Studies of the pathogenesis of experimentally induced bovine sarcocystosis

Dean Barnett
Iowa State University

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STUDIES OF THE PATHOGENESIS OF EXPERIMENTALLY INDUCED BOVINE SARCOCYSTOSIS

Iowa State University

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Studies of the pathogenesis
of experimentally induced
bovine sarcocystosis

by
Dean Barnett

A Dissertation Submitted to the
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DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

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GENERAL INTRODUCTION

Preamble

Sarcocystosis (sarcosporidiosis) in animals has been recognized for more than a century. The parasitism is common in domestic ruminants and has been found in numerous mammalian, avian and reptilian hosts. Sarcocysts, encysted protozoa within striated myofibers, traditionally have been the only recognized evidence of the infection. The life-cycle and mode of transmission of the parasite were demonstrated within this decade. Clinical disease and tissue inflammation with experimental sarcocystosis have been demonstrated. Simplified diagnostic methods must be developed before the economic significance of natural sarcocystosis in domestic animals can be determined.

A two-host, prey-predator type of protozoan life-cycle has been established for the genus *Sarcocystis*. *Sarcocystis cruzi* requires bovine and canine hosts for its life-cycle (Fig 1). The ox, *Bos taurus*, is the intermediate host and accommodates only asexual multiplication of the protozoan. The dog, *Canis familiaris*, is the definitive host and accommodates sexual reproduction of the parasite. Sporocysts, infectious for cattle, are produced by sexual reproduction of *S. cruzi* in the lamina propria of the canine intestine and are shed in feces. Bradyzoites in sarcocysts within bovine myofibers are the final product of 2 phases of asexual multiplication of *S. cruzi* in bovine tissues and are infectious for dogs.

Acute bovine sarcocystosis is the systemic febrile disease that accompanies schizogony, the initial phase of *S. cruzi* multiplication in all
Fig 1—Life cycle of *Sarcocystis cruzi*. Bradyzoites in sarcocysts ingested with muscle by dogs or related carnivores initiate sexual reproduction to produce sporocysts. Sporocysts ingested by cattle cause 2 phases of asexual reproduction. Merozoites, produced by schizogony in all organs, infect myofibers and give rise to bradyzoites in sarcocysts.
bovine organs. Merozoites develop within schizonts and are both initiators of and products of schizogony. Clinical signs of acute bovine sarcocystosis include fever, anorexia, weight loss, dehydration and muscle tremors. Severity of clinical signs range from mild to severe with recumbency and death. Abortion has been associated with acute bovine sarcocystosis.

Chronic bovine sarcocystosis is the wasting disease that accompanies the final phase of *S. cruzi* multiplication during sarcocyst development in bovine muscle. Metrocytes, the progenitors of bradyzoites, develop and multiply within cysts in myofibers. Sarcocyst maturation is accompanied by production of bradyzoites from metrocytes. Clinical signs of chronic bovine sarcocystosis include anorexia, cachexia, weakness, ataxia and patterned alopecia. No clinical signs have been associated with the enteric infection of dogs by *S. cruzi*.

Diagnostic confirmation of bovine sarcocystosis currently requires serological tests and histological recognition of schizonts in tissues. Interpretation of serologic titers is complicated by the ubiquity of *S. cruzi* in cattle. Schizonts are transient and often not demonstrable. *Sarcocystis cruzi* has never been cultured *in vitro*. Identification of a source of sporocysts provides epidemiological support for the diagnosis. A few cases of natural bovine sarcocystosis have been recently diagnosed. Development of direct, reliable and available diagnostic techniques is essential for further appreciation of the significance of natural bovine sarcocystosis.
Objectives

The objectives of this study were to (1) demonstrate and characterize developmental phases of *S. cruzi* in bovine tissues from the ingestion of sporocysts to development of mature sarcocysts, and (2) confirm the abortifacient effect of *S. cruzi* in pregnant cows and determine the pathogenesis of fetal death and abortion.

Thesis/Dissertation Format

This dissertation is presented in alternate format including 4 manuscripts to be submitted to scientific journals for publication. Two manuscripts will be submitted to American Journal of Veterinary Research, one to the Journal of the American Veterinary Medical Association, and one to Veterinary Pathology. All manuscripts are presented in the format required by American Journal of Veterinary Research except footnote notation which conforms to standard thesis format. References cited in each manuscript are included with that manuscript in journal format. The manuscripts are preceded by a general introduction to bovine sarcocystosis that includes a preamble, objectives of the studies, and a critical review of the literature. General discussion and conclusions from the studies follow the fourth manuscript. Additional literature cited refers to citations in the general introduction and general discussion and is listed in journal format consistent with that used in the manuscripts.

The PhD candidate, Dean Barnett, was the principal investigator for each of the studies and is the senior author for each of the manuscripts. Coauthors with direct but limited involvement in each study and specific
assistance from others are indicated in the by-line and footnotes of each manuscript.

Literature Review

Tubular cysts, containing numerous resident microorganisms, within striated muscle fibers were first described by Miescher in 1843.\(^1\) Miescher's tubules, sarcocysts, subsequently have been recognized in cardiac and skeletal muscle from numerous vertebrate hosts. The wide range of hosts is indicated in several comprehensive reviews of the literature.\(^2\)–\(^5\) The genus name *Sarcocystis* was introduced for these protozoan parasites of muscle by Lankester in 1882.\(^6\)

The coccidial nature of *Sarcocystis* was discovered when microgametogony, macrogametogony and cystlike bodies were found in cultured bovine kidney cells inoculated with protozoa from sarcocysts in grackle muscle by Fayer in 1972.\(^7\) An enteric phase of *Sarcocystis* was concurrently elucidated in Germany when isosporan oocysts and sporocysts were produced in carnivores fed sarcocysts in meat; in dogs fed sarcocysts in beef,\(^8\) in cats fed sarcocysts in beef,\(^9\) in man fed sarcocysts in beef or pork,\(^10\) and in cats fed sarcocysts in lamb.\(^11\) *Sarcocystis* schizonts in endothelial cells of several organs from calves fed sporocysts from dog feces were described in 1973 as evidence of systemic infection in cattle before muscle infection.\(^12\) An obligatorily heteroxenous, predator-prey type of life-cycle for the genus *Sarcocystis* was proposed,\(^13\) 130 years after the parasite was first described.

*Sarcocystis cruzi* (Hasselmann, 1926)\(^14\) has evolved, after years of nomenclature confusion, as the appropriate name for the *Sarcocystis* species
that has an alternating dog-cattle-dog cycle. Defense of the propriety of *S. cruzi* is presented in a review of the nomenclature of *Sarcocystis* in the ox and fecal coccidia of the dog by Levine. The name *S. fusiformis* (Railliet, 1897) commonly used for all sarcocysts in cattle originated with an erroneous citation; the originally reported host was the water buffalo, as noted by Babudieri in 1932. A biologically descriptive scheme for *Sarcocystis* nomenclature was proposed by German scientists to accommodate the enteric phase of infection in definitive hosts. The names proposed for *Sarcocystis* in cattle were *S. bovicanis*, *S. bovifelis* and *S. bovihominis* with application to the enteric phase in the definitive hosts: dogs, cats and man, respectively. Although these names have logical descriptive appeal, they violate the rules of precedent set forth in the International Code of Zoological Nomenclature. The 3 names for *Sarcocystis* in cattle defended by Levine are *S. cruzi* for the cattle/dog species, *S. hirsuta* (Moulé, 1888) for the cattle/cat species and *S. hominis* (Railliet and Lucet, 1891) for the cattle/man species. These names are also applicable to the enteric infections in the definitive hosts and the oocysts produced.

Sexual reproduction of *S. cruzi* occurs only in the definitive hosts. *Sarcocystis cruzi* bradyzoites encysted within myofibers of beef cause enteric infection that results in production of oocysts and sporocysts which are shed in canine feces. Bradyzoites apparently pass through the intestinal mucosa and enter cells in the lamina propria of the jejunum and ileum where they initiate gamogony. Gamogony is the production of macrogametes; single, spherical (8-9 μm diameter), haploid cells, and of microgametes; multiple (20-30), small, columnar (1 x 4-5 μm), flagellated,
haploid cells. Macrogametes, upon fertilization by microgametes, develop into oocysts. Sarcocystis cruzi gamogony has been compared in vitro in cultured cells and in vivo in dog intestine. Gamogony occurs very rapidly (<24 hours) either in vivo or in vitro. In contradistinction to many coccidia, gamogony of S. cruzi is not preceded by schizogony. Sporogony transforms the fertilized macrogamete into an isosporoid oocyst that contains 2 similar sporocysts which each contain 4 sporozoites. Sporogony has not been completed in vitro and occurs in situ, in vivo resulting in completely sporulated S. cruzi oocysts in the lamina propria of the canine small intestine. The several days required for sporulation result in a prepatent period of 9-33 days in canine sarcocystosis. Both oocysts and free sporocysts (16 x 10 μm), totaling from 1 to 20 million, are shed in the dog's feces during the 1 to several weeks of patent infection. The prolonged and variable prepatent and patent phases of canine sarcocystosis have been ascribed to the location of gamogony and sporogony in the lamina propria of the intestine. No clinical disease has been associated with canine sarcocystosis. Ingestion of S. cruzi bradyzoites by dogs after patent sarcocystosis reinitiates the infection. Ingestion of S. cruzi sporocysts by dogs does not cause canine sarcocystosis. Extraintestinal sarcocystosis apparently does not occur in the dog. The definitive host range for S. cruzi also includes coyotes, foxes, raccoons and wolves. Asexual multiplication of S. cruzi only occurs in the intermediate host and the ox, Bos taurus, is the only intermediate host known for S.
Bovine sarcocystosis is initiated when cattle ingest *S. cruzi* sporocysts from dog feces. *In vitro* excystation of sporozoites from *S. cruzi* sporocysts occurs during incubation with bovine bile but not with canine bile. That fact could contribute to the intermediate host specificity of *S. cruzi*. Sporozoites apparently penetrate the intestinal mucosa, presumably establish a parasitemia and cause systemically distributed schizogony. Schizonts have been found in vascular endothelial cells or reticuloendothelial cells in many organs of cattle 15 to 33 days, but not 40, 46 and 54 days, after oral inoculation of sporocysts. Schizonts are variably round to oval with the largest ones 52 μm across and contain from 3-200 discrete nuclei, 1-2 μm diameter. Size variations of schizonts and resident merozoites have been interpreted to represent multiple generations of schizogony but an equally attractive interpretation of the size variation is developmental stages within a generation of schizogony. Schizonts are a transient stage of *S. cruzi* and are sometimes not demonstrable in bovine tissues collected 15 to 33 days after inoculation. Ultrastructural study of *S. cruzi* schizogony has shown that the asexual multiplication occurs by endopolygeny, simultaneous formation of several daughter merozoites. Sarcocyst development within striated myofibers has been found as early as 33 and 34 days after cattle were fed sporocysts. The juvenile sarcocysts contain only metrocytes, the progenitors of bradyzoites found in mature sarcocysts. Sarcocyst maturation has been studied by electron microscopy and requires approximately 40 days; from 33 through 76 days after cattle are fed sporocysts. During that time the metrocytes divide into 2 daughter
metrocytes by endodyogony until the many metrocytes divide into bradyzoites that are infectious for the definitive hosts. Only bradyzoites were found in sarcocysts 76 or more days after calves were fed sporocysts. S. cruzi seems to show a predilection for cardiac muscle in cattle. Mature sarcocysts have occasionally been found in brains of cattle. Bradyzoites in beef do not infect dogs after the beef is heated to an internal temperature of 65-70 C or after it was frozen (-20 C) for more than 3 days. The ubiquity of Sarcocystis in beef is indicated by finding them in 98% and 100% of beef samples surveyed. Ultrastructural studies of bradyzoites in sarcocysts in beef have shown that the organisms have the features common to sporozoa in the subphylum Apicomplexa. The term bradyzoite as applied to S. cruzi in mature sarcocysts is apparently a descriptive synonym for merozoite, but its use will be continued here and the term merozoite will be restricted to those S. cruzi that are found in schizonts.

