were used as a source of infective blood for isolation of B. bigemina or B. argentina agglutinating antigens for the diagnosis of bovine babesiosis. By means of a rapid agglutination technique it was possible to detect specific Babesia spp. antibodies in serum of cattle experimentally infected with B. bigemina or B. argentina when the antigen was used within 20 days after preparation and stored at 3-5° C. However, non-specific agglutination was observed with antigen stored more than 20 days at refrigeration temperature. Experiments are in progress to evaluate different systems of antigen preservation and application of the rapid agglutination test for diagnosis of cattle naturally infected with babesiosis.

INTRODUCTION:

Although bovine babesiosis has been recognized as a serious economic disease of cattle for more than 80 years (Babes, 1888) a practical method of diagnosis and an effective control program have not yet been achieved. This hemoprotozoan malady causes considerable production and death losses of cattle especially in lowlands of subtropical and tropical areas of the world where there are ticks. The identification of the acutely infected animals has not presented a problem because the Babesia spp. parasites can be easily detected in Giemsa stained blood films. However, if animals are latently infected the diagnosis presents a difficult problem, since the blood from these animals does not contain sufficient number of Babesia spp. parasites upon which to base diagnosis. Therefore, a great deal of the past interest has been directed toward development of serologic techniques for the diagnosis of bovine babesiosis.

In the last decade several serologic tests have been developed for detection of specific Babesia spp. antibodies in cattle. Complement fixation (Mahoney, 1962; Mahoney, 1967; and Todorovic et al., 1971); agglutination (Curnow and Curnow, 1967; Lohr and Ross, 1969; and Goodger, 1971), fluorescent antibody (Ross and Lohr, 1968; Goldman et al., 1972; and Joyner et al., 1972), and precipitation (Todorovic et al., 1971) have been reported. These serologic tests showed a high degree of specificity for each Babesia spp. parasite, and due to the different sensitivity of each technique, antibodies can be detected for varying periods after infection depending on the test used. The complement fixation test which has the lowest sensitivity may reliably detect antibodies to B. argentina for 7 months and to B. bigemina for 4 months after a single infection (Mahoney and Ross, 1972). With indirect fluorescent antibody and passive hemagglutination tests, periods in excess of a year have been reported (Ross and Lohr, 1968; Johnston and Tammemagi, 1969; Goodger, 1971; and Joyner et al., 1972). Because of reinfection under natural conditions, the loss of detectable antibodies should rarely occur after the first infection has been established (Mahoney and Ross, 1972). All these tests are tedious to perform and considerable skills, techniques and equipment are required. Therefore, there is an evident need for a rapid serologic test which can be applied under field and laboratory conditions for the diagnosis of bovine babesiosis.

MATERIALS AND METHODS:

A. Babesia spp. Parasites:

Babesia bigemina and B. argentina were isolated from the naturally infected cattle in Texas (Kuttler, 1972). Blood was collected from these infected animals in citrate as an anticoagulant and stored at -196° C. before used in this

experiment. Freezing was accomplished by the following method. After separation of plasma, the infected erythrocytes were resuspended in sterile veronal buffer pH-7.2, and washed once with buffer. After washing the sedimented erythrocytes were resuspended in sterile 4M DMSO and frozen at -196° C. in a liquid nitrogen container.

B. Babesia spp. Parasitemia:

In order to produce a high percent of Babesia spp. parasitemia, a splenectomized calf (C-532) was inoculated (IM) with Azium* at a dosage of 0.8 mg/kg. Two days later, the calf was injected with the same drug at a dosage of 0.2 mg/kg of body weight. This treatment was repeated on 3 consecutive days. Six days after the first treatment with Azium, the calf was inoculated (IV) with 500 ml of citrate blood collected from a carrier calf (C-327) of B. bigemina. Twenty-four hours following blood inoculation, the calf first had B. bigemina in blood smears stained with Giemsa. Blood was collected from this calf every 3 to 4 hours and B. bigemina was recorded as follows in Table 1.

C. Isolation of B. bigemina Antigens:

Blood was collected from calf (C-532) at the time of 22% B. bigemina parasitemia by cannulation of arteric carrotis in EDTA** (1.2 g/liter) as an anticoagulant. Five liters of blood were collected. Blood was stored at refrigeration temperature (3-5° C.) before processing. Plasma was separated by centrifugation in a Sorvall refrigeration centrifuge at 3000 r.p.m. for 20 minutes. The sedimented infected erythrocytes were then washed twice in sterile physiologic saline (0.85% NaCl). The infected erythrocytes were lysed

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*Azium - Schering Corp., Bloomfield, N. J.

**EDTA - Baker Chemical Co., Phillipsburg, N. J.

Table 1. Development of B. bigemina Parasitemia in a Splenectomized Calf (C-532) Treated With Azium Before Infection.

TIME OF INJECTION (Hours)	PCV	PARASITEMIA
0	29	Negative
12	28	Negative
24	26	0.01% B. bigemina
26	26	0.1% B. bigemina
30	25	0.5% B. bigemina
40	25	1% B. bigemina
44	24	2% B. bigemina
48	24	5% B. bigemina
53	23	12% B. bigemina
56	21	15% B. bigemina
60	19	20% B. bigemina
62	18	22% B. bigemina

with 0.35% NaCl according to the technique described by Mahoney (1967). After lysis, the lysite was centrifuged at 6000 x g in the Sorvall centrifuge, the supernatant was discarded, and the sediment was washed once in a sterile antibiotic-saline solution (Pen + Strep). When this sediment was stained by the Giemsa technique it consisted of concentrated B. bigemina parasites. Antibiotic-saline solution was used for preservation of Babesia spp. parasitic suspension used for preparation of the card antigen. One portion of this suspension was stored at 3-5° C., while the other was frozen in a Revco freezer at -60° C.

D. Preparation of B. bigemina Test Antigen:

On the day of testing samples for the presence or absence of Babesia spp. antibodies, the Babesia card antigen was prepared from the frozen parasitic suspensions stored in the Revco deep freezer at -60° C. To each milliliter of parasitic suspension 2 volumes of sterile antibiotic-saline solution was added. This suspension was centrifuged in the Sorvall centrifuge at 5000 x g for 30 minutes. The resulting supernatant was discarded and the sediment was resuspended in 10 volumes of antibiotic-saline solution to which 0.025 ml of 1% Fast Green Dye* was added. After storage for 2 hours, with occasional agitation, the suspension was recentrifuged, supernatant discarded, and sediment used for preparation of Babesia spp. card antigen. This stained suspension was diluted from 1:1 to 1:64, and each dilution tested for specific Babesia spp. agglutination reaction with the known positive and negative serum samples.

E. Preparation of B. argentina Antigen:

A similar technique was used for the preparation of B. argentina card antigen. A splenectomized calf (C-328) was used for the production of B. argentina high parasitemia. The calf was inoculated (IM) with Azium 3 times at a dosage of 20 ml. After the second treatment, 500 ml of citrate blood was inoculated IV. Blood was collected from a splenectomized calf (C-553) acutely infected with B. argentina. After blood inoculation, the calf was examined every 3 to 4 hours for the presence of B. argentina parasites. Because of the high pathogenicity of the B. argentina parasites and early signs of cerebral babesiosis, the inoculated calf was followed very closely to collect infected blood before sudden death due to peracute infection.

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*Fast Green Dye - Allied Chemical Co., N. J.

After 24 hours B. argentina were detected in blood films stained with Giemsa. The development of B. argentina parasitemia is recorded in Table 2. At the time of highest parasitemia, the calf was exauguinated by canulation of arteria carrotis, and 6 liters of blood were collected.

Table 2. Development of B. argentina Parasitemia in Calf (C-328) Treated With Azium Before Infection.

TIME OF INJECTION (Hours)	PCV	PARASITEMIA
0	28	Negative
12	23	Negative
24	21	2% B. argentina
26	20	3% B. argentina
30	19	4% B. argentina
40	17	6% B. argentina
44	16	8% B. argentina
46	15	12% B. argentina
48	13	15% B. argentina
54	11	19% B. argentina

F. Performance of Rapid Agglutination Test:

A similar technique for the anaplasmosis card agglutination test described by Amerault and Roby (1968) was used for the performance of the Babesia rapid card agglutination test. A Brewer diagnostic card* was used to place and mix 2 drops of tested serum and 1 drop of Babesia spp. card

*Brewer diagnostic card - Hynson, Westcott & Dunning, Inc., Baltimore, Md.

antigen. The card was placed on a card test rotator* and agitated-rotated for 5 minutes and read for agglutination reaction. Serum samples were collected from cattle artificially or naturally infected with Babesia spp. It was also possible to collect samples before and after blood-borne infection. Normal sera were collected from cattle never exposed to Babesia spp. or other blood diseases. In order to determine the specificity of the agglutination reaction, tests were performed on sera collected from cattle with known Anaplasma marginale infection. In addition to these serum samples, sera from animals inoculated with a large amount of blood was also tested. Serum samples used in a babesiosis card rapid agglutination test were not inactivated. Plasma samples were ~~not~~ tested.

