Comparison of an Enzyme-Linked Immunosorbent Assay, an Indirect Fluorescent Antibody Test and a Direct Agglutination Test for Detecting Ovine Fetal Antibodies to Toxoplasma Gondii

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COMPARISON OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY, AN INDIRECT FLUORESCENT ANTIBODY TEST AND A DIRECT AGGLUTINATION TEST FOR DETECTING OVINE FETAL ANTIBODIES TO TOXOPLASMA GONDII

BY

SHERYL L. SEEFEILDT

A thesis submitted in partial fulfillment of the requirements for the degree Master of Science Major in Microbiology South Dakota State University 1988
COMPARISON OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY, AN INDIRECT FLUORESCENT ANTIBODY TEST AND A DIRECT AGGLUTINATION TEST FOR DETECTING OVINE FETAL ANTIBODIES TO TOXOPLASMA GONDII

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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SLS
INTRODUCTION

Toxoplasma gondii is a coccidian parasite which produces a disseminated infection and reproductive failure in sheep. It is a major cause of abortion in sheep in New Zealand, Australia, and England. Within the last 5 years, T. gondii has been reported to cause ovine abortion in the United States (21). Individual flocks in New Zealand may lose 15-20 percent and sometimes as high as 50 percent of the lamb crop as a result of T. gondii induced abortion (22). The prevalence of T. gondii and the economic impact of the disease in U.S. sheep flocks is unknown.

Several approaches have been employed to diagnose toxoplasmosis in ovine abortions (19). Inoculation of mice with fetal tissues can confirm the diagnosis but may require 3-8 weeks to detect the infection or isolate the organism (31). Microscopic examination of fetal tissues for lesions of toxoplasmosis is useful, but lesions are not always present, or they may not be identifiable in aborted lambs because of autolytic changes. The immune status of the ewe at the time of the abortion may indicate prior exposure of the ewe to T. gondii. However, this provides little information concerning the cause of abortion, because high antibody titers persist through several pregnancies (12).
Ewes experimentally infected with *T. gondii* often developed serum antibody titers to this organism after abortion or parturition (39). The serologic status of the aborted fetus appears to be useful in diagnosing *T. gondii*-induced abortions. Ovine maternal antibody normally does not cross the placental barrier (49). Therefore, antibody present in the fetus is of fetal origin, and detection of toxoplasma antibody in ovine fetal sera or body fluids is diagnostic. In contrast, the absence of specific antibody does not rule out the possibility of toxoplasmosis. The fetus may become infected prior to the age when fetal antibody production normally begins, or the strain of *T. gondii* may be so virulent that fetal death may occur before an immunocompetent fetus can produce an immune response (19, 39).

Serologic kits are available for detecting antibody to *T. gondii* in human serum. These kits can be modified for veterinary use with some reagent substitutions. These modified kits are convenient to use in veterinary diagnostics because the antigens and other reagents are standardized by the manufacturer and are consistent from lot to lot. This eliminates the need for diagnostic laboratory personnel to propagate antigen in mice or cats.
OBJECTIVES

The objectives of this research were to evaluate and compare the efficacy of 3 serologic techniques (i.e. direct agglutination, enzyme-linked immunosorbent assay, and indirect fluorescent antibody) for detecting antibody to *Toxoplasma gondii* in aborted ovine fetuses submitted to the South Dakota Veterinary Research and Diagnostic Laboratory, and to determine the prevalence of toxoplasma-induced ovine abortions.
LITERATURE REVIEW

**Toxoplasma gondii** is an intestinal coccidium of all species of cats. It causes a disseminated infection in other animal species and poses a serious health threat to humans and many animal species. However, its greatest economic impact is on the sheep industry. In sheep, the most serious form of toxoplasmosis is congenital infection which is characterized by placentitis, fetal encephalitis, abortion, and stillbirth (22, 35). Toxoplasmosis has wide geographic distribution and probably occurs in all countries where the sheep industry has developed. The disease is frequently incriminated in reproductive losses in sheep of Australia, New Zealand, Britain, Denmark, USSR, and Turkey (22, 29, 35). Although antibody to **T. gondii** is common in sheep in the United States (15, 44), the infection has only recently been incriminated with abortion in the United States (5, 19, 20, 21, 43).