Sarcocystis cruzi is pathogenic for cattle but clinical disease was not induced in calves fed sporocysts of S. hirsuta and S. hominis. Calves fed S. cruzi sporocysts became anorectic, weak, febrile (<42 C), mildly dehydrated and slightly anemic. Some of the calves became recumbent and died. Cows fed sporocysts developed muscle tremors, weakness, anorexia, pyrexia and cachexia and in some cows excessive saliva-tion, hair loss, abortion, recumbency and death also occurred. Gross lesions associated with bovine sarcocystosis include serous atrophy of fat, pallor of mucous membranes, lymphadenopathy, petechial and ecchymotic hemorrhage and serous effusion in the peritoneal, pleural and pericardial spaces.
Microscopic lesions found were multifocal hemorrhage and edema and diffuse perivascular infiltration of lymphocytes in heart, brain, liver, lung, kidney and striated muscle when schizonts were present in those organs. After the schizonts were gone, inflammation became subacute with perivascular accumulation of histiocytes, lymphocytes and a few plasma cells. Focal areas of Zenker's degeneration with some mineralization were found in heart and muscle tissues. Gross and microscopic lesions were similar in cows and calves with experimental sarcocystosis.

Diagnostic confirmation of natural bovine sarcocystosis involves (1) correlation of clinical signs and tissue lesions compatible with, but not pathognomonic for, the disease, (2) serologic tests, (3) epidemiologic studies and (4) histologic recognition of *S. cruzi* within tissues. The clinical signs and tissue lesions associated with bovine sarcocystosis have been reviewed. Serologic tests described for detection of antisarcocystis antibody include (1) complement fixation, which has not been developed for cattle sera, (2) indirect fluorescent antibody test, which detected serological titers of 1:40 to 1:640 in experimentally infected cattle, (3) indirect hemagglutination, which detected serological titers up to 1:39,000 in experimentally infected cattle and (4) agar gel diffusion, which fails to measure antibody titers. Results of serologic tests must be interpreted with caution because *S. cruzi* is ubiquitous in cattle and indirect hemagglutination titers up to 1:486 are found in sera from apparently normal cattle. Identification of the source of sporocysts provides epidemiologic support for the diagnosis. The *S. cruzi* schizont stage is
transient and may not be demonstrable in bovine tissues.\textsuperscript{39,55} Currently evidence most conveniently combined for the diagnosis is identification of \textit{S. cruzi} schizonts or developing sarcocysts in tissues from cattle with high serological titers of antisaarcocystis antibody.

A few cases of natural bovine sarcocystosis have been diagnosed. An outbreak in yearling dairy heifers was diagnosed on the basis of clinical signs, serological antisarcocystis titers, histologic tissue lesions and \textit{S. cruzi} infection and demonstration of sporocysts shed in feces of the farm dog.\textsuperscript{59,60} Fatal sarcocystosis was diagnosed by light and electron microscopic identification of \textit{S. cruzi} schizonts in tissues from a 2 week old calf.\textsuperscript{61} Sarcocystosis, with one fatal infection, was diagnosed in yearling steers by histologic study of tissue lesions and \textit{S. cruzi} schizonts and juvenile sarcocysts.\textsuperscript{62} Retrospective diagnoses of bovine sarcocystosis have been reported after identification of schizonts in bovine tissues.

Dalmeny disease was reported in dairy cows and calves in 1963.\textsuperscript{63} The causative protozoon has since been accepted to be \textit{S. cruzi} after study of similar bovine disease outbreaks.\textsuperscript{13,64–66} Abortion was associated with Dalmeny disease and \textit{S. cruzi} schizonts have been found in 6 aborted bovine fetuses or placentas.\textsuperscript{67}

Comparative aspects of \textit{Sarcocystis} and sarcocystosis in domestic animals have been presented by Dubey,\textsuperscript{29} and elegantly reviewed by Markus.\textsuperscript{68}
SARCOCYSTIS CRUZI IN BOVINE TISSUES DEMONSTRATED BY IMMUNOFLUORESCENCE

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The authors thank Ms. D. I. Buck, Ms. J. K. Y. Carter, Mr. L. R. Elliott and Mr. J. E. Gallagher (National Animal Disease Center) and Ms. P. J. Beach and Ms. M. C. Grindem (Veterinary Medical Research Institute) for technical assistance.

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Summary

Two cows and 2 calves were fed Sarcocystis cruzi sporocysts from canine feces. Nineteen weeks after inoculation, hearts from both cows contained mature sarcocysts. The sarcocysts were detected in myocardial tissue sections by HE and immunofluorescent stains. Sarcocysts were ultrastructurally identified as S. cruzi. Pepsin-HCl digestion released bradyzoites from myocardial sarcocysts. Smears of myocardial digest residue contained bradyzoites that were demonstrable, with difficulty, by Giemsa stain. Immunofluorescent stain enhanced the contrast of bradyzoites to muscle digest residue. Ultrastructural studies revealed no evidence of bradyzoite degradation associated with the digestion procedure. Sixteen and 20 days after inoculation, tissues from the calves contained S. cruzi schizonts that were stained by HE or Giemsa stains. The schizonts and individual merozoites in tissue were stained by immunofluorescent stain. Merozoites (1.5 x 4.5 μm) in tissue impression smears were quite distinguishable from bradyzoites (5 x 16 μm) in muscle digest residue smears. The immunofluorescent stain used bovine antisarcocystis serum (indirect hemagglutination titer was 1:39,000).

Introduction

Detection of microscopic sarcocysts within 5-7 μm muscle sections is limited in detection sensitivity by the small size of the sample examined. However, in spite of that limitation, Sarcocystis spp. have been found in several mammalian, avian and reptilian species since sarcocysts were first described in mouse musculature by Miescher (1843).
Various schemes to detect muscle parasites have been developed. Sensitivity, expediency and economy of trichinosis detection were combined by sequential muscle digestion, concentration of freed parasites and demonstration of the parasites.\textsuperscript{2,3,4} The procedure has been modified to detect \textit{Toxoplasma gondii} encysted within musculature.\textsuperscript{5}

The present study was conducted to evaluate the detection of \textit{Sarcozystis cruzi}\textsuperscript{6} encysted within bovine musculature by pepsin-HCl digestion, centrifugal concentration of freed bradyzoites and immunofluorescence of bradyzoites in the digest residue. Ultrastructural studies confirmed the identification of \textit{S. cruzi} and provided evidence of the resistance of bradyzoites to pepsin digestion. Immunofluorescence was found useful in detection of several developmental stages of \textit{S. cruzi}.

**Materials and Methods**

Feed for each of 2 Jersey cows was contaminated with 200,000 \textit{Sarcozystis cruzi} sporocysts (provided by Dr. R. Fayer, Animal Parasitology Institute, Beltsville Agricultural Research Center, Beltsville, MD). Nineteen weeks post inoculation (PI), the heart from each cow was harvested (provided by Dr. O.H.V. Stalheim, Bacteriology Research Laboratory, NADC, Ames, IA). Two 400 pound calves were each inoculated via the feed with 3 daily doses of 330,000 \textit{S. cruzi} sporocysts. Sixteen days PI, after the first inoculation of sporocysts, musculature, spleen, liver, kidney, lung, and bronchial lymph node were harvested from one calf (provided by Dr. D. E. Hughes, Bacteriology Research Laboratory, NADC, Ames, IA). The same tissues were harvested 20 days PI from the second calf.
The procedure described for excystment of *T. gondii* from musculature was used. Samples of musculature were chilled (4°C) until digestion was begun within 24 hours. Fifty grams of each muscle tissue were minced, suspended (1:10) in pepsin-HCl solution and incubated (37°C) with continual agitation (2 hrs). The digest residue was sedimented by low speed centrifugation (1,100 x G, 15 min).

Sections of frozen tissues, tissue impression smears and digest residue smears were mounted on glass slides, air dried and fixed in acetone (5 min). These preparations were then sequentially incubated (37°C, humid chamber) with bovine antisarcocystis serum (30 min), rinsed 3 times in buffered saline (5 min) and incubated (30 min) with anti-bovine globulins rabbit serum conjugated with fluorescein isothiocyanate (Sylvana Chemical Co., Orange, NJ). The bovine antisarcocystis serum was autologous, harvested from one of the cows 17 weeks after sporocyst ingestion, and had an anti-sarcocystis titer of 1:39,000 (determined by indirect hemagglutination by Dr. M. N. Lunde, National Institutes of Health, Bethesda, MD).