RESULTS AND DISCUSSION:

Preliminary results of using a Babesia spp. card agglutination test for the diagnosis of bovine babesiosis are encouraging. The agglutination reaction is clear and different from a negative reaction (Figure 1). More than 200 samples with known histories of Babesia spp. infection were tested using an antigen stored several days at 3-5° C. and the results are shown in Tables 1, 2, 3, 4, 5, 6, and 7. It appears that Babesia spp. card antigen is highly specific when stored for a week at refrigeration temperature and can be used in a test satisfactorily; however, stored longer, the of non-specific agglutination reaction occur, especially with sera from animals injected with large amounts of blood. The problem of antigen preservation is obvious. Experiments are in progress to test different techniques for antigen purification and preservation.

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*card test rotator - Hynson, Westcott & Dunning, Inc., Baltimore, Md.

Table 3. Results of Rapid Agglutination Test for Detection of *Babesia spp.* Antibodies With *B. bigemina* Antigen Stored at 3-5° C. for 2 Days.

ANIMAL NO.	DATE OF SAMPLE	HISTORY	RESULTS
132	Nov. 27, 1972	*A. marginale* and *B. bigemina* Infection	(2)++
132	Dec. 4, 1972	Treated with Imidocarb - Nov. 28, 1972	neg.
245	Oct. 26, 1972	*B. argentina* - recent infection	(3)+++
245	Nov. 13, 1972	*B. argentina* - old infection	S
327	Dec. 4, 1972	*B. bigemina* Infection	(1)+
327	Dec. 11, 1972	*B. bigemina* Infection	(1)+
327	Dec. 18, 1972	*B. bigemina* Infection	(2)++
327	Dec. 26, 1972	*B. bigemina* Infection	(3)+++
327	Jan. 8, 1973	*B. bigemina* Infection	(3)+++
327	Feb. 6, 1973	*B. bigemina* Infection	(4)++++
857	Dec. 28, 1972	Finley Ranch	neg.
328	Nov. 13, 1972	Normal Calf	neg.
342	July 17, 1972	*B. argentina* (hemolysed)	(1)+
544	Dec. 4, 1972	Hemolysed Serum - *B. bigemina*	(1)+
502	Sep. 25, 1972	*B. argentina*	S
502	Oct. 2, 1972	*B. argentina*	S
502	Nov. 6, 1972	*B. bigemina* and *B. argentina*	(3)+++
502	Nov. 13, 1972	*B. bigemina* and *B. argentina*	(4)++++
522	Jan. 8, 1973	*B. bigemina*	(1)++
Control +(327)	Feb. 6, 1973	*B. bigemina*	(4)++++
Control -(N)	Feb. 6, 1973		neg.

Table 4. Results of Rapid Agglutination Test for Detection of *Babesia spp.* Antibodies With *B. bigemina* Antigen Stored at 3-5° C. for 3 Days.

ANIMAL NO.	DATE OF SAMPLE	HISTORY	RESULTS
65	Feb. 5, 1973	Normal	Negative
66	Feb. 5, 1973	Normal	Negative
67	Feb. 5, 1973	Normal	Negative
68	Feb. 5, 1973	Normal	Negative
69	Feb. 5, 1973	Normal	Negative
70	Feb. 5, 1973	Normal	Negative
132	Feb. 5, 1973	*B. bigemina* Infection	(1)+
245	Feb. 5, 1973	*B. bigemina* Infection	(2)++
328	Feb. 5, 1973	Normal	Negative
340	Feb. 5, 1973	*A. marginale* Infection	Negative
342	Feb. 5, 1973	*B. bigemina* Infection	(3)+++
368	Feb. 5, 1973	Normal	Negative
370	Feb. 5, 1973	*A. marginale* Infection	Negative
373	Feb. 5, 1973	*A. marginale* Infection	Negative
460	Feb. 5, 1973	*A. marginale* Infection	Negative
503	Feb. 5, 1973	*A. marginale* Infection	Negative
507	Feb. 5, 1973	*A. marginale* Infection	Negative
327	Feb. 5, 1973	*B. bigemina* Infection	(4)++++
Normal Bovine Serum		Normal	Negative

Table 5. Results of Rapid Agglutination Test for Detection of *Babesia spp.* Antibodies With *B. bigemina* Antigen Stored at 3-5° C. for 6 Days.

ANIMAL NO.	DATE OF SAMPLE	HISTORY	RESULTS
73	Jan. 18, 1973	Never exposed to babesiosis	Negative
286	Jan. 18, 1973	Never exposed to babesiosis	Negative
287	Jan. 18, 1973	Never exposed to babesiosis	Negative
289	Jan. 18, 1973	Never exposed to babesiosis	Negative
291	Jan. 18, 1973	Never exposed to babesiosis	Negative
295	Jan. 18, 1973	Never exposed to babesiosis	Negative
298	Jan. 18, 1973	Never exposed to babesiosis	Negative
539	Jan. 18, 1973	Never exposed to babesiosis	Negative
540	Jan. 18, 1973	Never exposed to babesiosis	Negative
542	Jan. 18, 1973	Never exposed to babesiosis	Negative
543	Jan. 18, 1973	Never exposed to babesiosis	Negative
547	Jan. 18, 1973	Never exposed to babesiosis	Negative
549	Jan. 18, 1973	Never exposed to babesiosis	Negative
532	Jan. 18, 1973	Never exposed to babesiosis	Negative
900	Jan. 18, 1973	Never exposed to babesiosis	Negative
499	Jan. 18, 1973	Never exposed to babesiosis	Negative
901	Jan. 18, 1973	Never exposed to babesiosis	Negative
902	Jan. 18, 1973	Never exposed to babesiosis	Negative
327	Jan. 18, 1973	*B. bigemina* Infection	(4)++++
Normal	Jan. 18, 1973	Normal	Negative

Table 6. Results of Rapid Agglutination Test for Detection of *Babesia spp*. Antibodies With *B. bigemina* Antigen Stored at 3-5° C. for 10 Days.

ANIMAL NO.	DATE OF SAMPLE	HISTORY	RESULTS
516	Feb. 5, 1973	Normal	Negative
517	Feb. 5, 1973	Normal	Negative
522	Feb. 5, 1973	Normal	Negative
539	Feb. 5, 1973	Normal	(1)+
536	Feb. 5, 1973	Normal	Negative
538	Feb. 5, 1973	Normal	Negative
540	Feb. 5, 1973	Normal	(1)+
542	Feb. 5, 1973	Normal	Negative
543	Feb. 5, 1973	Normal	Negative
327	Feb. 5, 1973	*B. bigemina*	(4)++++
Normal Serum			Negative
132	Nov. 25, 1972	*B. bigemina*	(3)+++
544	Dec. 2, 1972	*B. bigemina*	(2)++
522	Dec. 15, 1972		(3)+++

Table 7. Results of Rapid Agglutination Test for Detection of *Babesia spp.* Antibodies With *B. bigemina* Antigen Stored at 3-5° C. for 12 Days.

ANIMAL NO.	HISTORY	RESULTS
2164	Caldwell Ranch	(2)++
2202	Caldwell Ranch	(4)++++
2161	Caldwell Ranch	Negative
2234	Caldwell Ranch	(3)+++
2220	Caldwell Ranch	Negative
2173	Caldwell Ranch	Negative
2163	Caldwell Ranch	Negative
2150	Caldwell Ranch	Negative
2216	Caldwell Ranch	Negative
2188	Caldwell Ranch	(2)++
2165	Caldwell Ranch	Negative
2149	Caldwell Ranch	Negative
327	*B. bigemina*	(4)++++
27	Normal	Negative
5	King Ranch	(3)+++
15	King Ranch	(2)+++
21	King Ranch	(3)+++
27 Samples	King Ranch	Negative

Table 8. Results of Rapid Agglutination (RA) Test for Diagnosis of Bovine Babesiosis. The Relationship Between Parasitemia (P) and Agglutinins.