Cats are the definitive hosts of **Toxoplasma gondii** while man and various other warm-blooded animals, including sheep, are intermediate hosts. Transmission occurs in one of 3 ways: ingestion of raw infected meat, ingestion of oocysts from infected cat feces, and transplacental. Cats
become infected by ingesting raw meat (e.g. aborted lambs or fetal placenta, mice, rats, or birds) containing tissue cysts or by ingesting sporulated oocysts excreted by another cat. Feline toxoplasmosis mainly occurs in young cats, but any age cat may acquire the infection if there has been no previous exposure to the organism. The clinical signs that sometimes occur in cats with acute disseminated toxoplasmosis include lethargy, anorexia, dyspnea, jaundice, and lymphadenopathy. In chronic toxoplasmosis of several weeks duration, the clinical signs include pyrexia, vomiting, anemia, abortion, dyspnea, lymphadenopathy, diarrhea, hepatic disease, blindness, and nervous derangement (6). When tissue cysts are ingested by a cat, the bradyzoites released by the digestive action of the intestine initiate the asexual merozoite stage to produce the gametocytes. The gametocytes undergo a sexual cycle in the feline intestinal mucosa in which the male gamont fertilizes the female gamont. A wall forms around the fertilized female gamont to form the oocysts (16). The prepatent period is 3 to 10 days from ingestion of tissue cysts, and the oocysts are shed by cats for one to 2 weeks (26). Cats may shed one thousand to one million oocysts per day (26). Two to 4 days later the oocysts sporulate and become infections. Humid conditions prolong the survival of the sporulated oocysts, enabling them to remain viable for
several months to a year (26). Sporulated oocysts are able to survive 28 days or more at -21 C (25).

The reproductive cycle of *T. gondii* is repeated if oocysts are ingested by a susceptible cat, sheep or other animal. Only cats produce oocysts. Cats that are immune to *T. gondii* are not likely to repeat the shedding of oocysts unless the cat becomes starved or immunosuppressed.

Sheep acquire infection by consuming feed that has been contaminated by cat feces containing oocysts (41). Following ingestion by a susceptible pregnant ewe, the wall of the sporulated oocyst is dissolved by digestive enzymes and the released sporozoites penetrate the gut wall and multiply. Parasitemia occurs during the first week following ingestion, and the organism is disseminated via blood and lymph to several organs, including brain, liver and heart. In these organs, the sporozoites enter cytoplasmic vacuoles and form groups of tachyzoites, and later, cysts of bradyzoites (35). Other than abortion, clinical signs in the ewe are absent except for slight fever and anorexia.

*T. gondii* infects the fetus during or after the second week following experimental inoculation of the ewe (13, 17). The organism multiplies in the placenta and invades the fetus (9). A fetal parasitemia ensues and tissue cysts containing bradyzoites form as in adult sheep.
The brain becomes the most heavily infected fetal organ, but the liver, heart, spleen, lymph nodes and blood are also infected (10, 32, 54). Lesions in the fetus are not pathognomonic. In twins, one or both fetuses may be affected (22).

The normal ovine placenta prevents transplacental passage of immunoglobulins from dam to fetus (49). Whether or not maternal immunoglobulins are transferred to the fetus when the placenta is damaged by infection is disputed. Most abortifacient infections of sheep (T. gondii, Chlamydia sp., Brucella sp., Campylobacter sp., Coxiella sp.) cause severe placentitis. Poitras et al. (42) inoculated pregnant ewes at 80 and 94 days gestation with human red blood cells (HRBC) to stimulate anti-HRBC antibody production. Placental damage was induced by injecting the ewes with either Aspergillus fumigatus spores at 100 days gestation or Escherichia coli endotoxin at 99 days gestation. Anti-HRBC antibody was detected in fetal body fluids of 7 of 16 fetuses. The damage inflicted on the placenta may have resulted in fusion of maternal and fetal circulations which permitted maternal antibody to cross the placental barrier and contaminate the fetal circulation. It is questionable whether the placental damage was entirely due to fungal spores or endotoxins, because control ewes as well as treated ewes spontaneously aborted. The possibility that an
unidentified infectious agent caused placental damage seems likely. Dubey et al. (18) conducted a similar experiment in which pregnant ewes were inoculated with chicken globulins (CG) and leptospira bacterin 2 months prior to oral inoculation with *Toxoplasma gondii* oocysts. All ewes developed high titers to CG but no appreciable amounts of anti-CG antibody were detected in their fetuses. Leptospira antibodies were detected in one fetus, although a poor response to the vaccine was noted among the ewes. Fetal response could have been caused by a congenital leptospira infection. These results suggest that it is unlikely for maternal antibody to cross even a severely damaged placenta.

Fetal sheep are capable of responding to antigenic stimuli and synthesizing specific antibody *in utero* (23, 46). The gestation period of the ewe is about 145 days, and the fetal immune system matures with increasing gestational age. Fahey and Morris (24) reported that fetal sheep are first able to produce detectable levels of antibody to many antigens between 64 and 82 days gestation. Immunoglobulin M is the only antibody class synthesized during this period. The capacity to synthesize IgG begins around 90 days gestation. Fetuses that become infected with *T. gondii* before 90 days are not capable of mounting an inflammatory response to the infection. Immunoglobulin G production and the inflammatory response seem to occur simultaneously and
may be related to the stage of development that the fetal immune system has reached at 90 days gestation (23, 46).