Formalin-fixed tissue sections and sections of frozen tissues were stained with hematoxylin and eosin (HE) or Giemsa stains. Smears of digest residue and tissue impression smears were air dried, fixed in 95% methanol (5 min) and stained with Giemsa stain.

Samples of intact tissues and tissue digest residue were processed for electron microscopy. Small samples (<1.0 mm³) were fixed in 2.5% glutaraldehyde (1 hr), rinsed twice in cacodylate buffer (1/2 hr), postfixed in 1% osmium tetroxide (2 hr) and rinsed twice in cacodylate buffer (1/2 hr).
Preparations were dehydrated in ethanol, embedded in epon\textsuperscript{10} and cut into ultrathin (70-80 nm) sections. Sections were stained with lead citrate and uranyl acetate\textsuperscript{11} and examined by transmission electron microscopy (Philips EM 200). Glutaraldehyde-fixed digest residue was also mounted on glass, dehydrated in ethanol and coated with gold for scanning electron microscopy (Cambridge Steroscan Mark II SEM).

Results

Centrifugation of pepsin-HCl digested myocardium from the cows (19 weeks PI) sedimented a residue that contained bradyzoites freed from sarcocysts. Digest residue smears stained with Giemsa stain contained bradyzoites (5 x 16 µm) that were differentiated, with difficulty, from residual myofiber nuclei by light microscopy (Fig 1A). Digest residue smears stained with immunofluorescent stain had many brightly stained bradyzoites (5 x 16 µm) that distinctly contrasted to nonstained components of the residue (Fig 1B & C). Glutaraldehyde-fixed digest residue contained bradyzoites discernible by scanning electron microscopy as cylindrical (3 µm diameter), slightly curved bodies (10.5 µm long) with rounded and tapered ends (Fig 1D). The rostral part of some bradyzoites were distinguished by longitudinal ridges interpreted as subpellicular microtubules (Fig 1E). Digest residue pelleted and sectioned for transmission electron microscopy included bradyzoites sectioned through various planes. Integrity of limiting membranes and internal organelles was similar in bradyzoites in digest residue (Fig 1F & G) and in bradyzoites sectioned within sarcocysts.

The cow hearts contained mature sarcocysts within myofibers. Sectioned sarcocysts were detected by HE (Fig 2A) and immunofluorescent stains (Fig
Fig 1—Bradyzoites freed from *Sarcocystis cruzi* infected bovine myocardium by pepsin-HCl digestion.

A—Bradyzoites were discernible in digest residue smears stained with Giemsa stain (arrows) but required cautious differentiation from residual nuclei and debris in the digest residue.

B & C—Immunofluorescent stain on bradyzoites provided distinct contrast to that of other residue components.

D & E—Scanning electron micrographs confirmed the slightly curved, cylindrical form of bradyzoites with a rounded posterior pole and a conical rostral pole.

F & G—Ultrathin sections of pelleted digest residue contained bradyzoites that contained internal organelles and had intact limiting membranes comparable with those of bradyzoites sectioned within sarcocysts (Fig 2).
Fig 2—Mature Sarcocystis cruzi sarcocysts within bovine myocardial myofibers 19 weeks after cows were fed S. cruzi sporocysts harvested from canine feces.

A—Sarcocyst in myocardium section stained with HE has a thin cyst wall and many bradyzoites.

B—Immunofluorescent stain on sarcocyst wall and resident bradyzoites; host myofiber and adjacent myofibers did not stain.

C—Ultrathin section of a sarcocyst. Mitochondria (M) and myofibrils (MF) of the host myofiber border the rarefied zone (RZ), which is traversed by tubular projections (TP) from the undulant primary membrane (PM). Ground substance (GS), subjacent to the primary membrane, extends into the cyst cavity and divides the cyst cavity into several internal compartments (IC), each of which contains several (< 15 in a plane of section) bradyzoites.
Sarcocysts were also found in ultrathin sections of glutaraldehyde-fixed myocardium (Fig 2C). The cysts were surrounded by complete walls. Wall components included a finely undulant primary membrane (PM) with multiple tubular projections (TP), a subjacent granular zone (GS) and a peripheral rarefied zone (RZ) traversed by the tubular projections. The tubular membrane projections were variable in orientation, quite uniform in diameter and contained granules but no fibrils. The peripheral rarefied zone separated the cyst membrane from the myofibrils (MF) of the host myofiber and contained very little granular material. The granular material subjacent to the membrane, cyst ground substance (GS), formed delicate septa that divided the cyst cavity into compartments and also extended into the tubular membrane projections. Cyst compartments (IC) contained slightly curved, cylindrical bradyzoites. Immunofluorescent stain detected both bradyzoites and cyst wall of sectioned sarcocysts. The cyst ground substance and an irregular zone peripheral to the cyst wall fluoresced with less intensity.

*Sarcocystis cruzi* in calf tissues 16 and 20 days PI are summarized in Table 1. Nine of 10 of the residue smears from calf muscle samples digested in pepsin-HCl contained bradyzoites. No sarcocysts were found in sections of 10 tissues from each calf. *Sarcocystis cruzi* schizonts were demonstrated by immunofluorescence in sections of spleen, kidney, lung and bronchial lymph node from the calves (Fig 3A). Individual merozoites, approximately one-third the size of bradyzoites were found in 3 of the muscle tissue sections 20 days PI. Merozoites were also present in 9 of 10
Table 1—Immunofluorescence of *Sarcocystis cruzi* in Tissues from Cattle with Experimentally Induced Sarcocystosis

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<th>Tissues</th>
<th>Cryostat Sectioned Tissues</th>
<th>Smears</th>
<th>Pepsin-HCl digest residue</th>
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<tbody>
<tr>
<td></td>
<td>Sarcocysts</td>
<td>Schizonts</td>
<td>Merozoites</td>
</tr>
<tr>
<td>Days after inoculation</td>
<td>16</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
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<td>Diaphragm</td>
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<td>Tongue</td>
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<tr>
<td>Skeletal muscle</td>
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<tr>
<td>Esophagus</td>
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<tr>
<td>Spleen</td>
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<tr>
<td>Liver</td>
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<td>Kidney</td>
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<td>Lung</td>
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<td></td>
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<tr>
<td>Bronchial lymph node</td>
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</tbody>
</table>

*No smears processed.*
sections of spleen, liver, kidney, lung and bronchial lymph node from the calves (Fig 3B). Merozoites were found in 7 of 10 direct impression smears from cut surfaces of the same tissues (Fig 3C).

*Sarcocystis cruzi* schizonts were recognized as round or oval structures containing several similar bodies (1-2 x 3-5 μm) that stained with fluorescent stain (Fig 3A). Individual merozoites were consistently rounded on one end and tapered to a blunt point at the other end (Fig 3B and C). Study of many ultrathin sections from the calf liver 20 days after inoculation fortuitously provided a merozoite for some ultrastructural characterization (Fig 3D).

**Discussion**

Bradyzoites freed from sarcocysts by pepsin-HCl digestion were identified in digest residue smears. In Giemsa-stained smears, bradyzoites could be distinguished from residual nuclei and debris in the digest residue but were indistinguishable in areas where the smear was thicker. Immunofluorescent stain provided distinct contrast for easy discrimination of bradyzoites from other digest residue components. The expediency of identification of bradyzoites with immunofluorescence after pepsin-HCl digestion suggests that development of a semiquantitative procedure is feasible. Size discrepancy between glutaraldehyde-fixed bradyzoites on scanning electron micrographs and larger bradyzoites detected by immunofluorescent stain was apparently due to shrinkage during glutaraldehyde fixation and to spreading of unfixed bradyzoites in adhering to glass. Ultrastructural study of the residue confirmed the presence of bradyzoites identical to
Fig 3—*Sarcocystis cruzi* schizonts and individual merozoites in tissues from calves fed *S. cruzi* sporocysts.

A—Schizont demonstrated by immunofluorescence (20 days PI).

B—Individual merozoites, demonstrated by immunofluorescence, in a cryostat section of bronchial lymph node (20 days PI).

C—Merozoite in an impression smear from the cut surface of liver (20 days PI).

D—Electronmicrograph of an individual *S. cruzi* merozoite in the cytoplasm of a host cell, adjacent to the cell nucleus (HN). The conoid (C) of the apical complex is at the rostral pole of the merozoite. Many micronemes (MN) are present between the conoid and the nucleus (N) of the merozoite. Subpellicular microtubules (ST) are subjacent to the pellicle (P), the limiting membrane of the merozoite.
those sectioned within sarcocysts. No evidence of bradyzoite membrane or organelle degradation after digestion was discernible.

Sectioned sarcocysts within myofibers were morphologically compatible with sarcocysts previously induced in calves by *S. cruzi* sporocyst inoculation. Bovine sarcocysts induced by feeding calves *Isospora bigemina* or *Isospora hominis* sporocysts, from feline or human feces respectively, have both thicker cyst walls and fibrils within tubular membrane projections of the cyst walls. Ultrastructural *Sarcocystis* species identification was essential because the serum used for immunofluorescent staining has not been adequately characterized for specificity. Immunofluorescence of the sectioned sarcocyst was shown with serum from an *S. cruzi* infected cow and provides evidence of which components of the sarcocyst a bovine host is immunologically sensitized to during the course of infection. Bradyzoites, cyst wall and cyst ground substance all have such antigens. The cyst ground substance is not ultrastructurally distinguishable from other granular material, and the fluorescent material peripheral to the cyst wall may be antigenically recognized metabolites or secretions from the sarcocyst.

