Development of an ELISA for the detection of antibodies to *Brucella canis* in dog serum

by

Barbara Maxwell Martin

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This is to certify that the Master's thesis of

Barbara Maxwell Martin

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

DEDICATION

I would like to dedicate this thesis to my family. Without your support I would not have been able to obtain this goal. I am fortunate to have a very supportive and caring husband who was willing to take over my family responsibilities while I concentrated on this work. Bruce's selflessness has enabled me to focus on the work at hand knowing that our children were well cared for. Our children, Andrea, Derek, and Alicia, have encouraged me with hugs, notes of support, and a willingness to give me the time needed to complete my studies. I owe a great deal to my parents, Gary and Pat Maxwell, who have always encouraged their children to set and achieve goals. I would also like to dedicate this thesis to my sisters and brother: Kathy Moser, Greg Maxwell, Lori Kunch, and Michelle Veren. I'm lucky to have siblings that are also my good friends. I am also grateful for my in-laws, Neil and Frances Martin. Their prayers, support, and encouragement have kept me going. I would like to thank all of my family for helping me to become the person I am today.

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INTRODUCTION

Brucella canis, an intracellular pathogen and a causative agent of reproductive disorders in dogs, is able to establish long term infections in tissues. Although clearance has been documented, the organisms may persist for many years even with long term antibiotic therapy.[1,2,3] Diagnosis of *B. canis* infection in breeder animals frequently results in a recommendation for removal of these dogs from the breeding stock and their euthanasia. Pet dogs are frequently spayed or neutered to reduce the possibility of zoonotic transfer resulting in human infection. Canine brucellosis, therefore, is especially devastating to commercial breeders since there is no vaccine to prevent *B. canis* infection and it is recommended that infected dogs be removed from the kennels to prevent additional infections.[4,5,6] Because of the insidious onset, the disease can spread unnoticed through a kennel before clinical signs are manifest. Although few clinical signs are typically observed in dogs of either gender, the most remarkable sign of infection is third trimester abortion in clinically normal bitches and mild epididymitis and decreased semen quality in males. Canine brucellosis can have a significant economic impact in a kennel where the disease can be transmitted through organisms present in vaginal discharge, aborted tissues, seminal fluid, and urine.[7,8,9]

Culture and identification of *B. canis* provide a definitive diagnosis, but failure to detect the agent in blood specimens does not provide sufficient evidence to rule out an infectious state or reproductive failure due to the organism, particularly when necropsy and/or biopsy specimens are not available. Bacteremia is usually transient, but may last from six to more than 60 months and negative hemoculture results from dogs infected

with *B. canis* are common.[10] Attempts to culture *B. canis* from various necropsy or biopsy tissues (lymph nodes, prostate, epididymis, spleen, and bone marrow) are commonly successful even though the dog may be hemoculture negative. Immunologic responses may clear the organisms from the blood months before the organisms are no longer detectable in other tissues. Culture results depend on isolation of the organisms from heparinized blood samples which are inoculated on solid media and streaked for isolation, or the blood samples may be added to an enrichment broth, frozen and then thawed to rupture the leukocytes containing the organisms. After incubation, the broth is inoculated onto solid media and streaked for isolation. Culture of tissues is accomplished by macerating collected samples and inoculating agar plates with the tissue macerates.

Because negative hemoculture results cannot rule out infection, reliable diagnosis of *B. canis* infection depends on serologic tests to aid in diagnosis. The three most commonly used serologic tests for canine brucellosis are the rapid slide agglutination test (RSAT), the 2-Mercaptoethanol tube agglutination test (2-ME-TAT), and the agar gel immunodiffusion assay (AGID). The RSAT and 2-ME-TAT whole cell antigens cross react with other rough Brucellae (*B. abortus* 4520, *B. ovis*, and *B. canis*) while the AGID antigen contains cytoplasmic antigens conserved throughout the genus.

The RSAT is a commercially-licensed test using heat-killed, Rose Bengal stained *Brucella ovis* cells.[11,12,a] The assay is a simple rapid test for the presumptive diagnosis of canine brucellosis, capable of detecting antibodies eight to twelve weeks after infection. Serologic responses remain positive up to three months after dogs are no longer

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[a]Symbiotic Corporation, 11011 Via Frontera, San Diego, CA 92127.

bacteremic. These attributes and the high sensitivity (≈99%) of the assay contribute to make the RSAT the most commonly used screening test in the United States. However, the specificity of the assay (the ability to differentiate immune responses due to the agent from those due to other organisms) is low and false positive reactions are frequently observed, resulting in a need for additional serologic and/or bacteriologic evaluation to support clinical findings.