The stage of immune system development affects the fetal response to infection by *T. gondii*. Infection of the ewe before 50 days gestation results in early embryonic death and resorption, probably because the fetus does not begin to develop immunologic competence until 60 to 70 days of gestation (13, 32). Infection of the ewe between 60 and 100 days gestation generally results in fetal death and abortion. Although fetal antibody synthesis has developed by this time, a T-lymphocyte mediated response to infection, which is necessary for protection, may be absent or ineffective (13). Infection of the ewe during the last 30 days of gestation is less likely to cause fetal death, but lambs born alive to such ewes may be congenitally infected (54). By late gestation, the fetal immune response is markedly enhanced compared to that of a fetus in mid-gestation.

Fetal body fluids or sera have been used in serologic tests to detect antibody to *T. gondii* (17, 19, 33, 37, 38, 39). Although ovine maternal antibody is unable to cross the placenta except under extraordinary conditions, many pathogens are capable of crossing the placental barrier and eliciting an immune response by the fetus (49). Therefore, anti-toxoplasma antibody in serum from aborted
fetuses is of fetal origin and its presence indicates that the fetus was infected by *T. gondii*. However, the absence of toxoplasma antibody does not preclude the possibility of toxoplasmosis, because the development of fetal antibody is dependent on the age of the fetus at the time of infection and the length of time between infection and fetal death (19).

The presence of anti-toxoplasma antibodies in a ewe that aborts provides little information as to the cause of the abortion. Infection of seronegative ewes with *T. gondii* stimulates a strong, persistent antibody response (22, 12). Experimentally infected ewes maintain high antibody titers to *T. gondii* for more than 3 years after initial infection (11). Ewes will usually have a normal gestation following infection and abortion and are resistant to reinfection (54). Ewes that contract toxoplasmosis and develop immunity before pregnancy will not abort and are resistant to infection during gestation (8).

Serologic tests for *T. gondii* have been developed for use in human clinical medicine. The Sabin-Feldman methylene blue dye test (DT) is considered the standard to which all tests are compared (45). Sabin found that the addition of alkaline methylene blue dye to a suspension of live *T. gondii* tachyzoites, stained the cytoplasm of the organism deep purple. Addition of fresh (not heat-
inactivated, thus containing complement) "normal" human serum did not affect staining of the organism by methylene blue, but fresh serum containing toxoplasma antibody prevented staining of the organisms. Thus, toxoplasma antibody plus complement prevents staining of the live tachyzoites. To perform the DT, sera must be heated to inactivate complement. Normal human serum that has not been heat-inactivated is used to dilute the antigen. The heat-inactivated test serum is added to the antigen-normal serum mixture, and any toxoplasma antibody present complexes with the complement factors provided by the normal serum. When the methylene blue is mixed with the suspension, staining of the parasite is prevented.

The DT has been used to detect toxoplasma antibody in sheep sera in the past but results may not be reliable. Te Punga attributed false-positive DT reactions in serum from apparently normal sheep to a nonspecific, heat-labile antibody which had anti-toxoplasma activity (47, 48). This heat-labile antibody is similar to complement except its activity is not inhibited when heated at 56 C for two hours, but its effect is markedly diminished when serum is heated at 60 C for one hour. Dubey (15) described a naturally-occuring antibody-like factor present in the sera of sheep and cattle (and probably other ruminants) not infected with T. gondii. Therefore, it is recommended that ruminant sera
be heat-inactivated at 60 C for at least 30 minutes before use in the DT. Serum may coagulate and become unsuitable for testing when heated at 60 C.

Another disadvantage of the DT is that it requires the use of live organisms which must be maintained through serial passage in mice. Maintenance of the cultures is time consuming and handling virulent *T. gondii* poses a health threat to laboratory personnel (45). Also the test requires a high degree of technical skill to perform.

The indirect hemagglutination test (HA) developed by Jacobs and Lunde (34) has been used to test sheep sera for toxoplasma antibodies (10, 11, 12, 30, 44). The HA test is based on the fact that soluble antigen can be adsorbed to tanned red blood cells (RBC's). In the HA test, toxoplasma antibodies bind to the soluble antigen adsorbed to the tanned RBC's and cause agglutination of the RBC's.

The direct agglutination test (DAT) described by Fulton et al. (27, 28) uses whole, killed toxoplasma tachyzoites for the antigen. Desmonts and Remington modified the DAT to increase its sensitivity and specificity (14). They prepared antigen in mice using a mixture of sarcoma cells and tachyzoites which resulted in production of a large quantity of pure antigen. They also enhanced the specificity of the test by incorporating 2-mercaptoethanol
into the serum diluent to inactivate any normal IgM that might be present to agglutinate *T. gondii* nonspecifically.

Gupta et al. (30) developed a rapid card agglutination test (CAT) that could screen undiluted serum in a short time. The antigen was whole formalinized tachyzoites. Although the CAT is not as sensitive as the HA it is simple to perform and results can be obtained within five minutes.