Soluble antigens extracted from *S. cruzi* bradyzoites, freed from bovine myocardium by digestion, have been used to determine bovine serologic antisarcocystis titers. The results of this investigation suggest morphologic localization of bradyzoite antigens used for serologic indirect hemagglutination or immunodiffusion tests. Immunofluorescent staining of bradyzoites offers proof of infection rather than indirect evidence acquired by demonstration of serum antibodies.
Schizonts shown by immunofluorescence were similar to *S. cruzi* schizonts previously described in HE stained sections of formalin-fixed tissues from calves with experimentally induced sarcocystosis.\textsuperscript{14,15} Demonstration of individual merozoites in sections of frozen tissues and in tissue impression smears is a unique result of this investigation. Immunofluorescent stain is currently the only technique successful in detecting individual *S. cruzi* merozoites. Demonstration of the merozoites in the absence of schizonts in several tissues clearly shows the additional sensitivity of merozoite detection over the sensitivity of schizont detection for demonstration of sarcocystosis in bovine tissues. Immunofluorescence of *S. cruzi* will be very useful in both study of and diagnosis of bovine sarcocystosis.

References


PATHOGENESIS OF BOVINE SARCOCYSTOSIS STUDIED BY IMMUNOFLUORESCENCE

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The authors thank Ms. J. D. Dunshee for technical assistance and Mr. G. L. Hedberg for the illustrations.
Summary

Sarcocystosis was induced in 28 cattle by oral inoculation of *Sarcocystis cruzi* sporocysts; 8 cattle were uninoculated controls. Tissues (spleen, liver, lung, kidney, adrenal gland, thymus, lymph nodes and muscle) from cattle killed at various days after inoculation were examined by immunofluorescent microscopy for presence of *S. cruzi*. Merozoites were systemically distributed in cattle examined 16-50 days post inoculation (PI). Merozoites persisted in lymph nodes but not in other tissues from cattle examined 55-120 days PI. Schizonts were common in all tissues except muscle from cattle examined 16-37 days PI. Schizonts were rarely found in muscle, whereas merozoites were common 20-50 days PI. Myofiber penetration was evident from 32-60 days PI. Sarcocysts containing few metrocytes were first found in muscle 37 days PI. The number of metrocytes increased with sarcocyst development, then decreased when metrocytes produced bradyzoites until mature sarcocysts, containing only bradyzoites, were present in muscle after 120 days PI.

Introduction

Sarcocystosis (sarcosporidiosis) is a protozoan infection characterized by 2 phases of asexual multiplication in the intermediate host.\(^1\) Cattle serve as intermediate hosts for *Sarcocystis cruzi*.\(^2\) Acute bovine sarcocystosis is the systemic febrile disease that accompanies schizogony, the initial phase of *S. cruzi* multiplication.\(^3\)\(^-\)\(^11\) Merozoites develop within schizonts and are both initiators of and products of schizogony.\(^12\) Chronic bovine sarcocystosis is the wasting disease that accompanies the final
phase of protozoan multiplication, which occurs as sarcocysts develop within bovine myofibers. \textsuperscript{13-17} Metrocytes, the progenitors of bradyzoites, develop and multiply within cysts in muscle. \textsuperscript{14,15,17} Sarcocyst maturation is accompanied by production of bradyzoites from metrocytes. Bradyzoites within mature sarcocysts are infectious for dogs and other definitive hosts of \textit{S. cruzi}. \textsuperscript{18-20}

Previous studies of \textit{S. cruzi} in bovine tissues have been limited to either schizonts within vascular endothelial cells or sarcocysts within myofibers. Schizonts are transient and are not consistently demonstrable during acute disease. \textsuperscript{3-7,9,10} In contrast, sarcocysts are persistent in muscle but develop after acute disease subsides in cattle. \textsuperscript{13-17} Individual merozoites between generations of schizogony or during myofiber penetration have not been studied.

Demonstration of individual \textit{S. cruzi} organisms by immunofluorescence recently was reported from this laboratory. \textsuperscript{21} The objective of this investigation was to characterize the chronological sequence of \textit{S. cruzi} development and concurrent tissue infection in cattle fed sporocysts. Bovine tissues had been collected periodically from sporocyst inoculation to sarcocyst maturation and were studied by immunofluorescence.

\textbf{Materials and Methods}

Tissues were collected from 36 cattle. Twenty-eight of the cattle were fed \textit{S. cruzi} sporocysts (Table 1). Those cattle were clinically normal and in good condition and ranged in age from 1 month to 4 1/2 years.
Table 1—Cattle with Experimentally Induced Sarcocystosis Necropsied for Collection of Tissues for Immunofluorescence Demonstration of *Sarcocystis cruzi*

<table>
<thead>
<tr>
<th>Cattle No.</th>
<th>Age</th>
<th><em>S. cruzi</em> inoculum</th>
<th>Necropsy</th>
<th><em>S. cruzi</em> in tissues</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>sporocysts per dose</td>
<td>No. of doses</td>
<td>Days post inoculation</td>
</tr>
<tr>
<td>1</td>
<td>1 mo</td>
<td>$3 \times 10^5$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>4½ yr</td>
<td>$1 \times 10^6$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1 mo</td>
<td>$3 \times 10^5$</td>
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<td>4</td>
</tr>
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<td>4</td>
<td>2½ mo</td>
<td>$1 \times 10^6$</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1 mo</td>
<td>$3 \times 10^5$</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>2½ mo</td>
<td>$1 \times 10^6$</td>
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<td>10</td>
</tr>
<tr>
<td>7</td>
<td>1 mo</td>
<td>$3 \times 10^5$</td>
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<td>12</td>
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<tr>
<td>8</td>
<td>8 mo</td>
<td>$3.3 \times 10^5$</td>
<td>$3^d$</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
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<td>20</td>
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<tr>
<td>10</td>
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<td>11</td>
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<tr>
<td>17</td>
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</tr>
<tr>
<td>(same cow)(^f)</td>
<td>1</td>
<td>$1 \times 10^5$</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>28</td>
<td>4½ yr</td>
<td>$5 \times 10^4$</td>
<td>20(^e)</td>
<td>150</td>
</tr>
</tbody>
</table>

\(^a\) Schizogony evidenced by schizonts or merozoites.

\(^b\) Days after first dose of sporocysts.

\(^c\) No smears examined.

\(^d\) Daily doses.

\(^e\) Semi-weekly doses.

\(^f\) Cow 27 was reinoculated with sporocysts 5 weeks after the multiple dose regimen.
when inoculated. Sporocysts used to initiate the investigation were provided by Dr. R. Fayer, Animal Parasitology Institute, Beltsville Agricultural Research Center, Beltsville, MD. The sporocysts had been harvested by flotation from feces of dogs fed *S. cruzi* infected beef and were suspended in distilled water. Dosages of sporocysts fed to individual cattle included single doses of $1 \times 10^5$ to $1 \times 10^6$ sporocysts and 3 multiple dosage regimens each totaling $1 \times 10^6$ sporocysts. Cow number 27 was fed $1 \times 10^6$ sporocysts, divided into 20 doses, over a 10-week interval and was reinoculated with $1 \times 10^5$ sporocysts in a single dose 5 weeks after completion of the first dosage regimen. Tissues studied were from cattle necropsied on various days (1-150) post inoculation (PI). The first sporocyst dose was fed on 0 days PI for all dosage regimens. Tissues from 8 cattle not fed sporocysts were included as control tissues. Control tissues were acquired when cattle comparable with the principal cattle were available for necropsy.

Immunofluorescence was used in all tissue studies. Samples (approximately 5 mm$^3$) of tissues collected at necropsy were frozen (-70°C), sectioned on a cryostat microtome and fixed by submersion (5 min) in acetone (4°C). *Sarcocystis cruzi* in the tissue sections were stained by immersion (30 min, 37°C) in antisarcocystis globulins-fluorescein conjugate, washed 3 times in phosphate buffered saline (pH 7.6, 25°C), rinsed in distilled water and examined by fluorescence microscopy. Tissues sectioned and examined from each of the cattle are summarized on Figure 1. The tissues have been divided into 3 groups with thymus included in the viscera category to simplify the results and discussion of this study. Direct
Fig 1—Distribution of *Sarcocystis cruzi* merozoites and schizonts, demonstrated by immunofluorescence, in tissues from cattle with experimentally induced sarcocystosis. *One cow was inoculated with a second dosage of *S. cruzi* sporocysts 15 weeks after the first dose.
Fig 2—Distribution of *Sarcocystis cruzi* merozoites on impression smears from several bovine tissues.

Impression smears on glass slides were taken from fresh cut surfaces of tissues as summarized on Figure 2. The smears were fixed in acetone and stained as tissue sections had been for *S. cruzi* immunofluorescence. Evans blue was used as counterstain on smears to minimize granulocyte autofluorescence.
Prior *S. cruzi* infection of cattle used in this investigation was determined by pepsin–HCl digestion of ventricular myocardium (50 gm) and detection of bradyzoites by immunofluorescence.  

**Results**

*Sarcocystis cruzi* merozoites and schizonts found in tissue sections by immunofluorescence are summarized on Figure 1. Merozoites (Fig 3a) were found in 78% (162 of 207) of all tissues examined from 14 cattle necropsied 16–50 days PI. Merozoites persisted in a few germinal centers in 15 of 26 lymph nodes (Fig 3b) from 5 cattle necropsied 55–120 days PI. Schizonts (Fig 4) were found in 41% (43 of 104) of sections from viscera and lymph nodes from cattle necropsied 16–37 days PI. The cow necropsied 140 days PI had been reinoculated with sporocysts 35 days before necropsy. Merozoites and schizonts were found in lung, kidney and 3 lymph node sections from that cow. Merozoites and schizonts were not found in tissues from 7 cattle necropsied 1–12 days PI, 1 cow necropsied 150 days PI and 8 cattle not fed sporocysts.

The only phase of *S. cruzi* demonstrated by immunofluorescence on direct impression smears from tissues was the merozoite (Fig 5). The tissues processed for direct impression smear examination and merozoites found in those smears are summarized on Figure 2.

In addition to the merozoites and few schizonts found in muscle (Fig 1), irregularly shaped fluorescent bodies were found apposing (Fig 6) or extending through the sarcolemma (Fig 7) of myofibers in muscle sections 32–60 days PI. The irregularly shaped bodies were interpreted as *S. cruzi*
Fig 3—Sarcocystis cruzi merozoites in bovine tissue demonstrated by immunofluorescence. Merozoites were numerous and systemically distributed 37 days PI (a). Single merozoites persisted in follicles of lymph nodes until 120 days PI (b).