Days Before and After Infection	Animal Identification Number						
	299	903	904	496	497	498	361
	RA----P	RA----P	RA----P	RA----P	RA----P	RA----P	RA----P
-31	neg-neg	neg-neg	neg- neg	neg-neg	neg-neg	neg-neg	neg-neg
-24	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg
-17	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg
-10	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg
- 3	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg
0	Infected by blood inoculation (523) *Babesia bigemina*						
4	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg
6	neg-neg	neg-neg	neg-neg	neg-neg	neg-neg	neg +	neg +
8	+ +	neg-neg	neg-neg	+ +	+ +	+ +	+ +
11	+ +	+ +	+ +	+ neg	+ +	+ neg	+ +
12	+ +	+ +	+ +	+ neg	+ +	+ neg	+ neg
13	+ +	+ +	+ +	+ neg	+ neg	+ neg	+ neg
14	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg
15	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg
18	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg
21	+ neg	+ neg	+ +	+ neg	+ neg	+ neg	+ neg
25	+ neg	+ neg	+ neg	+ +	+ neg	+ neg	+ neg
28	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg
31	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg
35	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg
38	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg
42	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg	+ neg

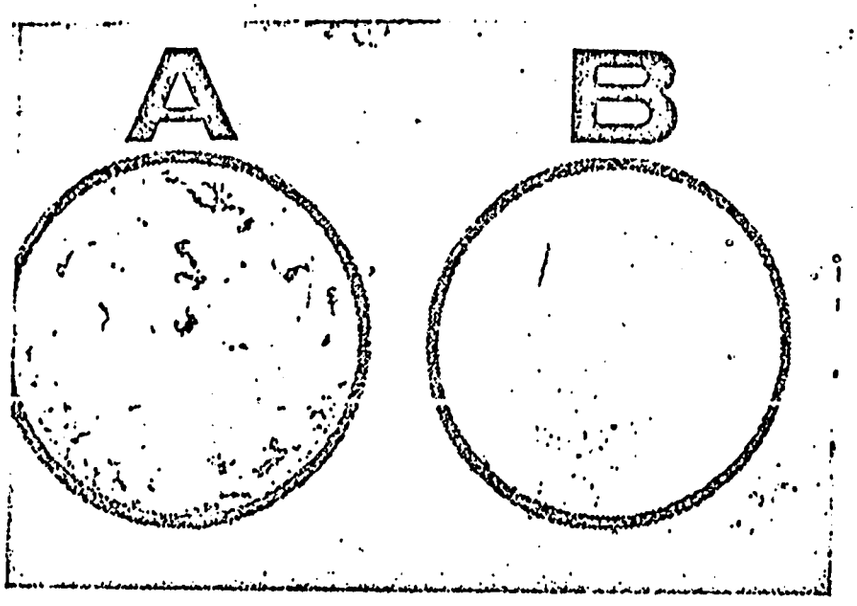

Figure 1. Pattern of agglutination reaction in a rapid agglutination test for the diagnosis of bovine babesiosis - positive (A), negative (B).

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Chemoprophylaxis (Imidocarb) Against *Babesia bigemina* and *Babesia argentina* Infections

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SUMMARY

Twenty-nine calves were used for the evaluation of chemoprophylactic effects of Imidocarb (3,3'-bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride) for bovine babesiosis. The compound was found to have prophylactic and therapeutic properties in calves artificially or naturally infected with Babesia bigemina and Babesia argentina of Colombian (South American) origin. Administered intramuscularly at a level of 2 mg./kg., Imidocarb suppressed the development of acute babesiosis in calves treated 46 days previously and later exposed to a lethal dose of Babesia spp. infected blood. Imidocarb failed to protect against Anaplasma marginale infection. Calves treated intravenously with 2 mg./kg. of Imidocarb and challenged 20 days later with a lethal challenge of Babesia spp. infected blood were protected. For 90 days after challenge, all calves were found negative for Babesia spp. parasitemia and also by blood-subinoculation into fully susceptible splenectomized calves. Calves intravenously treated 21 days previously with 3 mg./kg. Imidocarb resisted tick-borne challenge of Boophilus microplus. This resistance was evidenced for 15 weeks of field exposure by negative blood examinations and the mortality of non-treated calves from acute babesiosis. All calves treated with Imidocarb and subsequently exposed to blood or tick-borne Babesia spp. responded with a rise of complement fixing antibodies. Imidocarb readily controlled very severe acute infections with B. bigemina and B. argentina at dose rates of 1 mg./kg. by both intramuscular or subcutaneous routes. Acute signs of toxicity were observed following intravenous injections of 3 mg./kg. of Imidocarb. Three calves died following this injection with symptoms of embarrassed respiration, oral respiration, excessive salivation, muscular fasciculations, urination, defecation, incoordination, and prostration. Toxic signs were milder for intramuscular or subcutaneous injections of Imidocarb.

INTRODUCTION

The possibility of chemoprophylaxis against bovine babesiosis caused by Babesia divergens was investigated by Ryley[16], B. argentina by Newton and O'Sullivan[11], and B. bigemina and B. argentina by Callow and McGregor[5]. These investigators found that quinuronium derivatives had some prophylactic effect against Babesia spp., but not sufficient to prevent infection with these organisms in prophylactically treated cattle. Pipano[12] reported that diamidine derivatives given at a dose of 5 mg./kg. during the incubation period of infection with Babesia berbera had the effect of decreasing the severity of disease; however, the administration of the drug 4 days prior to the infection did not confer any protection.

Further search for the development of a new drug with longer prophylactic effect was obviously needed. Beveridge[2] reported that the compound now known as Imidocarb (3,3'-bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride, also known as 4A65-Burroughs-Wellcome Company) had a greater babesiacidal effect and a better chemoprophylactic index than quinuronium, diamidine, and amicarbalide derivatives against Babesia rodhaini in mice and rats. Imidocarb was selected from a series of carbanilides on the basis of screening tests against B. rodhaini by Schmidt et al.[17]

Following these laboratory observations, Imidocarb was found to have a high level of activity against B. bigemina and B. argentina in Australian cattle. Callow and McGregor[4] found that Imidocarb cured (sterilized) B. argentina at a dose of 2 mg./kg. but not at 1 mg./kg. It was found that B. bigemina was more susceptible to this compound. Imidocarb at a dose of 0.6 mg./kg. or more sterilized B. bigemina infections in splenectomized steers. The observations made by these authors that Imidocarb has a prophylactic effect has an important practical significance. Imidocarb injected at a dose of 2 mg./kg. reduced susceptibility of cattle to B. argentina infection for 33 days, and to B. bigemina infection for 10 weeks after treatment.

Roy-Smith[15] found that injection of 2 mg./kg. of Imidocarb at the time of tick-borne challenge prevented development of clinical sings of B. bigemina and B. argentina infections; however, treatment 36 days prior to infection was less effective. Brown and Berger[3] found that doses of 1 mg./kg. or more of Imidocarb given intramuscularly was effective in eliminating B. bigemina infection from cattle in Kenya. In Ireland, Wood[19] found that Imidocarb was very effective against B. divergens infections at doses of 1 mg./kg. for therapy and 2 mg./kg. as a prophylactic treatment. Hart et al.[7] reported that Imidocarb protected cattle from natural B. argentina infections for 44 days; however, protection against B. bigemina was not clearly demonstrated.

This paper describes the results of experiments carried out in Colombia to evaluate prophylactic effect, dosage, route of injection, and toxicity of imidocarb injected prior to infection with B. bigemina and B. argentina in cattle.

MATERIALS AND METHODS

Calves. Twenty-nine Holstein-Friesian calves 3 to 5 months of age were used as experimental animals. The calves were kept in a tick-free environment during the course of experimentation in Bogota (alt. 2,600 m.), but in Palmira, Cauca Valley, (alt. 1,000 m.), calves were exposed to infected ticks (B. microplus) 3 weeks after drug administration. Prior to the experimental or natural infection, all calves were screened for the presence of current or previous infection with B. bigemina, B. argentina, A. marginale, Trypanosoma vivax and Trypanosoma theileri, or other hemotropic infections. Blood samples were collected from all animals for complement fixation tests, preparation of stained blood films, and for inoculation into splenectomized calves.