The latex agglutination test (LAT) uses soluble antigen adsorbed to latex particles, but the test is less sensitive than the DT (7). Drops of undiluted or diluted test serum are mixed with the antigen suspension on a black glass tile and results are read 2 to 5 minutes later. Agglutination caused by the reaction of toxoplasma antibodies and antigen-coated latex particles indicates a positive reaction. Dubey et al. compared the DAT to DT, HA, and LAT and found the HA and LAT to be less sensitive than the DAT for detection of *T. gondii* antibody in ovine fetal sera (17).

An adaptation of the indirect fluorescent antibody (IFA) test developed by Kelen et al. (36) has been used to detect ovine fetal antibody to *T. gondii* (13, 33, 37, 39). One advantage of the IFA test is that both IgG and IgM class-specific conjugates are available so it is possible to assay for either IgG or IgM in ovine fetal sera.
Walls et al. (52) used an enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of toxoplasmosis in humans. O'Donoghue et al. (40) performed a serologic survey for *T. gondii* antibody in sheep to compare the HA test to the ELISA. There was significant positive correlation between the 2 tests. No comparison was made on fetal serum and the youngest lamb tested in the study was 5 months old. The ELISA uses soluble antigen adsorbed to wells in polystyrene plates. Test serum is placed in the wells and incubated. After addition of enzyme-labeled conjugate and substrate, a color change occurs if toxoplasma antibody was present to bind to the antigen in the well.

A variation of the ELISA method previously described to test sheep serum is the diffusion-in-gel enzyme-linked immunosorbent assay (DIG-ELISA) reported by Uggla (50). A soluble antigen is adsorbed to wells in a polystyrene plate and agar is then layered over the adsorbed antigen. Test serum is placed on the agar in the well followed by the addition of enzyme-labeled conjugate and substrate. Brown circular zones appear in areas with antibodies attached to antigen adsorbed to the plastic surface. The diameters of the reaction zones increase proportionately with the amount of antibody present.

Isolation of *T. gondii* from abortion tissue by mouse inoculation is the most specific method for confirming the
parasite as the cause of abortion, however it too has serious disadvantages. Inoculation of mice with abortion material is time consuming, sometimes requiring as long as 8 weeks to obtain results (31). Results may be obscured by interference from bacterial and mycotic contaminants in the inoculum that prevent positive identification of the parasite. For these reasons isolation of *T. gondii* is impractical for most laboratories to perform and also risks exposure of laboratory personnel to live organisms.

A direct fluorescent antibody (DFA) technique (1, 2, 3) has been used to detect *T. gondii* in abortion tissue but this has proven to be unsatisfactory on severely decomposed tissue, and usually only fragments of the parasite fluoresce when observed under the microscope, making identification difficult. A peroxidase-anti-peroxidase (PAP) technique was developed for visualizing *T. gondii* in formalin-fixed, paraffin-embedded tissues from abortion specimens (51). The DFA and PAP both require preparation of specific antisera to *T. gondii* which necessitates handling live organisms. The PAP is a very time-consuming test, requiring 1-2 days for fixation of tissue prior to actual performance of the PAP test. Both DFA and PAP results are highly subjective, and require experienced personnel to differentiate specific from nonspecific staining.
RESEARCH SUMMARY

A. Introduction

Toxoplasma gondii is an intestinal coccidium of all species of wild and domestic cats. The systemic form of toxoplasmosis poses a serious threat to humans and many animal species; however its greatest economic impact is on the sheep industry. The most serious form of toxoplasmosis in sheep is prenatal infection. Prenatal toxoplasmosis in sheep is characterized by placentitis, fetal encephalitis, abortion, and stillbirth (22, 35). Toxoplasmosis has wide geographic distribution and probably occurs in all countries where the sheep industry has developed. The disease is accountable for high reproductive losses among sheep in Australia, New Zealand, Britain, Denmark, USSR, and Turkey (22, 29, 35). Although antibody to T. gondii is common in sheep in the United States (15, 44), the infection has not been incriminated as a cause of abortion in sheep until recent years (5, 19, 20, 21, 43).

Since 1983, Toxoplasma gondii has been diagnosed with increasing frequency as a cause of ovine abortion among cases submitted to the South Dakota Animal Disease Research and Diagnostic Laboratory (19). Diagnosis by gross and
microscopic examinations is sometimes difficult because of the condition of the fetus. Autolyzed tissue often is unsuitable for histologic examination. Although lesions in the brain are quite specific, the brain undergoes autolysis early and is often unsuitable for examination (10). Gross placental lesions are quite characteristic, but often the fetal placenta is unavailable or is unsuitable for examination (9). Isolation of *T. gondii* from abortion material by mouse inoculation is impractical as a routine diagnostic procedure, because the test requires 3-8 weeks for completion and in addition, bacterial or mycotic contaminants may interfere with isolation (31).