Fig 4—Sarcocystis cruzi schizonts in bovine tissue demonstrated by immunofluorescence. Merozoites produced in schizonts were discernible in intact schizonts (a) and were clearly defined during release from schizonts (b).

Fig 5—Sarcocystis cruzi merozoites in bovine tissue impression smears, demonstrated by immunofluorescence (a). The nucleus (arrow) of many merozoites did not stain (b).

Fig 6—Sarcocystis cruzi merozoites apposed to bovine myofiber sarcolemma, the first step of myofiber penetration (41 days PI).

Fig 7—Sarcocystis cruzi merozoite, transverse plane, apposed to and extended through the bovine myofiber sarcolemma. Stained material subjacent to the sarcolemma (arrow) was interpreted to be antigenic secretion from the merozoite (45 days PI).

Fig 8—Sarcocystis cruzi sarcocysts demonstrated by immunofluorescence (70 days PI). Juvenile or developing sarcocysts contained large, irregular or ovoid metrocytes. Sarcocyst walls (arrows) also were stained.
penetrating myofibers. Sarcocysts first appeared within myofibers at 37 days PI and contained a few large oval metrocytes. Sarcocysts enlarged and metrocytes multiplied (Fig 8) until 60 days PI when metrocytes began to produce curved columnar bradyzoites. Metrocyte numbers diminished during bradyzoite production until only bradyzoites were found after 120 days PI. Only mature sarcocysts containing bradyzoites were found in muscle sampled at 140 and 150 days PI.

Pepsin-HCl digestion of muscle yielded bradyzoites. These were demonstrated by immunofluorescence in the digest residue from all cattle in the study except four 1-month-old calves and one 2 1/2-month-old calf (#6, Table 1).

Discussion

Schizogony, evidenced by presence of schizonts and merozoites, occurs in viscera and lymph nodes 16-50 days PI. The advantage in detection of individual merozoites in tissues (78%) over detection of schizonts (23%) is clearly established by the results of this study.

Consistent presence of merozoites and absence of schizonts in muscle during acute sarcocystosis indicates that merozoites function in myofiber penetration to establish sarcocysts but do not initiate additional generations of schizogony.

The persistence of individual merozoites within lymph nodes 55-120 days PI has not been detected in previous studies of sarcocystosis. Whether the persistent merozoites in lymph nodes could reinitiate systemic infection of cattle under influences not included in this study is an intriguing question. The presence of merozoites expands the interval for diagnostic application of immunofluorescence to 120 days PI.
Sarcocysts within myofibers were described first by Miescher in 1843. The morphology of juvenile and mature sarcocysts with resident metrocytes and bradyzoites, respectively, has been described from ultratstructural studies. Metrocytes and bradyzoites are also discernible by immunofluorescence, and the technique provides a unique advantage in demonstration of myofiber penetration by *S. cruzi* (Fig 6 and 7).

Myocardium digest residue from all cattle in this study, except the youngest calves, contained bradyzoites and thus confirmed *S. cruzi* infection pre-inoculation; nevertheless, infection was induced in all of the cattle fed sporocysts. Furthermore, the cow necropsied 140 days PI had been reinoculated with sporocysts 5 weeks after completion of a regimen of 20 doses over 10 weeks. Merozoites and schizonts in lung, kidney and lymph node from that cow indicate that infection did result from the last dose of sporocysts. These facts provide considerable evidence that lasting immunity against reinfection does not develop in cattle during infection with *S. cruzi*.

Results of this study show that developmental phases of *S. cruzi* and concurrent distribution of the protozoa in host tissue conform to a consistent chronological pattern (Fig 9). The variety of sporocyst dosages used (Table 1) and the range of ages of cattle in the study did not seem to influence the sequence of protozoan development and tissue infection. The chronology of bovine sarcocystosis derived from this study and the application of immunofluorescence for demonstration of *S. cruzi* infection of bovine tissues will be valuable to the veterinary diagnostician confronted with natural bovine sarcocystosis.
Fig 9—Schematic summary of the chronological sequence of *Sarcocystis cruzi*, demonstrated by immunofluorescence, in tissues from cattle with experimentally induced sarcocystosis.

We can conclude that immunofluorescence is a reliable, practical technique for demonstration of individual merozoites between generations of schizogony or during myofiber penetration and for demonstration of schizonts and sarcocysts in bovine tissues.
References


FETAL DEATH AND ABORTION CAUSED BY SARCOCYSTOSIS IN CATTLE

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Summary

*Sarcocystis cruzi* sporocysts were fed to 7 cows in midterm of second gestation. Febrile disease typical of acute sarcocystosis developed in all cows. Cows were killed and necropsied 25, 28, 30, 32, 35, 36, and 37 days post inoculation (PI). Fetuses from cows necropsied 32, 35, and 36 days PI were dead, reddened, and anasarcous; 2 of the dead fetuses were aborted. Live fetuses were collected from the other four cows. The cow necropsied 37 days PI was moribund. Allantochorionic membranes were thickened by edema and placentomes were small in all inoculated cows. *Sarcocystis cruzi* schizonts and merozoites were demonstrated within caruncles from all cows except the one necropsied 25 days PI. Evidence of fetal infection was sparse and was detected in only the 3 dead fetuses. The results of this study indicate that *S. cruzi* infection is abortifacient in pregnant cows and that infection of the fetus is an agonal event.

Introduction

Sarcocystosis (sarcosporidiosis) is a protozoan infection characterized by 2 phases of asexual multiplication in the intermediate host. Acute bovine sarcocystosis is the systemic febrile disease that accompanies schizogony (merogony) of the parasite within vascular endothelial cells in all organs of the host. Merozoites are both initiators of and products of schizogony. Chronic bovine sarcocystosis is the debilitating disease that can accompany the final phases of multiplication as sarcocysts develop within myofibers. Bradyzoites are resident protozoa within mature sarcocysts.
Sarcocystosis, experimental and natural, has been implicated in bovine fetal deaths and abortions. Fetal death and abortion occurred in half of the pregnant cows included in 2 studies of experimentally induced bovine sarcocystosis. In Dalmeny disease, retrospectively considered sarcocystosis, more than half of the pregnant cows aborted. Protozoan schizonts resembling Sarcocystis were found in fetuses or placentas from 6 natural bovine abortions. Schizonts within uterine and fetal tissues were direct evidence of sarcocystosis in field cases. Serologic antisarcocystis titers developed in experimentally infected cows, but protozoa were not demonstrated within uterine or fetal tissues.

The objectives of this investigation were to induce fetal deaths and abortions by feeding Sarcocystis cruzi sporocysts to pregnant cows and to demonstrate coincident protozoan infection in uterine and fetal tissues.

Materials and Methods

Eight cows in midterm of second gestation were used for this investigation. They were bred by artificial insemination (AI) and all conceived on first service. Pregnancy was confirmed by physical examination before sporocysts were fed 154-167 days after AI. The cows were housed in concrete isolation barns and fed a complete pelleted ration supplemented with alfalfa cubes. Cows were observed daily for evidence of abortion.

Inoculum was Sarcocystis cruzi sporocysts (Sarcocystis cruzi sporocyst suspension was provided by Dr. R. Fayer, Animal Parasitology Institute, Beltsville Agricultural Research Center, Beltsville, MD.) collected from feces of dogs fed infected bovine heart. Sporocysts were suspended
in tap water and stored at 4C until fed. Appropriate volumes of the aqueous suspension of sporocysts were sprinkled upon pelleted rations for individual cows. Sporocyst dosages were a single dose of 100,000 \((1 \times 10^5)\) for 1 cow and 200,000 daily on 5 consecutive days \((5 \times 2 \times 10^5)\) for 6 cows.

Tissues collected for demonstration of protozoan infection were processed for immunofluorescent examination. Small pieces (approximately \(5 \text{mm}^3\)) of uterus, placentomes, placental membranes, and several fetal tissues (Table 1) were rapidly frozen (-70C) for cryostat sectioning. Tissue sections were stained with antisarcocystis globulins-fluorescein conjugate and were examined by fluorescence microscopy.

Additional surveillance procedures were used for detection of previous Sarcocystis infection and concurrent infection by abortifacient viruses or bacteria. Musculature (50 g) from each cow and fetus was digested in pepsin-HCl and the residue screened for bradyzoites freed from mature sarcocysts as proof of previous sarcocystosis. Sera from the cows were evaluated for serologic evidence of concurrent brucellosis or leptospirosis. Samples of placentomes and fetal stomach contents were cultured for isolation of viruses or bacteria. Samples were incubated on blood-agar under microaerophilic conditions \((6\% O_2, 5\% CO_2)\) for 4-7 days to isolate Brucella abortus and Campylobacter fetus. Samples were also inoculated onto bovine turbinate cells for isolation of infectious bovine rhinotracheitis, bovine viral diarrhea, and parainfluenza-3 viruses. Apparently negative cultures were transferred for 3-4 blind passages and material from each passage was
checked for interference with a test virus. Cow kidney samples were cultured for *Leptospira* spp.

**Results**

Consequences of experimentally induced sarcocystosis in 6 pregnant cows fed $5 \times 2 \times 10^5$ sporocysts are summarized in Figure 1. Transient febrile disease, pyrexia (40–42°C), anorexia, and dyspnea were recorded in the third week (16–21 days) and recurred during the fifth week (28–32 days) post inoculation (PI). One cow developed persistent excessive salivation. A wobbly gait was consistently noted coincident with the second phase of febrile disease. The cows were killed for necropsy examination 28–37 days PI. A dead fetus was found in utero in the cow necropsied 32 days PI. Hours before necropsy, the 35-day PI cow aborted a dead fetus, and the 36-day PI cow was aborting a dead fetus when necropsied. Dead fetuses were reddened and anasarcous. Live and apparently normal fetuses were collected from the other 3 cows. Placentomes were small and allantochorionic membranes were thickened by yellow gelatinous edema in all 6 cows. The 37-day PI cow was moribund.