Medication. The Burroughs-Wellcome Company experimental compound Imidocarb (3,3'-bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride) was used in these experiments. The drug, received as a water soluble salt, was dissolved in sterile distilled water as a 10% solution and used within 24 hours after preparation. This solution was administered intravenously, intramuscularly, or subcutaneously at the dose of 1 mg. to 3 mg./kg. according to the experimental approach of treatment.

Chemoprophylaxis. Experiment 1: Three 85 ±8 kg., male, 4-month-old Holstein calves at the Veterinary Medical Research Laboratory of the Colombian Agricultural Institute in Bogota were used in the preliminary trial for the prophylactic evaluation of Imidocarb. Two calves were given 3 mg./kg. of Imidocarb intravenously; the other calf was not injected and was used as a control. The 2 injected calves and the non-treated control calf were challenged 21 days after injection of Imidocarb with 100 ml. of blood containing 0.1% B. bigemina from a splenectomized calf acutely effected. At the time of blood collection, the calf had a packed cell volume (PCV) of 26%. Blood was collected in ethylenediaminetetraacetic acid (EDTA) disodium salt at 2 mg./ml. of blood as anticoagulant and was intravenously injected into all calves immediately after collection.

Experiment 2: Five 90 kg., male, 4-month-old Holstein-Friesian calves at the Veterinary Medical Research Laboratory of the Colombian Agricultural Institute in Bogota were used in this experiment. Four calves were given 2 mg./kg. of Imidocarb intramuscularly; 1 calf was not injected and was used as a control. All 5 calves were each challenged 46 days after drug treatment with 100 ml. of pooled blood containing 0.5% B. bigemina, 0.01% B. argentina, and 1% A. marginale. Blood was collected from 2 splenectomized calves infected with Babesia spp. and A. marginale respectively. The calf infected with Babesia spp. had a 23% PCV, and the calf infected with A. marginale had an 18% PCV at the time of blood collection for

challenges. Blood was collected in EDTA and injected intravenously into all calves immediately after collection.

Experiment 3: Four 90 ±7 kg., male, 3-month-old Holstein-Friesian calves at the Veterinary Medical Research Laboratory of the Colombian Agricultural Institute in Bogota were used in this experiment. Three calves were intravenously injected with 2 mg./kg. of Imidocarb, and the remaining calf was not inoculated, hence, served as a control. All calves were challenged 21 days after drug treatment with 100 ml. of blood containing 0.5% *B. bigemina* and 0.1% *B. argentina*. Blood was collected from a splenectomized calf which had a 20% PCV at the time of blood collection. The calf was acutely effected; blood was collected with EDTA as anticoagulant and was intravenously injected into all calves immediately after collection.

Experiment 4: Eight 80 ±6 kg., male, 4-month-old Holstein-Friesian calves at the Colombian Agricultural Institute Palmira Experiment Station in Palmira, Cauca Valley, were used for a field evaluation of Imidocarb. Seven calves were given 3 mg./kg. of Imidocarb intravenously, and the ramining calf was not injected and used as a control. All calves were kept in tick-free units for 21 days after drug treatment, and thereafter were exposed to field challenge with *B. microplus* ticks which were naturally infected with *B. bigemina*, *B. argentina*, and *A. marginale*.

Chemotherapy. Experiment 5: This experiment was carried out to determine the therapeutic effect of Imidocarb against bovine babesiosis caused by *B. bigemina* and *B. argentina*. These organisms were isolated from natural field cases located in enzootic areas for bovine babesiosis in Fusagasuga, Cauca Valley, and Monteria, Colombia. The blood was collected in EDTA solution from infected cattle and inoculated into splenectomized calves at the Veterinary Medical Research Laboratory of the Colombian Agricultural Institute in Bogota. When symptoms of acute babesiosis infection were evidenced by anemia (decreased PCV), parasitemia, hemoglobinuria,

and temperature elevation, the calves were treated with Imidocarb. Three calves were injected intramuscularly with 1 mg./kg.; 2 calves were injected intramuscularly with 2 mg./kg.; and 1 calf was injected subcutaneously with 1 mg./kg. Three infected calves were not treated with Imidocarb and used as controls.

Determination of Drug Efficacy. The prophylactic effect of Imidocarb was determined by resistance to artificial and natural infection with *B. bigemina* and *B. argentina*. The therapeutic effect of Imidocarb was determined by recovery from acute infection after drug administration. The recovery from *Babesia spp.* infection was determined by a return to the normal parameters measured. All claves were examined daily and weekly before and after treatment with Imidocarb for the following parameters: hematocrit values (H), percentage of parasitemia (P), temperature (T), complement fixing antibody titers (AT) when applicable, and mortality. The complement fixation antibody test was performed according to techniques described by Todorovic *et al.*[18] Observations were made on drug toxicity at the time of treatment and during the period of the experiment. Inoculation of 500 ml. of blood into susceptible splenectomized calves was carried out to determine the presence or absence of *Babesia spp.* infection before or after treatment.

RESULTS

Chemoprophylactic Trials. The results of these trials are recorded in Figures 1, 2, 3, and 4. Graphic representation of measured parameters and their relationships to the prophylactic effects of Imidocarb as related to the artificial and natural challenge with *Babesia spp.* are shown on each graph.

Experiment 1: After intravenous injection of Imidocarb at a dose of 3 mg./kg., all calves exhibited excessive salivation, lacrimation, micturation, dispnoea, defecation, muscular tremor, coughing, and prostration. The first signs were

Fig. 1. Hematologic responses of calves given (intravenous administration) imidocarb 21 days before blood-borne challenge with Babesia bigemina (Experiment 1). Graphic representation of anemia (H), parasitemia (P) and their relationships to the prophylactic effect of drug and blood-borne challenge. Calves 1 and 3 were chemoprophylactically treated and calf 2 not treated died 16 days after blood-borne challenge (Experiment 1).

Fig. 2. Hematologic and serologic responses of 4 calves given (intramuscular administration) imidocarb 46 days before blood-borne challenge with Babesia bigemina and Babesia argentina and Anaplasma marginale (Experiment 2). Graphic representation of mean values for anemia (H), parasitemia (A. marginale Pa), rectal temperature (Tam or Tpm), Treatment (T) and Babesia spp. CF antibody titer (AT), and their relationships to the prophylactic effect of imidocarb and blood-borne challenge (Experiment 2).

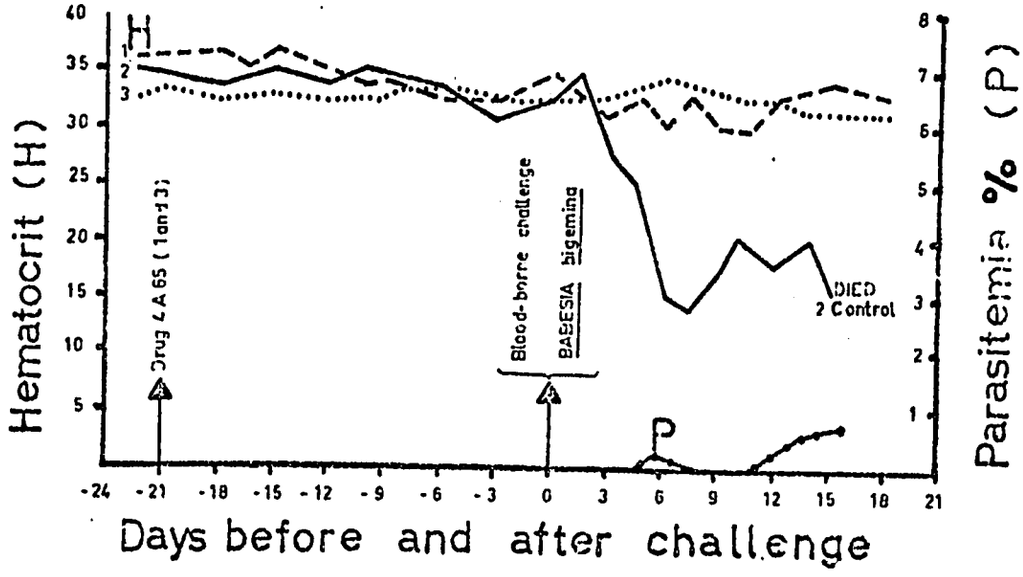
Hematocrit (H)
Parasitemia % (P)
Days before and after challenge
H
P
Drug LA 65 (1 and 3)
Blood-borne challenge
BABESIA bigemina
DIED
2 Control