The AGID is the most specific and the least sensitive of the serologic tests.[10,13,b] Interpretation of test results is complex and subjective, resulting in high variation in test results both between laboratories and within laboratories. Antibodies have been detected by twelve weeks post-infection and up to thirty-six months after the animals are no longer bacteremic.

The 2-ME-TAT was developed at the National Veterinary Services Laboratories (NVSL) in 1971.[14,c] The test is semi-quantitative and results are reported as negative, suspicious, or positive. The test antigen, produced and provided by the NVSL, is made of heat killed whole cells of *B. canis*. Because of the rough nature of *B. canis* bacteria, cells readily autoagglutinate and complicate the subjective interpretation of test results. The sensitivity of the test antigen varies widely among production lots, and test antigens are frequently unsatisfactory and must be discarded due to autoagglutination. The 2-ME-TAT is less sensitive and more specific than the RSAT (greater accuracy of the test).

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[b]Diagnostic Laboratory, P.O. Box 5786, Upper Tower Road, Cornell University, Ithaca, NY 14852-5786.

[c]NVSL, P.O. Box 844, Ames, IA 50010.

Antibodies have been detected eight to ten weeks after infection and up to 3 months after dogs are no longer bacteremic.

Although the available serologic tests provide valuable data for clinicians, no single assay provides optimal sensitivity and specificity. The focus of the study reported here is the development of an inexpensive, easy-to-perform serologic assay with increased sensitivity, increased specificity, and the ability to determine a diagnostic titer early after infection and lasting for a significant period after the animal is no longer bacteremic. Improved serologic assays would provide increased accuracy in the serologic status of individual dogs and thereby aid in the determination of the infection status.

LITERATURE REVIEW OF *BRUCELLA CANIS*

History of *Brucella*

The microbiological history of the Brucella genus dates to the initial 1897 report of Bruce who isolated a micrococcal organism, now designated *Brucella melitensis*, from infected humans on the island of Malta.[15] Ten years later Bang reported from Denmark concerning a bacillus believed to cause abortions in cattle.[16] This was the first report of the organism now classified as *Brucella abortus*. In 1914 Traum reported from the United States on a swine pathogen which was later classified as *Brucella suis*.[17] In 1912, Alice Evans provided evidence that the three organisms, although morphologically different and isolated from diverse animal hosts, were biologically related and recommended they be regarded as one genus based on a number of biochemical and growth characteristics.[18] By 1925, the genus Brucella was established and consisted of three species: *B. melitensis*, *B. abortus*, and *B. suis*. The report of Stoenner added the specie *Brucella neotomae* to the genus; while the reports of McFarland et al. (1952) and Simmons et al. (1953) added *Brucella ovis*.[19,20,21] A gram negative coccobacillus that causes abortions in marine mammals is currently under investigation as a member of the genus *Brucella*.[22]

The genus *Brucella* currently consists of six species: *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. neotomae*, and *B. canis*. However, some proposals would limit the genus to only the one specie, *B. melitensis*, with the other five strains as sub-species.[23] Members of the genus are known to cause abortions and infertility, which result in significant economic losses to livestock producers. All currently defined species except *B. neotomae* and *B. ovis* are zoonotic agents capable of establishing long term infections in human hosts.

Discovery of *Brucella canis*

The most recent specie identified in the Brucella genus is *B. canis*. The first report of disease associated with *B. canis* was in June of 1966 when the agent was isolated from dogs in a beagle kennel suffering from reproductive problems including abortion, dead and weak pups, infertility, and reproductive tract pathologies.[1,24] The disease was thought to be geographically widespread in the United States, but had only been reported in beagles. Scientific evidence had not previously been presented concerning a canine brucellosis epizootic. The initial report sparked a rapid response from the canine industry and the veterinary diagnostic community. Numerous articles were published that provided additional information about the agent, clinical symptoms, diagnosis, and immune response.[2,3,7,8,9,25,26,27,28,29,30,31,32,33,34,35]

Epizootiology

Canine susceptibility

Dogs were implicated as possible carriers of *Brucella* on numerous occasions prior to 1966 in reports of sporadic transmission to dogs of *B. abortus*, *B. melitensis*, and *B. suis* from the normal preferential hosts of cattle, sheep or goats, and swine.[36,37,38] Although dogs are known to be susceptible to *B. abortus* infection, they are not thought to be major carriers for transmission of the organisms. It has been assumed that infection in dogs occurs following ingestion of aborted bovine, caprine, or ovine fetuses and placentas harboring the agent.[4,39,39,40]