The cow fed $1 \times 1 \times 10^5$ sporocysts (low-dose cow) was necropsied 25 days PI. Her live fetus was apparently normal although the allantochorionic membrane was slightly edematous. The eighth cow served as an unexposed control, was not fed sporocysts, and was necropsied at 197 days of gestation. The uterus, placentomes, placenta, and fetus from the unexposed control cow were normal. Febrile disease comparable with that of the 6 principal cows was observed in the low-dose cow but not in the unexposed control cow.
Fig 1—Reproductive consequence of experimentally induced sarcocystosis in 6 pregnant cows. Cows were fed $2 \times 10^5$ *Sarcocystis cruzi* sporocysts daily on 5 consecutive days and were necropsied 28–37 days after the first dose of sporocysts. Age of the fetuses necropsied ranged from 189 to 197 days. The cow necropsied 37 days after inoculation was moribund.
Results of uterine tissue examination for presence of *S. cruzi* are tabulated in Table 1. Numerous merozoites were demonstrated by immunofluorescence in all caruncle tissue sections from the 6 principal cows. Schizonts were present in caruncle sections from 4 of the principal cows. *Sarcocystis cruzi* were demonstrated by immunofluorescence in 3 of 4 uterus sections from principal cows. Protozoa were not demonstrated in uterus or caruncle sections from either the low-dose cow or the unexposed control cow.

The only *S. cruzi* demonstrated in fetal tissues (Table 1) were from the 3 dead fetuses. The amnion section from 1 contained 2 schizonts, the lung section from 1 contained 1 merozoite, and the lung section from the other contained 1 schizont. No protozoa were demonstrated in sections from 65 other fetal tissues.

Pepsin-HCl digestion of musculature from all 8 cows yielded *S. cruzi* bradyzoites that were demonstrated by immunofluorescence. Myocardium was consistently positive for bradyzoites, whereas presence of bradyzoites was variable in other cow musculature as summarized in Table 2. Bradyzoites were not demonstrated in musculature from any of the 8 fetuses.

Neither maternal serologic tests nor culture of placentomes, fetal stomach contents, or cow kidney provided any evidence of concurrent bacterial or viral infection of the 8 cows or fetuses.

**Discussion**

Successful induction of fetal deaths and abortions by feeding *S. cruzi* sporocysts to pregnant cows complements previous reports of sarcocystosis
TABLE 1—*Sarcocystis cruzi* Merozoites (M) and Schizonts (S) Demonstrated by Immunofluorescence on Maternal and Fetal Tissue Sections from Pregnant Cows after Experimental Induction of Sarcocystosis

<table>
<thead>
<tr>
<th>Tissues examined</th>
<th>Days after <em>S. cruzi</em> sporocyst inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25(^a)</td>
</tr>
<tr>
<td>Maternal tissues</td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>0(^d)</td>
</tr>
<tr>
<td>Caruncle</td>
<td>0</td>
</tr>
<tr>
<td>Fetal tissues</td>
<td></td>
</tr>
<tr>
<td>Cotyledon</td>
<td>0</td>
</tr>
<tr>
<td>Allantochorion</td>
<td>...</td>
</tr>
<tr>
<td>Amnion</td>
<td>...</td>
</tr>
<tr>
<td>Spleen</td>
<td>...</td>
</tr>
<tr>
<td>Liver</td>
<td>...</td>
</tr>
<tr>
<td>Lung</td>
<td>...</td>
</tr>
<tr>
<td>Thymus</td>
<td>...</td>
</tr>
<tr>
<td>Kidney</td>
<td>...</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>...</td>
</tr>
<tr>
<td>Hepatic ln.</td>
<td>...</td>
</tr>
<tr>
<td>Heart</td>
<td>0</td>
</tr>
<tr>
<td>Tongue</td>
<td>0</td>
</tr>
<tr>
<td>Muscle</td>
<td>0</td>
</tr>
</tbody>
</table>

Age of fetus, days 188 195 195 197 197 194 189 197

\(^a\)Cow inoculated with 1x10\(^5\) *S. cruzi* sporocysts.

\(^b\)Cows inoculated with 5x2x10\(^5\) *S. cruzi* sporocysts.

\(^c\)Gestation-matched control cow.

\(^d\)No *S. cruzi*.

\(^e\)Tissue not examined.
<table>
<thead>
<tr>
<th>Tissues examined</th>
<th>Days after S. cruzi sporocyst inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maternal musculature</td>
<td></td>
</tr>
<tr>
<td>Myocardium</td>
<td>B</td>
</tr>
<tr>
<td>Tongue</td>
<td>B</td>
</tr>
<tr>
<td>Esophagus</td>
<td>B</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>B</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0</td>
</tr>
<tr>
<td>Fetal musculature</td>
<td></td>
</tr>
<tr>
<td>(pooled)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cow inoculated with 1x10<sup>5</sup> S. cruzi sporocysts.
<sup>b</sup>Cows inoculated with 5x2x10<sup>5</sup> S. cruzi sporocysts.
<sup>c</sup>Gestation-matched control cow.
<sup>d</sup>No demonstrable bradyzoites on 4 smears of digest residue.
<sup>e</sup>Five muscle samples from each fetus pooled into 1 sample (50g).

in pregnant cows. Death of fetuses has consistently accompanied sarcocystosis in pregnant cows. Experimental infection of pregnant cows in 2 previous studies was accompanied by death of 2 of 4 fetuses<sup>5</sup> and 4 of 7 fetuses.<sup>6</sup> Natural sarcocystosis in pregnant cows has been reported only once as Dalmeny disease, and 10 of the 17 infected pregnant cows aborted dead fetuses.<sup>8</sup> In the present study, 3 of 7 fetuses died and presumably
those in cows necropsied 25, 28, and 30 days PI would have succumbed if the cows had been allowed to live a few more days. Demonstration of merozoites and schizonts in uterine caruncles and in fetal tissues coincident with fetal death in this study clearly establishes S. cruzi as an abortifacient pathogen in cattle.

The sporocyst dosage and the necropsy schedule were selected to cause fetal death and to provide tissues from pregnant cows before and after fetal death. Fetal death and abortion had previously been induced by inoculation of 150,000 to 500,000 sporocysts for pregnant cows but was more consistent with $1 \times 10^6$ sporocysts.\textsuperscript{5,6} Single doses of $5 \times 10^5$ and $1 \times 10^6$ S. cruzi sporocysts have caused mortality in some cows.\textsuperscript{5,6} Division of $1 \times 10^6$ sporocysts into 5 equivalent daily doses was intended to reduce the risk of cow mortality. The 28 to 37-day range of the necropsy schedule preceded and included the earliest previously reported fetal death (35 days PI).\textsuperscript{6} Abortions had previously been induced by feeding sporocysts to cows between 5 and 5 1/2 months of gestation.\textsuperscript{5,6}

Numerous merozoites and fewer schizonts in maternal uterus and caruncle were typical of the infection in most organs from cattle with acute bovine sarcocystosis.\textsuperscript{4} Absence of demonstrable protozoa in tissues from live fetuses was suggestive of a placental barrier to fetal infection by S. cruzi. Sparse but demonstrable infection of the 3 dead fetuses was suggestive of failure of the barrier just before death of the fetus. Multiplication and systemic distribution of S. cruzi within fetal tissues was apparently precluded by the agonal condition of the fetus when infected. Agonal infection of fetuses coupled with placentome and allantochorion
changes before infection and death of fetuses is suggestive of a mechanism of fetal death related to maternal, not fetal, sarcocystosis.

Immunofluorescence examination of placentome sections revealed numerous *S. cruzi* within maternal caruncles; this is in contrast to the absence of protozoa in adjacent fetal cotyledons. Results of examination of aborted placentas and fetuses for *S. cruzi* infection suggest that demonstration of the protozoon even in very low numbers may be difficult but diagnostic. Numerous *S. cruzi* in uterine caruncles coincident with fetal death indicate a diagnostic advantage in examination of caruncle rather than fetal tissues.

Uniformly positive results of the cow muscle digestion procedure for detection of bradyzoites from sarcocysts clearly suggests that systemic infection, fetal death, and abortion can be induced in pregnant cows previously infected with *S. cruzi*. Bradyzoites begin to develop within sarcocysts 60-70 days PI and continue to develop over several weeks. Bradyzoites detected 25-37 days PI were the result of previous *S. cruzi* infection. Possible preinvestment sources of *S. cruzi* sporocysts include the hay, straw used as bedding in barns, or sporocysts shed by dogs, foxes, or raccoons that occasionally gain access to drylots.

Two problems that remain unresolved are (1) the interval of gestation when fetuses succumb to sarcocystosis in pregnant cows, and (2) the minimum dosage of *S. cruzi* sporocysts required to cause death of bovine fetuses.
References


PATHOGENESIS OF BOVINE ABORTION INDUCED BY SARCOCYSTIS CRUZI

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Veterinary Pathology

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No product endorsements are implied herein.
Summary

Seven cows were fed Sarcocystis cruzi sporocysts during midgestation and necropsied 25, 28, 30, 32, 35, 36, and 37 days post inoculation (PI). Live fetuses with no discernible lesions were found in cows killed within 30 days PI. Three fetuses from cows necropsied 32–36 days PI were dead and autolyzed. Placentomes from all inoculated cows were diminished in size due to involution of uterine caruncles. Chorionic villi of the fetal cotyledons had been expelled from caruncle crypts as the caruncles regressed. Diffuse edema thickened the allantochorions in all 7 cows. Corpora lutea were infected by S. cruzi and luteal cell regression corresponded with the severity of infection which was maximal 35 days after inoculation. Plasma progesterone concentration was diminished in correlation with ovarian luteolysis, caruncle involution and fetal death. Results of this study indicate that bovine abortion induced by sarcocystosis is mediated through protozoan infection of corpora lutea. Infection causes luteolysis and reduction of plasma progesterone concentration to a level too low for maintenance of pregnancy.