Maternal antibody does not cross the ovine placenta (49) except in instances where severe placental damage results in fusion of maternal and fetal circulations (42). However, such damage does not occur during toxoplasma infection. Serologic tests to detect anti-toxoplasma antibody from an aborted fetus can aid diagnosis because specific antibody present in the toxoplasma-infected fetus is of fetal origin (18, 19). Serologic tests have been developed to detect specific antibody to *T. gondii* in humans. With the exception of the Sabin-Feldman methylene blue dye test (DT) (45), these tests can easily be adapted for use in veterinary medicine. It is difficult to adapt the DT for use with ovine sera, because a complement-like
factor in ovine sera may cause false-positive results (15, 47, 48). This factor differs from normal complement in that it must be heated to 60 C for 30 minutes before it is inactivated. The live organisms used in the DT are also difficult to propagate and a hazard to laboratory personnel. This along with the modified serum treatment procedure and the fact that some sera may coagulate and become unsuitable for testing when heated to 60 C are major disadvantages of the DT.

In recent years, several commercially manufactured serologic tests to detect antibody to *T. gondii* in human serum have become available. These tests may be adapted for veterinary use. This report is a comparison of the methods and results of 3 modified commercial serologic tests used to detect *T. gondii* antibody in ovine fetal sera. The tests were: (1) a direct agglutination test (Toxo-screen, DA; Hynson, Westcott & Dunning, Baltimore, MD; production of this test was discontinued in 1986 but antigen for the test was kindly provided by Dr. J. P. Dubey, USDA ARS, Beltsville, MD), (2) an enzyme-linked immunosorbent assay (Toxoelisa; Microbiological Associates Bioproducts, Walkersville, MD), and (3) an indirect fluorescent antibody test (antigen slides manufactured by Gull Laboratories, Salt Lake City, UT). The results of the tests were evaluated and compared for detection of toxoplasma antibody in ovine fetal
thoracic fluid or sera obtained from aborted fetuses submitted during 1985 and 1986 to the South Dakota Animal Disease Research and Diagnostic Laboratory.

**B. Materials and Methods**

**Sera.** At necropsy, fluids were aspirated from thoracic cavities or blood was collected from the hearts of aborted lambs. Sera were not heat-inactivated and were stored at 4°C.

**Direct Agglutination Test (DAT).** Serum samples and control sera were diluted 1:8 and 1:800 with phosphate buffered saline solution (PBS), pH 7.2 (140 ml of 0.2 M NaH$_2$PO$_4$, 360 ml of 0.2 M Na$_2$HPO$_4$, 500 ml distilled water and 8.5 g NaCl). Twenty-five microliters of each test serum dilution was added to one of 2 wells in a U-bottom microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA). Twenty-five microliters of 0.2 M 2-mercaptoethanol (2-ME) (50 microliters 2-ME in 3.5 ml PBS, pH 7.2) was added to each test well (making the final dilution of the serum samples 1:16 and 1:1600). This was followed by 50 microliters (containing approximately 20,000 organisms per microliter) of toxoplasma antigen diluted in freshly prepared 0.1 M borate buffered saline, pH 8.9 (1.753 g NaCl, 0.773 g H$_3$BO$_3$, 6 ml of 1.0 N NaOH, 1.0 g bovine serum albumin, 0.25 g NaN$_3$ in 250 ml distilled water). Positive and negative goat sera were used to titrate each lot of
antigen to determine the working dilution and were also used as controls in each test. These control sera were furnished with the commercial kits; however, any serum known to have a high titer to *T. gondii* could serve as a positive control, and any serum known to be free of toxoplasma antibody could serve as a negative control. An antigen control (consisting of PBS, 2-ME, and diluted antigen) was included in each test to check for autoagglutination of antigen.

The plate was sealed with a transparent adhesive sheet. The contents of the plate were mixed one minute on a microtitration plate mixer at a setting of 4 (Syntron Jogger, Homer City, PA). The plate was then allowed to stand undisturbed at least 6 hours at room temperature (20-25 C) before it was examined. The reaction was stable up to 72 hours and could be successfully examined up to that time provided the plates were not disturbed.

The results of the test were examined visually with a Microtiter mirror (Cooke Engineering, Co., Alexandria, VA). Results were considered positive when the antigen formed a mat covering more than half of the well base. Mats that shrank around the edge to produce irregularly-shaped rings were regarded as positive if more than 50% of the well bottom was covered. A result was equivocal when the agglutination mat covered 50% or less of the well base but did not form a distinct button or ring. A negative result
was determined by the formation of a button or ring similar to that formed by the negative or antigen control. Test sera producing positive or equivocal reactions were titrated to the end point.

**ELISA.** The ELISA was performed as indicated by the instructions in the kit except that alkaline-phosphatase-labeled rabbit anti-sheep immunoglobulin G (IgG) (Kirkgaard and Perry Laboratories, Inc., Gaithersburg, MD) was substituted for the anti-human IgG conjugate furnished with the kit. All tests were performed at room temperature (20-25°C). The kit plate consisted of removable well strips in which alternating wells were coated with toxoplasma antigen. Each test serum required both an antigen well and a control well. The removable well strips allowed one to use only the number of wells necessary for the number of sera to be tested.