In 1922 Daregin and Plazy reported Malta fever when *B. melitensis* was isolated from seven naval officers who had not consumed goat milk or cheese; furthermore, goats and sheep in the area were reported to be free of *B. melitensis*. The officers had shared their living space

with a dog who had whelped three stillborn pups whose blood contained antibodies to *Brucella*. The dog was destroyed prior to any bacteriologic investigation. No further reports of Malta Fever were reported in the area and the authors presumed the dog had been the source of infection.[41]

Brucella suis was isolated from a testicular abscess of a fox terrier that had been lethargic for six weeks and exhibited some pain and stiffness in the hind legs. Serologic tests were positive at a 1:500 dilution.[42] In 1940, Nolan reported a positive agglutination titer in serum from a dog that had consumed meat from horses that had been experimentally challenged with *B. suis*.[43]

Geographic distribution

Brucella canis was originally reported in commercial beagle kennels in the north eastern United States.[1] It has since been isolated from numerous purebreds and mixed breeds throughout the United States. Stray dogs have been shown to be the principal reservoirs of infection.[7,9,44,45,46,47,48,49] Estimates of the incidence of canine brucellosis in the U.S. vary from 1.5% to 6.6%.[29,50] However, prevalence in commercial kennels known to be infected with *B. canis* has been reported to be as high as 86%.[35]

Although first reported in the U.S., canine brucellosis has been identified world-wide. Reports from Quebec identified significant serologic titers in 1.6% of pet dogs surveyed.[51] In Mexico City, 11.8% of 59 stray dogs were infected with *B. canis*.[52] Canine brucellosis has been reported in the South American countries of Peru,[53] and Brazil.[54,55] It's also been reported in Czechoslovakia,[56] Britain,[57] Germany,[58,59] Madagascar,[60] Tunisia,[61] New Guinea,[62] Sri Lanka,[63] Australia,[64] Japan,[65,66,67,68,69,70,71] and China.[72,73,74]

Transmission

Canine brucellosis is most commonly transmitted by the ingestion or inhalation of the organisms from aborted tissues or postabortion discharges that are contaminated with the organisms.[5,25,30,75] Infection can also occur through contact with other mucous membranes (nasal, conjunctival, and genital) and skin abrasions.[25,75] Aborted fetuses and vaginal secretions may contain as many as 10^{10} viable organisms per ml.[76] *Brucella canis* is also found in milk and in salivary and nasal secretions.[38,75] The organisms are present in the sperm of infected dogs in large numbers for three to eleven weeks post-infection; by sixty weeks post-infection, the organism can no longer be cultured from seminal fluid samples.[77] Vaginal secretions, urine, and semen are thought to be likely sources of infection due to the elevated concentration of organisms usually found in these samples.[26,75,78] Brucellae are known to establish long term intracellular infective states in the epididymis and prostate gland, where they have been reported to persist more than two months after dogs were no longer bacteremic.[79] Urine from acutely infected male dogs may contain 10^3 to 10^6 organisms per ml, while lower concentrations of the organisms are found in the urine of infected female dogs.[78] It has been reported that the minimum infectious oral dose is 10^6 organisms and the minimum conjunctival dose is 10^4 to 10^6 organisms.[76,77]

Transmission of canine brucellosis occurs during mating as well as when same gender dogs are housed together. In one study, adult female canine sentinels became infected after four to five months of cohabitation with infected nonpregnant adult female dogs. No estrus was observed during this time. Infection was transmitted between infected and noninfected male dogs housed together for four to six months, while infection of female noninfected

juvenile dogs occurred only after 10 to 12 months of cohabitation with male infected juvenile dogs.[76]

Human Infections with *B. canis*

More than thirty-five cases of human infection with *B. canis* have been reported.[80,81,82] Laboratory acquired infections, diagnosed when laboratory personnel developed serologic responses to the agent or developed symptoms, reemphasize the importance of the use of biologic safety cabinets when handling *B. canis* infected tissues. Occupational exposure has also resulted in reports of infections in veterinarians and animal handlers.[83] Transmission from pet dogs to their owners has resulted in additional human infections.[34,84,85]

Humans appear to be relative resistant to infection with *B. canis* and symptoms reported have been much milder than those due to *B. suis*, *B. melitensis*, and *B. abortus*.[6,79] The major symptoms in clinical cases have included fever, chills, malaise, headache, anorexia, fatigue, and generalized lymphadenopathy.[79,85] Surveys of human populations for serologic evidence of exposure to *B. canis* have resulted in diagnosis of asymptomatic infections.[86,87] Serious complications, although rarely encountered, can cause serious disease and may include arthritis, endocarditis, hepatitis, meningitis, and visceral abscesses.[4]