Fig. 1

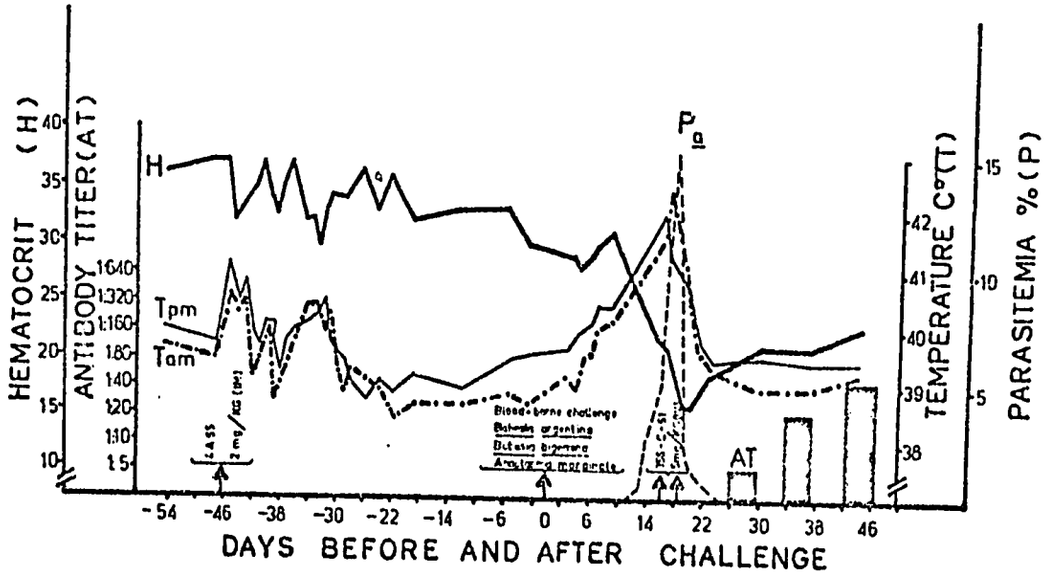
HEMATOCRIT (H)
ANTIBODY TITER (AT)
TEMPERATURE C°(T)
PARASITEMIA % (P)
DAYS BEFORE AND AFTER CHALLENGE
H
Tpm
Tam
Pa
AT

Fig. 2

Fig. 3. Hematologic and serologic responses of 4 calves given (intravenous administration) imidocarb 20 days before blood-borne challenge with Babesia bigemina and Babesia argentina (Experiment 3). Graphic representation of mean values for anemia (H), parasitemia (P), temperature (T), and Babesia spp. CF antibody titer (AT) and their relationships to the prophylactic effect of drug and blood-borne challenge. Control calf not chemoprophylactically treated died of babesiasis 42 days after challenge exposure (Experiment 3).

Fig. 4. Hematologic and serologic responses of 6 calves given (intravenous administration) imidocarb 21 days before tick-borne (Boophilus microplus) challenge. Ticks were naturally infected with Babesia spp. and Anaplasma marginale (Experiment 4). Graphic representation of mean values for anemia (H-continued line, and circles represent individual variation), parasitemia (Babesia spp.-Pb, and A. marginale Pa), and Babesia spp. CF antibody titer (AT) and their relationships to the prophylactic effect of drug and tick-borne challenge. Control calf not chemoprophylactically treated died of babesiasis 17 weeks after tick-borne challenge exposure (Experiment 4).

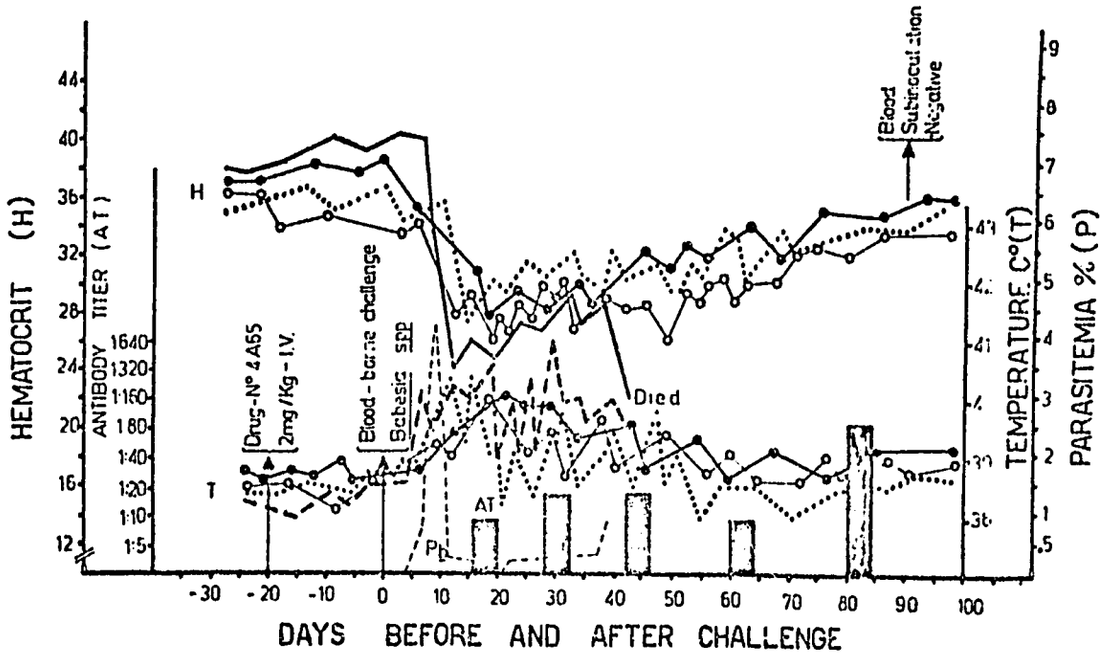
HEMATOCRIT (H)
ANTIBODY TITER (AT)
TEMPERATURE C°(T)
PARASITEMIA % (P)
H
T
Drug-N° 4 A65
2mg/Kg - I.V.
Blood - borne challenge
Babesia spp
Died
Blood Subinoculation Negative
AT
Pb
DAYS BEFORE AND AFTER CHALLENGE

Fig. 3

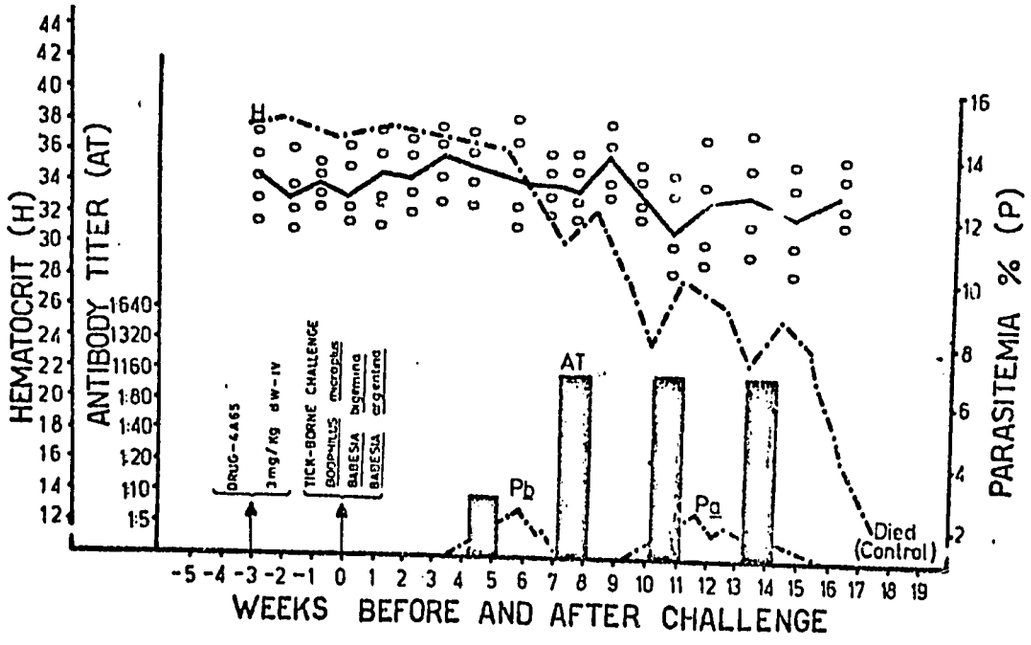
HEMATOCRIT (H)
ANTIBODY TITER (AT)
PARASITEMIA % (P)
H
DRUG-4A65
3mg/kg s.w-IV
TICK-BORNE CHALLENGE
BOOPHILUS microplus
BABESIA bigemina
BABESIA argentina
AT
Pb
Pa
Died (Control)
WEEKS BEFORE AND AFTER CHALLENGE