Wells were washed 3 times with PBS (pH 7.2) containing .05% (v/v) Tween-20 solution to reduce non-specific binding. After the wash solution was completely removed by inverting the plates on paper towels and gently tapping the wells, 250 microliters of serum diluent (furnished with the kit, composition unknown) was added to each well. Ten microliters of test serum was added to a well coated with *T. gondii* antigen, and the same amount was added to the diluent in an adjacent control well. The final
serum dilution was 1:26. To ensure adequate mixing with the serum diluent, each serum addition was withdrawn and expelled from the delivery pipette 10 times. When all serum diluent and test sera had been added, the plate was rotated 2 minutes on a micromixer (American Rotator V, R4140, American Dade, Miami, FL) at 75 rpm. The plates were incubated 45 minutes at 20-25 C in a humid chamber. The diluted serum was removed from the wells by inverting the plates and the wells were washed 3 times with PBS-.05% Tween-20. Two hundred and fifty microliters of alkaline-phosphatase-labeled rabbit anti-sheep IgG (diluted 1:800 as predetermined by checkerboard titration with known positive serum) was added to each well, and the plates were incubated 45 minutes at room temperature under the same conditions as above. The conjugate was removed from the wells by inverting the plates and the wells were washed 3 times with PBS-0.5% Tween-20. The substrate, para-nitrophenyl phosphate (furnished with the kit), was prepared no more than 30 minutes before use, and 250 microliters were added to each well. The plates were incubated 45 minutes, and the reaction was stopped by the addition of 50 microliters of 1.0 N NaOH to each well. The plates were read on a Titertek Multiskan MC (Flow Laboratories, Maclean, VA) at a wavelength of 405 nm.
The OD value for each serum was calculated by subtracting the OD of the control well from the OD of the antigen well. Because positive ovine control sera with known antibody levels were not available, the threshold value for a positive result was arbitrarily established at twice the average OD value of 2 negative controls.

**Indirect Fluorescent Antibody Test (IFA).** Glass microscope slides with 10 printed circular areas covered with Rh strain *T. gondii* (about 20 tachyzoites per 400X microscope field) were used (Gull Laboratories, Salt Lake City, UT). Serum samples were diluted 1:16 and 1:256 in 0.01 M phosphate-buffered saline solution (PBS), pH 7.6, and 20 microliters of one test serum dilution was placed on one circular area while the other dilution was added to the adjacent circular area. The slides were incubated 45 minutes at room temperature in a moist chamber. The slides were rinsed 10 minutes with 0.01 M PBS (pH 7.6), and then were air dried. The reaction sites were covered with 20 microliters of fluorescein isothiocyanate conjugated rabbit-anti-sheep IgG (diluted 1:30) or IgM (diluted 1:20) (Cappel, Malvern, PA). The conjugate dilutions were titrated using ovine fetal sera with and without antibody to *T. gondii* that had been screened by both DAT and ELISA. The slides were again incubated 45 minutes, rinsed 10 minutes in 0.01 M PBS (pH 7.6), and air dried. One drop of mounting fluid (Bacto-
FA Mounting Fluid, pH 9.0, Difco Laboratories, Detroit, MI) was placed on each reaction site and a coverslip was added. Positive and negative controls consisted of ovine fetal sera that were positive or negative for antibody to *T. gondii* by both DAT and ELISA. An antigen control overlaid with conjugate only was run on each slide to check for autofluorescence of the antigen. The slides were examined at a magnification of 400 X using a fluorescent microscope (Leitz Ortholux microscope and a xenon light source with 490 nm excitation filter and 530 nm blocking filter). Positive sera produced complete bright peripheral fluorescence. Staining at one end (capping) of the parasite, partial staining of the cell wall and faint staining were considered to be negative.

C. Results

Results of DAT, ELISA, and IFA are compared in Figure 1. A total of 377 ovine fetal fluid samples was screened by all 3 tests. Sixty-seven samples were positive by DAT, 58 were positive by ELISA, and 62 were positive by IgG-IFA. Five samples were positive by IgM-IFA and all 5 were also positive by IgG-IFA. The correlation of the results from the DAT, ELISA and IgG-IFA tests is given in Table 1.
Control reactions for each test were consistent for every test run. Test sera titers in the DAT ranged from 1:16 to greater than 1:65,536.

The DAT and ELISA detected toxoplasma antibody in 7 autolyzed fetuses as shown in Table 2. Five of the autolyzed fetuses were positive by IgG-IFA and had DAT titers ranging from 1:32 to greater than 1:32,768.