In 1970, Swenson became the first to isolate *B. canis* from the blood of a human patient. The woman had been diagnosed with endocarditis caused by *Streptococcus viridans* and had been placed on long term antibiotic therapy. After six months on 500,000 units of penicillin daily, the patient discontinued antibiotic therapy. Within ten days she developed fever, chills, and headaches. *Brucella canis* was isolated from seven of twelve blood cultures drawn over the next week. The patient was treated with streptomycin and ampicillin for five

weeks and remained well following termination of antibiotic therapy. The patient owned two dogs, both of which were clinically normal, but one had a titer of 1:500 to *B. canis*. None of the other twelve family members had serologic titers to *B. canis*, nor did they report any symptoms indicative of the disease.[88]

Human clinical laboratory analyses include both serologic testing and hemoculture attempts.[87] In humans, as in dogs, *B. canis* is capable of establishing long term infections with intermittent, transient bacteremia. In addition to the transient nature of the bacteremia, concentrations of the microorganisms in the blood are thought to be very low. For these reasons, attempts to culture *B. canis* rely on sequential blood samples taken over a period of months. In addition, cultures may be incubated for extended periods of time to allow detection of these slow growing pathogens. Treatment with tetracycline has been proven effective, while relapses have occurred after ampicillin treatment.[4,80]

Non-canine Infections with *B. canis*

Experimental infections of cattle, swine, sheep, mice rabbits, rats, and guinea pigs have been attempted.[3,75] No clinical signs were detected in cattle, pigs, or sheep; however, *B. canis* was isolated from the lymph nodes of pigs and sheep and the blood of sheep following exposure by the conjunctival route to a large numbers (1×10^{10} colony forming units).[75] Virulence for laboratory animals has appeared to be low, even when large doses of *B. canis* were administered. Guinea pig spleens were only slightly enlarged, but rabbits developed peritoneal abscesses and orchitis after experimental exposure to large numbers of live *B. canis*.[30,75]

Nonhuman primates (*Macaca arctoides*) were experimentally infected with *B. canis* by

the intravenous and oral-conjunctival routes. Monkeys were bacteremic for 5 to 7 weeks and lesions were found in liver, spleen, and lymph node specimens.[89] Studies with wildlife species have been limited. Red foxes inoculated orally became bacteremic within 4 weeks and were still bacteremic at the time of necropsy (14 weeks). Serological surveys of of approximately 800 wild carnivores and opossums found significant titers in 2 coyotes, 1 bobcat, and 1 red fox. Gray foxes, opossums, striped skunks, and wolves surveyed were serologically negative.[90]

Clinical Diagnosis

Clinical signs

Dogs infected with *B. canis* manifest few clinical signs.[2,8,29,30,32,91] Even dogs experimentally challenged with high numbers of organisms usually remain afebrile, which is thought to be due to the low levels of endotoxin in the cell walls of *B. canis*.[7] The most remarkable clinical sign is third trimester abortion in clinically normal bitches. Infected females may abort two to three successive litters. Although spontaneous abortions occur at any stage, most reported abortions occur between 45 and 55 days of gestation.[5] Most infected pups are dead or die within a few hours after birth; surviving pups are reported to be bacteremic a minimum of two months. Diagnosis of failure to conceive may be reported in cases of embryonic death and spontaneous abortion.[7] Infected males exhibit mild epididymitis, scrotal dermatitis, infertility, and testicular atrophy. The quality of semen from infected dogs decreases by five weeks post-infection and is markedly abnormal by eight weeks post-infection. Orchitis has been observed, but is not commonly reported.[7,8,92,,93]

Isolation of the organism

Because a definitive diagnosis of Brucellosis is based on the isolation of *B. canis* and the organisms are often present transiently in low numbers in the sampled fluids and tissues, care must be taken to obtain quality samples to increase the possibility of isolating the organisms from infected animals.[94] The most commonly collected specimen is whole blood, which is aseptically collected by jugular venipuncture in heparinized containers. Collection of urine samples by cystocentisis and tissue samples using aseptic technique will prevent overgrowth by more rapidly growing bacteria.[8,76,95] Aborted tissues, vaginal or uterine fluids, and seminal fluid are commonly collected from dogs when infection with *B. canis* is suspected; the most commonly collected tissues at necropsy are lymph nodes, male and female reproductive organs, spleen, liver, and bone marrow.[8,80]