Introduction

Acute bovine sarcocystosis is the febrile clinical disease that accompanies schizogony of Sarcocystis cruzi\(^1\) within vascular endothelial cells throughout the bovine host.\(^2,3,4\) In pregnant cattle, abortion is often associated with sarcocystosis and occurs 30–40 days after sporocysts are ingested.\(^5,6,7\)
In experimental studies, fetal death was a consequence of acute sarcocystosis in more than half of the pregnant cows.\textsuperscript{5,6,7} In natural infection, reported as Dalmeny disease, the incidence was similar.\textsuperscript{8,9} The distribution of \textit{S. cruzi} within maternal tissues coincident with death of fetuses suggests that fetuses succumb to systemic infection of the cow and that infection of fetuses is an agonal event.\textsuperscript{5} The pathogenetic mechanisms responsible for death of the fetus are unknown.

Lesions attributable to sarcocystosis have not been characterized in bovine reproductive organs or fetuses. The objective of this investigation was to characterize sarcocystosis induced lesions and dysfunction of bovine reproductive organs and fetuses.

\textbf{Materials and Methods}

Experimental design: Seven cows bred by artificial insemination were in midgestation when inoculated with \textit{S. cruzi} sporocysts \textit{per os}. Heparinized plasma samples were collected periodically for progesterone assay. Cows were killed for examination of reproductive organs and fetuses 25, 28, 30, 32, 35, 36, and 37 days post inoculation (PI, 188-197 days of pregnancy). One uninoculated cow was the source of control plasma samples and uninfected tissues for examination (197 days of pregnancy).

Seven pregnant (5-5 1/2 months) cows were fed \textit{Sarcocystis cruzi} sporocysts from the feces of dogs fed infected beef hearts\textsuperscript{10} (sporocysts suspended in water were provided by Dr. R. Fayer, Animal Parasitology Institute, Beltsville Agricultural Research Center, Beltsville, MD). Tissues were collected from cows necropsied 25-37 days PI. Uninfected gestation-matched tissues were taken from an eighth cow not fed sporocysts. Clinical
reproductive consequences of acute sarcocystosis in these pregnant cows and the distribution of *S. cruzi* within uterine and fetal tissues were reported elsewhere.\(^5\)

Tissue samples collected for light microscopy were fixed by immersion in Bouin's fluid, trimmed, washed in tap water, dehydrated in an automated tissue processor and embedded in paraffin. Tissues were sectioned at 6 µm and stained with hematoxylin and eosin. Maternal tissues sampled for this study were endometrium, uterine body and gravid horn; cervix, proximal and distal; ovary with corpus luteum; pituitary and mammary glands. Fetal tissues sampled were lung, kidney, liver, spleen, thymus, lymph node, small intestine, colon, spinal cord, tongue, esophagus, diaphragm, skeletal muscle, heart, umbilical cord, amnion, and allantochorion. Several placentaomes were sampled from each cow and they provided sections with both fetal cotyledon and maternal uterine caruncle.

Plasma progesterone concentration was measured by radioimmunoassay (\(^{125}\)I Progesterone Radioimmunoassay Kit, Diagnostics Biochem International, Inc., 4616 W. Buffalo Avenue, Tampa, Florida 33614). Plasma was harvested from heparinized blood samples collected periodically by jugular venipuncture. Plasma samples were frozen (-70°C) until progesterone concentrations were determined.

**Results**

Live and apparently normal fetuses were found in cows killed 25, 28, 30, and 37 days PI and in the control cow. No gross or microscopic lesions
were detected in tissues from live fetuses. Fetuses from cows killed 32, 35, and 36 days PI were dead, reddened and anasarcous. All tissues from dead fetuses were autolyzed.

Diffuse edematous swelling of the allantochorion was consistent in inoculated cows with live fetuses. The swelling was maximal (3-4 cm) 30 and 32 days PI. Swelling was minimal in the allantochorion from cows with aborted fetuses (35 and 36 days PI).

Placentomes from inoculated cows were smaller than those from the control cow. Regression of the maternal caruncles was consistent in inoculated cows (Figs 2-6). Crypts were shortened and the interstitium was dense and less vascular with necrosis at the periphery of the caruncles. Crypts were very sparse 35 and 36 days PI. Diminished crypt epithelium correlated with contracted interstitium and was very sparse 35 and 36 days PI. Size of fetal cotyledons was consistent and the chorionic villi had been extruded by caruncle crypt regression. Distal parts of a few chorionic villi, deepest within the caruncle crypts, remained unchanged through 35 days PI whereas the proximal parts of the villi were degenerate 32, 35, and 36 days PI. Placentomes from the cow necropsied 37 days PI had diffuse change similar to, but of lesser extent than the placentomes 32, 35, and 36 days PI.

Histologic findings in the corpora lutea are summarized on Table I. The control tissue was uniform and consisted of polygonal large luteal cells, small luteal cells and delicate vascular stroma (Fig 7). Large luteal cells were consistently smaller and had large cytoplasmic vacuoles in corpora lutea from inoculated cows (Figs 8 and 9). A unique feature of the
Figs 1-6—Placentomes from cows with experimental sarcocystosis and from a gestation-matched control cow.

Fig 1—Control cow (197 days after insemination). Chorionic villi on fetal cotyledons are fronds of vascular mesenchyme covered with a simple layer of trophoblast (arrow). Crypts lined with simple cuboidal epithelium penetrate the vascular connective tissue of the uterine caruncle.

Fig 2—25 days after inoculation (188 days after insemination). Crypt epithelium is slightly shortened and crypt interstitium is thickened.

Fig 3—28 days after inoculation (195 days after insemination). Crypt epithelial cells are shrunken and several have pyknotic nuclei.

Fig 4—30 days after inoculation (195 days after insemination). Necrosis of interstitium in septae between highest order crypts (arrow). Necrosis is subjacent to intact epithelium. Interstitium is thickened.

Fig 5—32 days after inoculation (197 days after insemination). Caruncle has contracted and is predominantly dense connective tissue. Residual crypts are partially lined with flattened epithelium.

Fig 6—35 days after inoculation (197 days after insemination). Crypt epithelium is sparse and flattened in residual crypts and chorionic villi within crypts are necrotic.
Table 1—Lesions and *Sarcocystis cruzi* Schizonts in Corpora Lutea from Pregnant Cows Fed *S. cruzi* Sporocysts.

<table>
<thead>
<tr>
<th>Corpus luteum (uninoculated)</th>
<th>Control cow (uninoculated)</th>
<th>Days after sporocyst inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cruzi</em> schizonts per cm² in corpus luteum section (6 μm)</td>
<td>none</td>
<td>1.6 5.2 8.1 59.2 0.7 none</td>
</tr>
<tr>
<td>Large luteal cell</td>
<td>50%</td>
<td>35% 20% 10% 0-20%&lt;sup&gt;a&lt;/sup&gt; 20% 30%</td>
</tr>
<tr>
<td>Large luteal cell to stroma ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large luteal cell morphology</td>
<td>Polygonal, approx 40 μm dia (&lt;55 μm) Stellate, approx 20 μm dia (&lt;35 μm)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Small cytoplasmic vacuoles (2-3 μm) Large cytoplasmic vacuoles (&lt;10 μm)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Patchy variation in the ratio.

<sup>b</sup>Several hypertrophied large luteal cells (65 x 130 μm) were randomly distributed in the corpus luteum from the cow necropsied 28 days after sporocyst inoculation.
Fig 7-10—Corpora lutea from cows with experimental sarcocystosis and a gestation-matched control cow.

Fig 7—Control cow (197 days after insemination). Corpus luteum is comprised of large luteal cells, small luteal cells and delicate vascular stroma.

Fig 8—28 days after inoculation (188 days after insemination). Large luteal cell degeneration (luteolysis) and large cytoplasmic vacuoles are present (arrow). Note multifocal accumulations of lymphocytes.

Fig 9—32 days after inoculation (197 days after insemination). Large luteal cells are fewer than normal. Stroma and lymphocytes are prominent.

Fig 10—S. cruzi schizonts in corpus luteum taken 35 days after inoculation (197 days after insemination). Endopolygeny (a), the first phase of multiple division of S. cruzi nucleus in the host cell. Schizonts with numerous protozoa of 2 different sizes (b and c). Dark, eccentric host cell nucleus is included in a and b.
corpus luteum 28 days PI was the presence of several large, randomly distributed cells interpreted to be hypertrophied large luteal cells. Protozoan schizonts were present 25-36 days PI and were numerous in the tissue taken 35 days PI (Fig 10). The large luteal cell-to-stroma ratio was inversely proportional to the severity of infection. Stromal components were more prominent in infected corpora lutea due to large luteal cell regression. Prominence of foamy cytoplasm of histiocytes corresponded to regression of large luteal cells. Lymphocytes were present in all sections and were more numerous in infected tissue. A few neutrophils were associated with focal degenerative lesions in corpora lutea from the cows necropsied 25 and 28 days PI.

Progesterone concentration in 82 plasma samples from 7 pregnant cows with sarcocystosis and the gestation-matched control cow are summarized on Table II. Progesterone concentrations ranged from 5.9 to 0.3 ng/ml in the plasma assayed. Abortions corresponded with the only 2 progesterone concentrations that were less than 0.9 ng/ml.

In pituitary glands focal perivascular accumulations of neutrophils were found in capsules 28, 32, and 36 days PI. In adenohypophyses from cows 32, 35, 36, and 37 days PI a few acidophil cells with pyknotic nuclei and hyalin cytoplasm and focal accumulations of lymphocytes were present. Focal gliosis and lytic lesions in the neurohypophysis were found 28, 32, 35, 36, and 37 days PI. Mild focal neutrophilic vasculitis was also present 36 and 37 days PI. Schizonts were present in the neurohypophysis 32 days PI.