The gestational ages of 5 fetuses positive in the IgM-IFA were estimated to be greater than 4 months as shown in Table 3. DAT titers for this group ranged from 1:128 to greater than 1:32,768. One fetus in this group did not have IgG antibody that was detectable by ELISA.
Figure 1. Total number of ovine fetal sera screened by DAT, ELISA, and IFA and the total number positive for toxoplasma antibody in each test in 1985 and 1986.
Table 1. Comparison of the DAT, ELISA, and IgG-IFA for detecting toxoplasma IgG antibody from 377 aborted ovine fetuses.

<table>
<thead>
<tr>
<th>Reference Test</th>
<th>DAT</th>
<th>ELISA</th>
<th>IgG-IFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT</td>
<td>67 (100)</td>
<td>57 (85)</td>
<td>58 (86)</td>
</tr>
<tr>
<td>ELISA</td>
<td>57 (98)</td>
<td>58 (100)</td>
<td>56 (96)</td>
</tr>
<tr>
<td>IgG-IFA</td>
<td>58 (93)</td>
<td>56 (90)</td>
<td>62 (100)</td>
</tr>
</tbody>
</table>

Note: Figures in parentheses are % correlation between tests, i.e., ELISA and IgG-IFA corresponded 85% and 86% respectively with DAT results. The differences between results of the tests were not significant.
Table 2. DAT toxoplasma antibody titers, ELISA results, and results of IFA in autolyzed ovine fetuses.

<table>
<thead>
<tr>
<th>Case #</th>
<th>DAT titer*</th>
<th>ELISA*</th>
<th>IgG-IFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>85-706</td>
<td>64</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>85-1011</td>
<td>4,096</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>85-1803</td>
<td>&gt;32,768</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>85-2465</td>
<td>16</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>85-3314</td>
<td>&gt;32,768</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>86-73</td>
<td>1,280</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>86-440</td>
<td>32</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*IgG antibody only.
Table 3. Estimated age, DAT toxoplasma antibody titers, ELISA results and IFA results in ovine fetuses that were IFA positive for toxoplasma-specific IgM.

<table>
<thead>
<tr>
<th>Case #</th>
<th>Age</th>
<th>DAT titer*</th>
<th>ELISA</th>
<th>IgG-IFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>85-759</td>
<td>Term</td>
<td>&gt;32,768</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>85-1474</td>
<td>Term</td>
<td>&gt;32,768</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>86-2097</td>
<td>Term</td>
<td>128</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>86-13964</td>
<td>Term</td>
<td>256</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>86-14545</td>
<td>4 mo.</td>
<td>1,024</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*IgG antibody only.
D. Discussion

Experimental data show that infection of the fetus with *T. gondii* occurs about 2 weeks after infection of the ewe (13). Fetuses from ewes infected prior to 50 days gestation usually die and are resorbed; or the ewe aborts, but the abortion goes undetected (32). Ewes infected during mid-gestation (70-90 days) usually abort or have lambs that are born alive but are congenitally infected (53, 54). Abortion generally does not occur until 4 weeks or more after experimental inoculation of the ewe with *T. gondii* (39). Appreciable amounts of anti-toxoplasma IgM and IgG antibody could only be detected in fetuses from ewes examined 30 days or more after experimental inoculation of the ewe with *T. gondii* (13, 17).

The ovine fetus begins to develop immunocompetency between the 60th and 70th day of gestation, producing only IgM during this time (24). As the fetus matures it becomes more immunocompetent and develops the ability to synthesize IgG around 90 days gestation (23, 46). It is possible for the fetus to be in the late stages of IgM synthesis at the time the ewe is infected, and by the time the fetus becomes infected it may be able to synthesize IgG, which dominates the immune response (38). The absence of toxoplasma antibodies in an aborted fetus does not preclude the possibility of toxoplasmosis, because the development of
fetal antibodies depends on the age of the fetus at the time of infection and the time between infection and death or examination (19).

The IgG class of antibody was detected more frequently than IgM (Figure 1); however, only one method was used to detect IgM. Anti-toxoplasma IgG was present in all of the fetuses that had IgM antibodies to *T. gondii*. The results of the IgM-IFA test were significantly (P < .05) different from those of the other 3 tests.

Whole killed tachyzoites are used for the antigen in the DAT (14). This test measures only IgG activity because the 2-mercaptoethanol added to the sera to prevent nonspecific agglutination of the parasite by "normal" IgM also neutralizes any specific IgM that might be present. It is necessary to screen each serum sample at 2 dilutions to avoid false negative results caused by the "prozone phenomenon" (4). The prozone phenomenon occurs when an excess of specific IgG is present and saturates binding sites on the antigen. This prevents cross-linking between antigen and antibody and agglutination cannot occur. In sera with large concentrations of anti-toxoplasma IgG, the prozone effect usually occurs at the lower dilution (1:16), while at the higher dilution (1:1600), agglutination occurs because the antigen:antibody ratio is more favorable to cross-link formation. The lowest fetal DAT titer considered
significant is 1:16 (J. P. Dubey, personal communication). In the current study, all sera in which a prozone effect occurred at the 1:16 dilution had titers much higher than 1:1600, therefore, there is little likelihood that a serum which produced the prozone effect at 1:16 would be negative at the 1:1600 dilution.