Blood and fluids may be added to an enrichment broth (Trypticase soy, Tryptose, or Tryptose serum broths) and incubated aerobically for 4-7 days at 37±2°C before inoculation onto selective media containing antibiotics (cycloheximide, bacitracin, polymyxin B sulfate, vancomycin, and nystatin) and dyes (ethyl violet), which decrease the numbers of rapidly growing contaminants. Swabs and macerated specimens may be inoculated directly onto selective media and incubated aerobically at 37±2°C . Growth can often be detected by 48 to 72 hours; colonies appear 48 to 72 hours after inoculation of media plates and incubation at 37±2°C and are 1.0 to 1.5 mm after 4 to 5 days. Growth is especially abundant on media enriched with serum. Individual colonies are smooth, glistening, and transparent. After several days incubation, colonies become mucoid on solid media or ropy in liquid media. Microscopic examination reveals *B. canis* to be a small, non-motile, non-sporulating, gram

negative coccobacillus. Unlike other members of the genus *Brucella*, *B. canis* growth is inhibited by an atmosphere of 5% carbon dioxide. *Brucella canis* is rapidly urease positive, catalase positive, weakly oxidase positive, and hydrogen sulfide negative. It grows in the presence of thionin but most strains are inhibited by basic fuchsin.[80,94] Safety should always be a primary concern when manipulating specimens suspected to contain *Brucella* species. All work should be performed in biological safety cabinets, thereby decreasing the possibility of laboratory infections due to the organisms.[94]

Serologic diagnosis

Reliable diagnosis of *B. canis* infection in dogs depends on serologic tests to aid in diagnosis. Many assays have been developed that are used infrequently including: counterimmunoeloctrophoresis,[96] complement fixation,[97,98] indirect immunofluorescence,[99] and ELISA.[100,101,102] The three most commonly used serologic tests for canine brucellosis are the RSAT, 2-ME-TAT, and the AGID. None of the assays is able to differentiate infections due to *B. canis* from infections caused by other members of the genus *Brucella*. Although the available serologic tests provide valuable data for clinicians, no single assay provides optimal sensitivity and specificity.[13,80]

2-ME-TAT The 2-ME-TAT was developed at the National Veterinary Services Laboratories (NVSL).[14,103,c] The assay is less sensitive and more specific than the RSAT. Although it is described as an agglutination test, results rely on observation for clearing of the antigen-serum mix rather than on agglutination. When first developed, the assay was performed without the addition of 2-ME. Addition of 2-ME was found to reduce the number of false positives, presumably by breaking down the less specific IgM molecules. Serum

dilutions of 1:50, 1:100, and 1:200 are assayed and after a 48 hr incubation at 37C±2°C at which time the individual dilutions are observed for clearing. Test results are reported in 3 categories: negative, suspicious, and positive. When no clearing is observed in the 1:50 dilution of antigen-serum mixture is observed when compared to the negative control, the test results are reported as negative. Results are considered suspicious if clearing is incomplete or complete in the 1:50 or 1:100 dilutions. Incomplete or complete clearing of the test antigen by a 1:200 dilution of serum is considered a positive result. Antibodies have been detected eight to ten weeks after infection and up to 3 months after dogs are no longer bacteremic.[4,79]

The test antigen, produced and provided by the NVSL, is made of heat killed whole cells of *B. canis*. Because of the rough nature of *B. canis*, cells readily autoagglutinate and complicate the subjective interpretation of test results. In addition, the sensitivity of the test antigen varies widely between production lots; production lots of test antigen are frequently unsatisfactory and must be discarded due to autoagglutination.

RSAT The RSAT is more sensitive and less specific than the 2-ME-TAT. It was originally developed by George and contained heat-killed, Rose Bengal-stained *B. ovis* cells with a packed cell concentration of 6%.[11] *Brucella ovis* cells were used to avoid the autoagglutination problems frequently encountered with antigens made with *B. canis* cells. The antigen compared favorably with the TAT and results were available in 2 minutes instead of 48 hours. The antigen was commercially licensed and made available to veterinarians. In 1981, Badakhsh reported an improved RSAT that utilized the same antigen as the standard RSAT, but involved brief treatment of serum samples with 2-ME. This modification decreased the number of false positive test results, and the test kit was modified to include the

use of 2-ME.[12,a]

The assay is a simple rapid test for the presumptive diagnosis of canine brucellosis, capable of detecting antibodies eight to twelve weeks after infection. Serologic responses remain positive up to three months after the dogs become abacteremic.[4,79] These attributes and the high sensitivity (≈99%) of the RSAT contribute to its being the most commonly used screening test in the United States. However, the specificity of the assay is low and false positive reactions are frequently observed, resulting in a need for additional serologic and/or bacteriologic evaluations to support clinical recommendations.