Endometrial lesions ranged from epithelial cell anisocytosis, vacuolation and sloughing 32 days after inoculation to fibrinouspurulent endometritis with histiocytic infiltration of the submucosa 36 days PI. Cervical seals
Table 2—Progesterone Concentration in Plasma from Pregnant Cows Inoculated with *Sarcocystis* *cruzi* Sporocysts

<table>
<thead>
<tr>
<th>Cows</th>
<th>Days after inoculation</th>
<th>0</th>
<th>3</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>17</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated control cow</td>
<td></td>
<td>2.1</td>
<td>1.9</td>
<td>1.9</td>
<td>1.8</td>
<td>1.7</td>
<td>2.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Inoculated cows with live fetuses</td>
<td></td>
<td>3.3</td>
<td>5.9</td>
<td>4.4</td>
<td>3.6</td>
<td>3.2</td>
<td>3.1</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2</td>
<td>2.3</td>
<td>1.9</td>
<td>2.5</td>
<td>2.4</td>
<td>4.9</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.8</td>
<td>ns</td>
<td>4.7</td>
<td>3.1</td>
<td>1.7</td>
<td>2.2</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.9</td>
<td>1.2</td>
<td>1.3</td>
<td>1.6</td>
<td>0.9</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>2.3</td>
<td>3.1</td>
<td>3.1</td>
<td>2.7</td>
<td>2.1</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>sd$^f$</td>
<td>.81</td>
<td>2.01</td>
<td>1.49</td>
<td>.74</td>
<td>.85</td>
<td>1.30</td>
<td>1.26</td>
</tr>
<tr>
<td>Inoculated cows with dead fetuses</td>
<td></td>
<td>3.6</td>
<td>5.6</td>
<td>4.6</td>
<td>2.6</td>
<td>4.9</td>
<td>5.2</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.6</td>
<td>4.1</td>
<td>4.2</td>
<td>2.9</td>
<td>2.2</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ns</td>
<td>3.4</td>
<td>3.2</td>
<td>2.2</td>
<td>1.5</td>
<td>3.0</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>3.1</td>
<td>4.4</td>
<td>4.0</td>
<td>2.6</td>
<td>2.9</td>
<td>3.4</td>
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<tr>
<td></td>
<td>sd</td>
<td>.50</td>
<td>.92</td>
<td>.59</td>
<td>.29</td>
<td>1.47</td>
<td>1.34</td>
<td>.81</td>
</tr>
</tbody>
</table>

$^a$ Sampling days were grouped to include days with single samples.

$b$ No sample.

$^c$ Inoculated with $1 \times 10^5$ *S. cruzi* sporocysts.

$d$ Inoculated with $1 \times 10^6$ *S. cruzi* sporocysts.

$^e$ Mean progesterone concentration from each group of inoculated cows.

$^f$ One standard deviation from the mean.
<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Necropsy schedule</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>days after inoculation</td>
</tr>
<tr>
<td>24</td>
<td>28-29(^a)</td>
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<tr>
<td>1.4</td>
<td>2.4</td>
</tr>
<tr>
<td>4.7</td>
<td>ns(^b)</td>
</tr>
<tr>
<td>2.9</td>
<td>3.4</td>
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<tr>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>0.9</td>
<td>1.2</td>
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consisted of clear mucus 25 and 28 days PI. A few linear tracts of necrotic cells were in the mucus 30 days PI and tracts were more numerous 32 days PI. Uniform simple columnar epithelium was subjacent to clear cervical mucus whereas anisocytosis, swelling and focal sloughing of cervical epithelial cells was found 30 and 32 days PI. Neutrophils, histiocytes and plasmacytes were present in the submucosa 30 and 32 days PI. *Sarcocystis cruzi* schizonts were found in some sections of endometrium but none were found in sections of cervix.

Sections of mammary tissue from the 7 inoculated cows were similar to the control tissue. Some lymphocytes and fewer plasmacytes were present within the interstitium of all 8 mammae. Mild accumulation of interstitial edema was found in interlobular connective tissue of glands from the 3 cows with dead fetuses.

**Discussion**

The mechanism of fetal death induced by bovine sarcocystosis is presumably failure of vital maternal-fetal exchange processes. Swelling due to fluid accumulation in allantochorions is the only lesion found in either fetuses or placentae before fetal death. Transplacental infection of fetuses by *S. cruzi* is minimal and was demonstrated only in dead fetuses. Changes in the placentome are interpreted as premature involution of the caruncle causing gradual extrusion of chorionic villi and finally necrosis of the fetal cotyledon. Placentome atrophy in inoculated cows is disproportionate between the maternal caruncles and the fetal cotyledons. The maternal caruncle diminishes in size and extrudes the chorionic villi which
apparently do not regress in size but degenerate. *S. cruzi* were demonstrated in sectioned caruncles but the interstitial contraction and crypt epithelium regression occurs with minimal inflammatory response.

Degenerative changes in large luteal cells of corpora lutea from inoculated cows is remarkable and surely must correspond to diminished function of those cells. Luteolysis and abortion can be induced in cattle in mid-gestation by parenteral administration of prostaglandin F₂α. Abortion can be induced in pregnant cattle until the 200th day of gestation by surgical extirpation of corpora lutea. Sarcocystosis is apparently abortifacent due to infection and luteolysis of corpora lutea. If this hypothesis is valid, the abortifacent efficacy of sarcocystosis in cattle should be diminished later in pregnancy, after 200 days.

Plasma progesterone concentration was lowest (<0.5 ng/ml) in cattle sampled 35 and 36 days PI. The lowest progesterone concentrations corresponded with abortion of dead fetuses. The necropsy protocol for these 7 cows eliminated infected cows beyond 37 days PI. The progesterone assay results complement the morphologic degeneration found in corpora lutea. These progesterone assays are comparable to previously described progesterone concentrations in plasma from pregnant cows. Plasma progesterone concentration in the control cow did not diminish during the interval of gestation studied.

*Sarcocystis cruzi* infection and mild multifocal inflammation were consistent in sections of pituitary gland from inoculated cows but distribution and extent of the lesions in adenohypophyses and neurohypophyses were not
consistent. The lesions might cause endocrine imbalance but comprehensive evaluation of function was beyond the scope of this investigation.

Degenerative changes described in endometrial and cervical tissues were associated with dead fetus expulsion. We believe the changes did not cause, but were the result of, fetal death. Mild accumulation of interstitial edema with no cellular exudate in mammary tissue from cows with dead fetuses is probably endocrine mediated. Interstitial lymphocytes and plasmacytes are normal in nonlactating cows during the interval of gestation studied. Conclusions based on these results are (1) corpora lutea are infected by *S. cruzi*, (2) the infection causes diffuse luteolysis and diminished plasma progesterone concentration, (3) premature involution of uterine caruncles is induced, (4) vital exchange processes in placentomes diminish and the fetus dies, (5) an autolyzed fetus is aborted.
References


GENERAL DISCUSSION AND CONCLUSIONS

Results of this investigation include the first successful application of immunofluorescent stain to visualize asexual stages of *S. cruzi* in bovine tissues. The technique was used to chronologically characterize the distribution of various stages of *S. cruzi* within various tissues during bovine sarcocystosis. The third part of the investigation confirmed that *S. cruzi* is an abortifacient pathogen in pregnant cows. *Sarcocystis cruzi* infection and lesions found in reproductive organs and plasma progesterone assays indicate that fetal death is caused by infection of corpora lutea in pregnant cows.

The immunofluorescent stain used in this investigation was made from autologous serum. Bovine serum with an antisarcocystis titer of 1:39,000, determined by indirect hemagglutination, was used in conjunction with rabbit antibovine globulins conjugated with fluorescein. Globulins from the same bovine serum were conjugated with fluorescein to expedite further studies. *Sarcocystis cruzi* were resolved individually by immunofluorescent stain and each stage of *S. cruzi* had sufficient uniformity for recognition. Autofluorescence of bovine granulocytes was an annoyance, but not a serious problem, encountered in the bovine tissue studies. Visualization of *S. cruzi* in lesions of eosinophilic myositis by use of immunofluorescent stain would require more sophisticated techniques than were employed in this investigation.
The immunofluorescent stain used was not characterized in regard to specificity except that it did not stain *Toxoplasma gondii*. No frozen bovine tissues known to be infected with *S. hirsuta* or *S. hominis* were available to evaluate the species specificity of the stain.

The void in the results of the study of developmental phases of *S. cruzi* in various tissues was an early phase. *Sarcocystis cruzi* was not found during the first 12 days after sporocysts were fed to cattle. Where and how do sporozoites leave the lumen of the intestine to enter the host tissues? Does a trophozoite phase precede the first generation of schizogony? Hematogenous spread of the infection is indicated by the systemic distribution of schizonts in endothelial cells. Neither extracellular nor intracellular *S. cruzi* have ever been recognized in bovine blood smears.

The abortion study results clearly establish *S. cruzi* as an abortifacient pathogen in pregnant cattle. Gestation times of the cows studied were intentionally clustered (154-167 days when inoculated) to accommodate comparison of reproductive tissues among the cows. The disadvantage of the clustering is that large intervals of the 280 day bovine gestation remain to be experimentally challenged by sarcocystosis. Results of the abortion study indicated that, although caruncles are infected, fetal death is caused by infection of the corpus luteum and luteolysis which result in diminished plasma progesterone concentration. If the hypothesis is valid, then administration of exogenous repositol progesterone would be expected to minimize the number of abortions during an outbreak of sarcocystosis in a herd of pregnant cows.
Conclusions derived from these studies are: (1) immunofluorescence is a reliable, practical technique for demonstration of *S. cruzi* in bovine tissues, (2) immunofluorescence is currently the only technique for demonstration of individual merozoites between generations of schizogony or during myofiber penetration, (3) individual merozoites persist in lymph nodes of cattle for several weeks after systemically distributed schizogony subsides, (4) sarcocystosis does not confer lasting protective immunity against re-infection, (5) *S. cruzi* is an abortifacient pathogen in pregnant cows, (6) *S. cruzi* infects the corpora lutea of pregnant cows and causes luteolysis accompanied by diminished plasma progesterone concentration, and (7) fetal death, due to premature involution of uterine caruncles, precedes abortion.

Considerations for further investigation include: (1) characterization of the relative specificity of the antisarcocystis conjugate which would require tissues infected with other *Sarcocystis* species and related protozoa, (2) elucidation of the mechanism of infection of cattle during the first 15 days after sporocyst inoculation, (3) reactivation of schizogony in bovine tissues by residual merozoites in lymph nodes, perhaps by dexamethasone treatment of cattle, (4) establish the interval of bovine pregnancy susceptible to the abortifacient effect of sarcocystosis, and (5) establish the significance of natural bovine sarcocystosis by diagnostic survey of cases.
ADDITIONAL REFERENCES CITED


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