Fig. 4

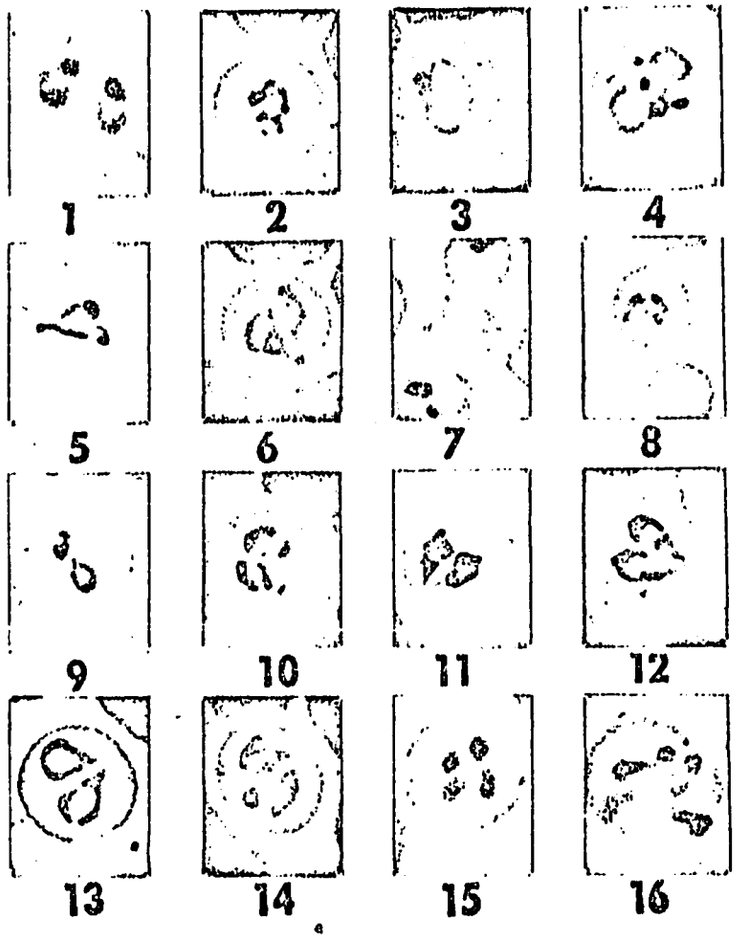

Fig. 5. Stained films of peripheral blood of Colombian cattle acutely infected with Babesia bigemina and Babesia argentina. The typical paired piriform body of Babesia bigemina lies outside of the erythrocyte (legend 1). Various forms, amoeboid, rounded or irregularly shaped of Babesia bigemina (legends 2, 3, and 4). Multiplication of Babesia bigemina in the erythrocytes occurs more frequently by budding than by binary fission (legends 5 and 6). Babesia argentina is a smaller form which lies in the center of erythrocytes; paired parasites form an obtuse angle (legends 7, 8, and 9). Babesia bigemina is a larger parasite; may assume various shapes, but most characteristically it is pear shaped, forming an acute angle in the erythrocyte (legends 10, 11, 12, 13, and 14). Multiple infection of a single erythrocyte with Babesia argentina (legends 15 and 16). Stained by Giemsa X 1000.

Table 1. Treatment of Acute Babesiosis with Imidocarb

Animals infected with *Babesia spp.*	Isolates of *Babesia spp.*	PCV %	Dose & Route	Observations
Calf 85 kg. *B. bigemina* - 6% *B. argentina* - 0.1%	Fusagasuga	13%	1 mg. S/C	Tremor, salivation, and urination at 8 minutes after injection of drug. Died the day following treatment.
Calf 76 kg. *B. bigemina* - 1% *B. argentina* - 0.5%	Fusagasuga	19%	2 mg. IM	Lacrimation, nasal and oral salivation, urination, defecation, dyspnoea, and cough at 10 minutes after injection of drug. Recovered with PCV of 34% 15 days following treatment. No parasites were observed day after the treatment.
Calf 54 kg. *B. bigemina* - 8% *B. argentina* - 0.5%	Cauca Valley	11%	1 mg. IM Repeated the day after	Lacrimation, nasal secretion, salivation, urination, increased dyspnoea, and cough at 10 minutes after injection of drug. Recovered with PCV of 29% 25 days after treatment. No parasites were observed the day after treatment.
Calf 83 kg. *B. bigemina* - 0.5% *B. argentina* - 0.01%	Cauca Valley	17%	2 mg. IM	Slight lacrimation and urination at 5 minutes after injection of drug. Recovered three weeks following the drug treatment with PCV of 30%
Calf 90 kg. *B. bigemina* - 12% *B. argentina* - 0.01%	Monteria	11%	1 mg. IM	Splenectomized calf. Dyspnoea, convulsion, and urination at 5 minutes after treatment. Died 15 minutes after injection of drug.
Calf 90 kg. *B. bigemina* - 5% *B. argentina* - 0.1%	Monteria	14%	1 mg. IM	Slight lacrimation, salivation, and urination. Recovered with PCV of 35% 4 weeks after drug treatment.

observed 1 to 2 minutes after drug inoculation. Calves were continuously watched for several hours after drug injection and thereafter watched sporadically for drug toxicity. Acute toxic effects abated at 12 hours in all calves, but they remained with roughened coats and refused food for a further 10 to 12 hours. No death losses due to drug toxicity occurred in this experiment. Daily examination of hematocrit values remained at normal levels. At day 0, or 21 days after drug treatment, all calves were injected intravenously with 100 ml. of blood infected with B. bigemina. At the time of challenge, all calves had hematocrit values of 35 to 36%. Four days after blood-borne challenge, the calf not injected with Imidocarb had a parasitemia of 0.5% with B. bigemina which persisted until day 16 when this calf died. Anemia was evidenced by low hematocrit values of 15% which coincided with the Babesia spp. parasitemia. The necropsy findings were typical for an acute babesiosis infection. Two calves treated with Imidocarb 21 days before blood-borne challenge with B. bigemina did not have any demonstrable parasitemia, and their hematocrit values remained at normal levels during the course of the experiment (Fig. 1).

Experiment 2: Calves injected intramuscularly with 2 mg./kg. doses appeared to tolerate Imidocarb better than calves injected intravenously. All calves had varying signs of toxicity, however, less pronounced than calves treated with 3 mg./kg. It was evidenced only with slight salivation and lacrimation. Apart from some roughness of the hair coat, they also showed a 1 degree Celcius rise of body temperature on the day of drug treatment and during the first week of treatment. A slight drop of hematocrit values at the time of temperature elevation was also observed. On day 0, or 46 days after drug treatment, all calves were exposed to blood-borne challenge with B. bigemina, B. argentina, and A. marginale. At the time of challenge, the calves had average hematocrit values of 30%, morning temperatures of 38° C., and afternoon temperatures

of 39° C. Thirteen days after challenge, all calves had an A. marginale parasitemia which reached 15% on day 18 when all calves were treated with compound 356C61 twice. Body temperature rose to 42° C. and hematocrit values dropped to 16% at the time of highest parasitemia. No demonstrable parasitemia was observed in peripheral blood films of all the calves treated with Imidocarb. The first rise of Babesia and Anaplasma complement fixing antibody titers was noted 26 days after blood challenges. Titers of 1:10 were cetected on 26 and then 36 and 46 days later (Fig. 2). The control calf, not treated and challenged with the same blood as the treated ones, died 17 days after challenge with signs of acute babesiosis and anaplasmosis and had a 4% parasitemia with B. bigemina, 0.3% with B. argentina, and 0.3% with A. marginale.

Experiment 3: Calves injected intravenously with 2 mg./kg. doses of Imidocarb had acute signs of toxicity. All calves had dispnoea, oral respiration, excessive salivation, lacrimation, muscular fasciculations, acute incoordination, urination, defecation, and coughing with mucous or bloody discharge from both nostrils immediately after drug treatment; symptoms were transient and disappeared within 24 hours. Calves refused food at the time of treatment. On day 0, or 20 days after drug treatment, all calves were exposed to blood-borne challenge with B. bigemina and B. argentina. Four days later, B. bigemina was found in the peripheral blood films of the non-treated calf; however, no detectable parasitemias were observed in all the treated calves. All calves had a drop in hematocrit values on day 10 after challenge and gradually recovered from this transient anemia. Body temperature was elevated and coincided with symptoms of anemia. Elevation of temperature was also transient and coincided with the temperature of the control calf which was infected with Babesia spp. The first complement fixing antibodies were detected at 15 days after challenge at a titer of 1:10 and this titer persisted during the time of observations. At 90 days of challenge, blood taken from each calf was inoculated into a

splenectomized calf and was not found to be infected with Babesia spp. The control calf, not treated with Imidocarb but challenged with the same dose of blood, died 40 days after challenge with symptoms of acute babesiosis (Fig. 3).