AGID The AGID is the most specific and the least sensitive of the serologic tests.[13,b] The antigen used in this assay is predominantly cytoplasmic proteins extracted from *B. canis*, and contains small amounts of cell wall components that are found in other *Brucella* species. Interpretation of test results is complex and subjective, resulting in high variation in test results both between laboratories and within laboratories. Antibodies are detected by twelve weeks post-infection and up to thirty-six months after the animal is no longer bacteremic.[13]

Prevention and Treatment

Canine brucellosis is not a reportable disease in the U.S. It is, however, economically important in commercial kennels. No vaccine is available, although several attempts have been made to produce both live and killed vaccines.[7] The necessity of a vaccine has been questioned because of the possibility of further complicating diagnosis with immunologic response to a vaccine. An effective vaccine would ideally provide lasting immunity while not confounding serologic diagnosis.[4,79]

Prevention of *B. canis* infection requires monitoring, proper care of animals, and strict attention to hygiene.[5,86] New dogs should be serologically evaluated before or at purchase and isolated until a minimum of two serologic tests spaced one month apart are negative. Annual serologic evaluation as well as immediate serologic evaluations upon detection of reproductive problems serve as an adequate monitor for most kennels. Serologic evaluations of females should be performed two to three weeks prior to breeding. This schedule will allow time for repeat testing if results are questionable. Studs brought in for breeding should be evaluated for serologic evidence of exposure to *B. canis* as should animals that have been exposed to situations in which infection from outside sources may have occurred.[5,79]

An immediate response when a kennel experiences an increase in failure to conceive or abortion will limit losses due to *B. canis* infection. Samples (semen, vaginal swabs, aborted fetuses) from affected animals should be cultured. Upon isolation of *B. canis* from a kennel dog, all dogs in the kennel should be evaluated serologically, and all serologically positive animals should be removed. Repeat tests performed monthly until three successive monthly tests reveal no additional positive animals are generally thought to be necessary to ensure control of the agent.[5,79]

MATERIALS AND METHODS

Assay Development

Antigen production

Brucella canis strain RM66/6 and *Brucella ovis* strain 3572 were used for antigen production. Lyophilized cultures were provided by the Diagnostic Bacteriology Laboratory of the National Veterinary Services Laboratories.[c]

2-ME-TAT antigen Lyophilized cultures of *B. canis* strain RM66/6 were reconstituted with distilled water and 0.1 ml aliquots were streaked for isolation onto TSA and incubated at 37±2°C for 48 hours. The growth from each slant was harvested in 5.0 ml sterile phosphate buffered saline (PBS, pH 6.4). The cell suspension was used to inoculate Roux bottles (5ml/Roux bottle) containing TSA. Roux bottles were incubated in an inverted position for 48 hours at 37±2°C. The cell growth was washed from the agar surface using 50 ml of PBS/Roux bottle. Harvested cell suspensions were pooled and purity tests performed. The pooled cell suspension was heat killed at 70±2°C for a minimum of 60 minutes. After sterility had been demonstrated, the cell suspension was washed 2 times with PBS (8000 X *g* for 30 minutes). Cell concentration was standardized to 4.5% cells and suspensions were stored at 4±2°C.

Heat extracted ELISA antigens Lyophilized cultures of *B. canis* strain RM66/6 or *B. ovis* strain 3572 were reconstituted with distilled water and 0.1 ml was streaked for isolation onto TSA. Plates containing *B. canis* were incubated at 37±2°C for 48 hours and plates containing *B. ovis* were incubated 72 to 96 hours in a 10% CO_2 atmosphere. The growth from each slant was harvested in 5.0 ml sterile PBS and inoculated onto TSA in Roux

bottles. Roux bottles were incubated in an inverted position for 48 hours (*B. canis*) or 72 to 96 hours with 10% added CO_2 (*B. ovis*). The cell growth was harvested in 50 ml sterile saline/Roux bottle. Cell suspensions were pooled and centrifuged at 12,000 X *g* for 30 minutes. Cell pellets were resuspended in 100 ml of sterile saline. Centrifugation and cell resuspension were repeated for a total of three washes. The cells were diluted with PBS so that a 1:10 dilution of cells had an optical density (OD) of 0.48 at 550 nm. The cell suspension was autoclaved for 20 minutes, allowed to cool, and centrifuged at 8,000 X *g* for 20 minutes. The supernatant fluid was decanted and stored at 4±2°C.