The DAT required the least amount of time to perform. All reagents were added to the wells during the initial set-up and allowed to react. The DAT results were evaluated visually and subjectively and depended on the interpretative skills of the technician. The DAT required no special instrumentation for visualizing the results. Although the Chi square test showed no significant difference in the results of the DAT, ELISA and IgG-IFA, more fetuses were positive by the DAT than by the other 2 tests (Table 1). Dubey et al. compared the DAT to the DT, indirect hemagglutination (HA), and latex agglutination test (LAT) and found the HA and LAT to be less sensitive than the DAT for detection of *T. gondii* antibodies in ovine fetal sera (17).

The antigen for the IFA is whole, killed tachyzoites affixed to microscope slides. The screening test may be performed using only one serum dilution (1:16) although the instructions provided with the slides suggested the use of 2 dilutions (1:16 and 1:256). The IFA test can be used to
screen ovine fetal sera for IgM as well as IgG antibody classes. The IFA requires use of a fluorescent microscope for visualization of the organisms to determine the specificity of their fluorescence. Test results are evaluated visually and subjectively, but they were not difficult to evaluate.

Munday obtained good results using 1:16 as the lowest dilution for examining serum samples from aborted fetuses (37). However, Munday and Dubey found that toxoplasma antibody in autolyzed fetuses could not be detected by IFA (39). In the present study, anti-toxoplasma IgG was found in 7 autolyzed fetuses by DAT and ELISA. The IgG-IFA did not detect toxoplasma antibody in 2 of these 7 fetuses. Anti-toxoplasma IgM was not detected by IFA in any of these 7 fetuses.

At the time of examination, the estimated gestational age of 5 fetuses that were positive in the IgM-IFA test was greater than 4 months. Less mature fetuses may not be capable of producing detectable quantities of anti-toxoplasma IgM, or high levels of specific IgG may prevent binding of specific IgM due to competition. Using the IFA to selectively screen for IgM only would not be practical, because more positive IgG results were obtained from fetuses ranging in gestational age from 3 months to term. The IgM-
IFA appears to serve little purpose as a routine screening test for toxoplasma antibody in aborted ovine fetuses.

The ELISA employs soluble toxoplasma antigen adsorbed to microplate wells and could be used to detect either IgG or IgM antibody. Preliminary trials indicated the ELISA test for anti-toxoplasma IgM would not be productive, and only the test for IgG was used in this trial. The ELISA was the most labor intensive of all the tests. It required that reagents be made up immediately prior to their addition to the wells and many steps were required to complete the test. Reactions in control and antigen wells occasionally were inconsistent.

Interpretation of results for the ELISA test was objective. Once the threshold value was established, test serum OD values fell into either the positive or negative range. The ELISA requires use of a photometer to determine absorbance values of the wells because visual examination of the wells for color change could only distinguish negative reactions from strong positive reactions.

None of the tests used in this study required sera to be heat-inactivated because complement was not necessary for any of the reactions. Therefore, use of these tests avoids the interference caused in the DT by the naturally occurring complement-like factor present in some ovine sera (15, 47, 48). Also live organisms are not used in the 3
tests, thus all are safe for laboratory personnel. The antigen in the IFA and ELISA tests has been standardized by the manufacturer to assure uniformity from lot to lot, and each kit is ready to use immediately. Antigen for the DAT presently cannot be purchased in this country although it can be bought in France. If sufficient demand for the antigen develops, perhaps it will be marketed again in the U.S.

The DAT was preferred over the ELISA and IFA test because of the following: (1) it was the least labor intensive, (2) it was convenient for single samples, (3) the results were evaluated visually and required no special instrumentation, (4) the DAT could be used to test sera from any species, because species-specific conjugates are not used, (5) autolysis appeared to have a minimal effect on the results of the DAT whereas it seemed to interfere with the IFA results in some cases.
CONCLUSIONS

1. Although the Chi square test showed no significant difference among the DAT, ELISA, and IgG-IFA for detecting toxoplasma IgG antibody in aborted ovine fetuses, more fetuses tested positive by the DAT than by either of the other two tests.

2. The results of the IgM-IFA differed significantly from those of the DAT, ELISA and the IgG-IFA.

3. Five fetuses out of 377 tested had a positive reaction on the IgM-IFA, and all but one (which was negative in the ELISA) were positive by the other three tests. Therefore, the IgM-IFA was of little value for routine screening of aborted ovine fetuses for toxoplasma antibody.

4. The DAT and ELISA were more effective than the IgG-IFA for detecting toxoplasma antibody in severely autolyzed fetuses.

5. Because of its simplicity, sensitivity, and the fact that it can be used to test serum from any species without modification, the DAT was the preferred test.
LITERATURE CITED