Experiment 4: Eight calves injected intravenously with 3 mg./kg. doses of Imidocarb had acute signs of drug toxicity. Three calves died immediately after drug treatment with symptoms of excessive salivation, lacrimation, dispnoea, oral respiration, muscle tremor, acute incoordination, prostration, urination, defecation, and coughing with bloody discharge from the nostrils. Five calves which recovered from drug toxicity refused food for 24 hours. On day 0, or 21 days after drug treatment, all calves were exposed to tick-borne challenge with B. microplus. At the time of exposure, all calves had hematocrit values in the range of 32 to 36%. After 25 days of tick exposure, B. bigemina and B. argentina were found in blood films taken from the non-treated calf. However, neither B. bigemina nor B. argentina were found in the peripheral blood films of calves treated with Imidocarb. All calves had a low level of A. marginale infection. The first complement fixing antibody titers were detected at 5 weeks after challenge at 1:10 and therafter 1:160 which persisted throughout the experiment. The untreated control calf had a Babesia spp. parasitemia followed by an A. marginale parasitemia, and finally died 17 weeks after tick exposure with acute babesiosis and anaplasmosis (Fig. 4).

Chemotherapy. Experiment 5: The results of this experiment are shown in Table 1. Six calves with acute babesiosis caused by B. bigemina and B. argentina were treated with Imidocarb. One calf treated with an intravenous dose of 1 mg./kg. and 3 calves treated with 2 mg./kg. doses of Imidocarb injected intramuscularly recovered from acute infection. The calves had symptoms of excessive salivation, lacrimation, dispnoea, muscular fasciculations, and prostration. Two calves

treated intramuscularly or subcutaneously with Imidocarb at doses of 1 mg./kg. died at the time of treatment due to the drug, or the effect of hemotropic disease, or both. Calves which recovered from babesiosis were not tested after treatment for the presence or absence of Babesia parasites. Calves did not relapse with babesiosis during the 3 month period of observations. Three calves acutely infected with the same isolates of B. bigemina and B. argentina and not treated with Imidocarb died from acute babesiosis.

DISCUSSION

Bovine babesiosis is still of great importance as a threat to the livestock industry throughout the world. Since the natural transmission of Babesia spp. parasites is dependent on ticks, disease can be eradicated only by an adequate tick control program.[13] In many countries a vector program can not be implemented for various reasons. At the present time, the only measure which can control the disease and prevent losses is an effective vaccination or chemotherapeutic program.[10]

The search for a new drug which can be administered into susceptible cattle as a prophylactic measure in shorter or longer terms before exposure is obviously needed. The introduction of such a drug as a prophylaxis of babesiosis will have a great practical application in the countries where babesiosis is prevalent.[16]

The mechanism of action of the majority of chemotherapeutic drugs available against babesiosis has not been studied sufficiently and their action is not known.[8] The same statement could be said for the new experimental compound Imidocarb which was found to have dual properties: (1) therapeutic and (2) chemoprophylactic. These properties undoubtedly exert multiple actions on the Babesia parasites as well as the infected host through various tissue mechanisms

which are not presently understood.[2]

Chemoprophylactic effects consist of the administration of a compound with prolonged residual action into cattle before exposure with Babesia parasites. This procedure prevents the development of the Babesia infection, completely stops the establishment of parasites in tissues, and protects the bovine host from invading Babesia parasites. Following the administration of Imidocarb to cattle, residues were found which persisted for long periods of time, and this probably resulted in the prophylactic effect observed. This could be an explanation of the prophylactic mechanism of Imidocarb. Wood[19] reported that Imidocarb has prolonged residual properties. He found that the drug persisted with a concentration of 0.01 μg./g. in bovine adipose tissue up to 8 weeks, and up to 12 weeks in muscle at 0.03 μg./g., kidney at 0.51 μg./g., and liver at 0.34 μg./g. He also studied the residue in bovine fetuses and found that Imidocarb persisted for 4 weeks in the liver at 2.4 μg./g. and kidney at 1.1 μg./g.

Although several compounds are known to be useful in the treatment of clinical babesiosis, there is not a commercially available drug showing prophylactic properties. In Australia, Callow and McGregor[4] recently reported that cattle treated with 2 mg./kg. of Imidocarb were refractory to infection with B. bigemina or B. argentina up to several weeks after treatment. This work confirmed their observation on the prophylactic effect of Imidocarb against B. bigemina and B. argentina of South American origin.

Pipano[12] reported that Berenil (Ganaseg) given to calves in doses of 5 mg./kg. during the incubation period of infection with B. berbera had the effect of lengthening the period of incubation and infection, and decreasing the severity of the disease. One or 2 doses did not interfere with the co-infectious immunity of recovered calves. The administration of the drug 4 days prior to infection did not produce any protection.

Ryley[16] tested the ability of sparingly soluble salts of quinuronium derivatives with known therapeutic activity to form a drug depot when subcutaneously injected into rats. The drugs which formed a depot were tested for their ability to prevent clinical babesiosis in splenectomized calves when the animals were later challenged with B. divergens. Encouraging but not satisfactory results were obtained with quinuronium suraminate, quinuronium 5,5'-methylene bis-salicylate, and quinuronium embonate.

Barnett[1] studied the chemotherapy of B. bigemina in a series of experiments in splenectomized cattle in which the effects of treatment, subsequent relapses, and immunity were noted. Amicarbalide in doses of 10 mg./kg. was effective, but the treated calves subsequently proved susceptible to reinfection. Co-infectious immunity followed the administration of 7.5 mg./kg. or less, but some fatal relapses occurred. Homidium bromide was active at 1 mg./kg. or more, but reduction of the number of parasites after treatment was slow and one severe relapse occurred after treatment. Phenamidine isothionate at 13.3 mg./kg. acted rapidly; co-infectious immunity followed treatment and clinical relapse never occurred with doses at 8 to 10 mg./kg. Acaprin at 1 mg./kg. acted rapidly, producing co-infectious immunity with clinical relapses; but with a dosage of 0.75 mg./kg. a fatal relapse occurred after treatment. Berenil (Ganaseg) in doses of 3.5 mg./kg. or less caused a rapid elimination of the parasites which were not demonstrable thereafter. The calves were immune for up to 109 days after treatment. Berenil at 5 mg./kg. produced a sterile cure and the calf was completely susceptible to reinfection 68 days later.

Newton and O'Sullivan[11] found that a 20% suspension of the quinuronium compound, 5,5'-methylene bis-salicylate, in arachis oil administered subcutaneously in 1 g. doses had a satisfactory prophylactic effect against B. argentina in young calves. A 2 g. dose produced acute toxic effects with fatal nephritis in some calves; however, protective effects were demonstrated in

calves that recovered. Babesia argentina was established in calves infected by inoculation with infective blood or by ticks, but none of the 31 calves injected with the drug 7 to 35 days previously developed clinical babesiosis; the control calves did. The control calves had significantly higher febrile reactions and the differences in the hemoglobin levels and packed cell volumes were statistically significant.

Callow and McGregor[5] reported that cattle treated with the babesiacidal depot compound, quinuronium 5,5'-methylene bis-salicylate, within the period 4 weeks before to 1 week after inoculation with B. argentina live vaccine had lower parasitemias than the untreated controls. Treated and vaccinated steers had adequate immunity 15 weeks after vaccination. Steers and splenectomized calves treated at the times of inoculation and onset of primary parasitemias, respectively, were not sterilized of babesial infection. No drug resistance developed in B. argentina passaged through 7 calves treated with the depot compound. Renal damage was observed in 4 calves that died 2 to 10 weeks after treatment.