Ammonium sulfate precipitated antigen *Brucella canis* and *B. ovis* were grown from lyophilized cultures, subcultured in Roux bottles, harvested, washed, autoclaved, and centrifuged as described with the heat extracted ELISA antigens. Saturated ammonium sulfate was added to 20 ml of the heat extracted antigen to a final concentration of 70% (v/v). The precipitate was collected by centrifugation at 20,000 x g for 10 minutes at 4±2°C, dissolved in 20 ml of 5 mM NH_4HCO_3, and dialyzed against the buffer until the dialysate tested negative for sulfate ion. The antigen was stored at 4±2°C.

Triton X-100 extracted antigen An antigen extraction buffer containing t-Octylphenoxypolyethoxyethanol (Triton X-100)[d] was made as follows: 4.3 g tricine and 9.8 g tris were added to 500 ml of 5 mM EDTA. One ml of Triton X-100 was added and the solution was brought to a final volume of 1 L with additional 5 mM EDTA. The pH was adjusted to 8.6 and the solution was stored at room temperature until used. One Roux bottle of TSA was inoculated with *B. canis* RM66\6 and incubated for 48 hr at 37±2°C. The

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[d] Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547.

growth from the Roux bottles was harvested into 30 ml 0.1 mM PBS and the cell suspension was centrifuged at 5000 X g for 30 minutes. The supernatant fluid was discarded, 15 ml of the antigen extraction buffer was added to the cell pellet, and the cells were resuspended. The cells suspension was stirred overnight at 4±2°C, then filter sterilized through a .2μm filter and divided into 5 aliquots. Merthiolate (1:5000) was added to 2 of the aliquots. One aliquot with merthiolate and 1 without were stored at 4±2°C and -20±2°C. The remaining aliquot without merthiolate was stored at 25±4°C.

Reference ELISA

Antigen preparations were diluted in coating buffer and 100 μl was added to wells of an Immulon II 96-well microtitration plate. Plates were incubated for 60 ± 15 minutes at 37±2°C and then stored at 4±2°C overnight or longer. Plates were washed 3 times in PBST, blocked, and incubated at 37±2°C for 60 ± 15 minutes. After washing in PBST 3 times, positive and negative control sera were added and two-fold dilutions were made by serial transfer. The plates were sealed, and incubated at 37±2°C for 30 ± 5 minutes, and washed 3 times in PBST. Anti-species horse radish peroxidase labeled IgG (conjugate) was added to detect bound immunoglobulin and plates were sealed and incubated at 37±2°C for 30 ± 5 minutes. Plates were washed 3 times in PBST and substrate was added. Plates were rotated until color developed and the reactions were chemically terminated with 100 μl of a stop solution. An $OD_{450-650}$ was determined and the results graphed.

Buffers and solutions Antigen coating buffer (0.5 M carbonate buffer) was prepared by dissolving 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 liter deionized water. The pH was adjusted to 9.6, and the buffer was stored at 4±2°C for up to 1 week. Phosphate

buffered saline (PBS), pH 7.2, containing .05% Tween 20 (PBST) was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 1.15 G Na_2HPO_4, 0.2 g KH_2PO_4, and 0.5 ml Tween 20 in 1 liter deionized water and adjusting the pH to 7.2. Sodium dodecyl sulfate (%) or 2.5 M H_2SO_4 were used to terminate reactions.

Five serum dilution buffers were evaluated by use in the reference ELISA. The standard serum dilution buffer recipe was 0.015 M NaCl, 1 mM EDTA, 0.05 M Tris-HCl, and 0.5 ml Tween 20/L (SDB1). SDB2 consisted of SDB1 with the addition of 0.1% BSA. SDB3 was prepared by adding 0.1% BSA and 0.1 M 2-Mercaptoethanol to SDB1. SDB4 was prepared by adding 0.01 M 2-Mercaptoethanol to 3.5% NaCl. Phosphate buffered saline with 0.05% Tween 20 was evaluated as SDB5.