Callow and McGregor[4] reported that Imidocarb readily controlled very severe infections with B. argentina at dose rates of 1 mg./kg. to 20 mg./kg. Babesia bigemina infections were treated successfully with dose rates of 0.4 mg./kg. to 20 mg./kg. Signs of toxicity were acute following intravenous dosages of 10 mg./kg. and 20 mg./kg. although the calves survived. Toxic signs were mild or absent for subcutaneous dosages of 10 mg./kg. and less. The Imidocarb completely cured (sterilized) infections with B. argentina in clinically recovered steers treated subcutaneously at dose rates from 2 mg./kg. to 10 mg./kg., but not at 1 mg./kg. The steers treated at 10 mg./kg. carried B. bigemina as well, and these infections were also sterilized. Splenectomized calves with acute B. bigemina infections were sterilized when treated subcutaneously at 0.6 and 0.4

mg./kg. The compound also reduced the susceptibility of cattle treated with it before infection. Protection was evident in a group of steers treated subcutaneously with Imidocarb at 2 mg./kg. 33 days before inoculation with B. argentina and was pronounced in groups treated 24, 23, and 13 days before inoculation. In tests of prophylaxis against B. bigemina, 2 calves receiving 2 mg./kg. were strongly protected 12 weeks after treatment and 3 others given higher dosages were refractory to infection up to 10 weeks after treatment.

The chemoprophylactic activity of Imidocarb against B. rodhaini in the mouse and rat is not exactly comparable with that against bovine Babesia spp. infections. The differences in prophylactic activity between Imidocarb and amicarbalide were negligible, except perhaps when they were administered within 1 week of challenge. When given within this period, the effect of Imidocarb was better than of amicarbalide.[2] According to Hart et al.[7] and Roy-Smith,[15] Imidocarb protected cattle against B. argentina up to 44 days; however, protection against B. bigemina was not clearly demonstrated. In additon, there is an implication that the Babesia spp. in blood-borne infected cattle lose their virulence and resistance to chemotherapeutic agents when maintained by serial passage in cattle.[10] This decrease may occur only after the second or third passage in intact cattle and after the sixth passage insplenectomized cattle. Different authors obtained different results in regard to the action of Imidocarb because Babesia spp. of different drug resistances were probably used.

Results of the experiments in which antibody responses of calves treated with Imidocarb and exposed to B. microplus infected ticks were similar to those obtained by Roy-Smith.[15] Complement fixing antibody titers were detected in treated calves without detectable parasitemias with Babesia spp. The increase of complement antibody took place at the same time as infection of untreated control calves. This method of antibody stimulation is a matter of speculation

and worthy of detailed investigation.[15] One explanation possible is that invading parasites from the tick were killed at the site of entry and due to their large numbers they produced antigenic stimulation, whereby the host responded with the production of antibodies which were detected by the complement fixation test.

The results of the experiment under field conditions in Palmira confirm the laboratory findings in Bogota of the prophylactic effect of Imidocarb against B. bigemina and B. argentina. These results are not in agreement with findings of Roy-Smith[15] that B. bigemina is somehow Imidocarb resistant, in view of the fact that the parasitemia was detectable after drug administration. The authors observed that some cattle had a detectable B. bigemina parasitemia, but none of them showed clinical signs of disease. In contrast, calves in the control group did show clinical signs following parasitemia.

Roy-Smith[15] reported the results of a series of 4 field trials. The chemoprophylactic effect of Imidocarb at a dose rate of 2.0 mg./kg. subcutaneously against B. argentina and B. bigemina infections was evaluated in the field trials by exposing treated and untreated cattle to natural infection. In these circumstances, untreated cattle developed clinical babesiosis with B. argentina. Treatment with Imidocarb at the time of exposure prevented development of clinical signs of the disease but permitted the development of Babesia antibodies. Treatment of a small group of cattle, 36 days before exposure was less effective.

Imidocarb was found highly effective against acute infections of B. bigemina and B. argentina by a number of authors (Callow and McGregor,[4] Brown and Berger,[3] Wood[19]), and this was supported by the results of the experiment reported in this paper. The mechanism of Imidocarb action is not clear. Imidocarb apparently acts directly on the Babesia parasites causing an alteration in the number, size,

vacuolation, and morphology of the nucleus and cytoplasm. These changes in Babesia parasites apparently occur due to the malfunction of the metabolic and enzymatic processes which ordinarily end in the death of the organism. Simultaneously, the drug causes changes in the tissue reactivity, stimulates the active mesenchime and reticuloendothelial system, increases the phagocytosis or the number of macrophages, helps to produce defensive substances, and increases the immunological properties of the host. Imidocarb effectively controls parasitemia; the day following injection, Babesia parasites disappear.

Severe toxic reactions of calves treated with Imidocarb found in our experiments are not substantially supported by the results of other investigators in respect to dosage of drug. We found that 3 mg./kg. injected intravenously killed 3 of 10 calves and that 2 mg./kg. injected subcutaneously or intramuscularly caused severe toxic reactions but did not kill calves. Callow and McGregor[4] reported that only 50 mg./kg. injected intravenously might be severely toxic; however, they found that only 1 of 6 calves dying within several hours of treatment appeared to die from drug toxicity. This calf received 50 mg./kg. intravenously for a B. argentina infection. Other calves were considered to have died from either very acute babesiosis or the effects of procedures involved in the collection of the blood vaccine. Two calves given Imidocarb intravenously at 20 mg./kg. and 1 at 10 mg./kg. showed incoordination, dyspnoea, and salivation but they recovered within 24 hours. The 8 steer treated subcutaneously at 10 mg./kg. were not affected. A local reaction taking the form of a flat swelling up to 15 cm. in diameter was seen in some of the calves treated subcutaneously at 5 or 10 mg./kg. The swelling gradually subsided without any external evidence of necrosis.

Brown and Berger [3] found that Imidocarb at doses of 10 mg./kg. injected intramuscularly can be toxic. They reported that 1 of 12 calves injected with

the above dose died after treatment. Calves showed acute symptoms of toxicity with varying degrees of salivation, hyperventilation, and lacrimation, the severity of which correlated with the size of dosage. An additional 8 calves which received total subcutaneous or intramuscular doses of 25 to 35 mg./kg. as repeated daily doses of 5 to 10 mg./kg. died of cummulative toxicity 8 to 14 days later. Critical observations of 24 calves, 6 to 15 months old, and 22 steers after administration of slow (2-6 minutes) and fast (10 seconds) intravenous doses of 0.125 upward to 4 mg./kg. of base or dihydrochloride showed that reactions were similar with both preparations. There were no cases of fatal toxicity and there were no obvious differences in the susceptibility of calves and steers to either base or dihydrochloride. In 56 calves observed, transient symptoms of salivation, lacrimation, hyperventilation, coughing, and frequent micturation were severe at 2 and 4 mg./kg. and moderate or slight correlated with the size of the Imidocarb dose and the route of injection. At 3 mg./kg. aggregate symptoms were moderate in both calves and adults, salivation and micturation being more evident in adults and mild distress in calves. At 5 mg./kg. symptoms, although transient, were more severe in calves, showing a greater tendency to distress and hyperventilation, while repeated micturation was more marked in adults.

Kuttler[9] also found that Imidocarb was responsible for excessive salivation, lacrimation, and labored breathing when injected intravenously into cattle. These side effects appear less severe when the drug is administered intramuscularly or subcutaneously and the efficacy was not altered. No mortality was observed in cattle when Imidocarb was injected intramuscularly 3 times at 24-hour intervals at a dosage of 6 mg./kg. or when the compound was injected subcutaneously once at 15 mg./kg.

Carbrey _et al._[6] suggested that the alarming side effects of Imidocarb could

be diminished by dividing the daily dose into 2 parts given within an interim of 3 to 6 hours. Roby and Mazzola[14] also found that Imidocarb dihydrochloride caused marked swelling and inflammation at the injection site in cattle. The authors suggested that this is due to the high acidity of the aqueous solution of Imidocarb; however, another form of Imidocarb, diproprionate salt, did not cause local irritation.

The potential use of Imidocarb as a prophylactic drug is indicated in a number of circumstances. To protect susceptible cattle moving from a tick- and *Babesia*-free country through a tick infected area, Imidocarb can be used to replace hazardous vaccination procedures with virulent *Babesia spp.* parasites. Furthermore, application of Imidocarb and exposure of cattle to infected ticks with *Babesia spp.* might help in the development of natural co-infectious immunity without hazardous losses.

Finally, on the basis of our results discussed herein, Imidocarb has proven to be highly effective in the treatment of acute babesiosis caused by *B. bigemina* and *B. argentina* of Colombian origin. In addition, the prophylactic effect of Imidocarb was effective for a longer period than any babesiacidal drug tested. However, toxic effects were found which might limit its application, but need to be determined.

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