Blockers Various blockers including 5%nonfat dry milk (NFDM), 1% polyvinyl alcohol (PVA), 1% bovine serum albumin (BSA), 5% fish gelatin (FG), phosphate buffered saline with 0.05% Tween-20 (PBST), and phosphate buffered saline (PBS) were evaluated using the reference ELISA to determine if a blocker was necessary, and, if a blocker was necessary, which blocker reduced the background to acceptable levels without lowering the ability of the assay to detect a positive response. After determining the best blocker, the optimum blocker concentration was determined using various concentrations (1, 2.5, 5, 7.5, and 10%) of blocker.

Positive and negative serum pools for ELISA development Sera from 10 dogs with known exposure to *B. canis* and with known titers using the 2-ME-TAT serologic assay were pooled as a positive control. Sera from 10 additional dogs with no known exposure to *B. canis* and with negative results on the 2-ME-TAT serologic assay were pooled as a

negative control.

Conjugate Rabbit origin horse radish peroxidase conjugated, anti-canine heavy and light chain IgG was obtained from Jackson ImmunoResearch Laboratories (JIRL).[e] Goat origin horse radish peroxidase conjugated, anti-canine heavy and light chain IgG was obtained from Kirkegaard Perry Laboratories (KPL).[f]

Substrate Two substrates were evaluated using the reference ELISA; 3, 3', 5, 5', tetramethylbenzidine (TMB) and 2, 2'-azino-di-3-ethylbenzthiazoline (ABTS).[f]

Assay Evaluation

Serum panel for ELISA evaluation

In order to evaluate the assay, positive and negative serum panels were developed. Blood specimens were collected for culture and serology from 304 dogs known to have been naturally exposed to *B. canis* and dogs having no known exposure to *B. canis*. Jugular venipuncture samples for culture were collected aseptically in heparinized vacutainer tubes. Blood specimens for serologic testing were collected in a similar manner in serum separation tubes. Blood and serum were stored frozen at -20± 2°C until cultured or assayed. The 2-ME-TAT was performed on all sera sampled and the serologic and culture results were compared.

Media

The following standard isolation media were purchased as dry powder and made following manufacturers' recommendations: tryptose serum broth (TSB), tryptose serum agar

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[e] Jackson ImmunoResearch Laboratories,

[f] Kirkegaard Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879

(TSA), tryptose serum agar with antibiotics (TSA + antibiotics) (cycloheximide, 30 mg/L; bacitracin, 7,500 units/L; and polymyxin B sulfate, 1,800 units/L), tryptose serum agar with antibiotics and 1.4 μg/L ethyl violet (Ethyl Violet), Ewalt's medium[104], and Farrell's medium[105] [serum dextrose agar with: cycloheximide (100 mg/L), bacitracin (25,000 units/L), polymyxin B sulfate (5,000 units/L), vancomycin (20 mg/L), nalidixic acid (5 mg/L), nystatin (100,000 units/L)], serum dextrose agar, and thionic and basic fuchin dye plates [thionin or basic fuchin (1:50,000) added to serum dextrose agar].

Blood culture

Direct culturing of blood A 0.1 ml aliquot of blood collected in heparinized tubes was inoculated and streaked for isolation on agar plates (TSA, TSA + antibiotics, Ethyl Violet, Ewalt's, and Farrell's media). The plates were incubated at 37±2°C for 7 days.

Subculturing from broth A 5.0 ml aliquot of the heparinized blood was added to a tube of tryptose serum broth which was subsequently frozen, thawed, and incubated for 7 days at 37±2°C. A 0.1 ml aliquot of the blood-broth mixture was inoculated onto various agar plates (TSA, TSA + antibiotics, ethyl violet, Ewalt's, and Farrell's media) and streaked for isolation. Plates were incubated at 37±2°C for 7 days.

Bacterial isolate selection and characterization of *Brucella* isolates

Incubated plates were examined with a low-power stereoscopic microscope using obliquely reflected light.[106] Morphologically typical colonies were inoculated onto TSA + antibiotics and plates were incubated at 37±2°C for 3 days. Isolates were characterized by growth, biochemical, and antigenic characteristics.

Carbon dioxide (CO_2) growth requirement A heavy suspension of the test culture was prepared in 0.5 ml of sterile saline. Duplicate TSA slants were inoculated, streaked for isolation, and incubated at 37±2°C for 3 days. One tube was incubated in an aerobic atmosphere and the other was incubated in 5% CO_2 atmosphere. Slants were visually examined for growth.

Growth in the presence of thionin and basic fuchin A small amount of test culture was suspended in sterile saline and streaked for isolation onto serum dextrose agar plates containing thionin or basic fuschin (1:50,000) using sterile cotton swabs. Plates were incubated at 37±2° C for 4 days and visually examined for growth.

Urease test A 5% solution of urea containing 0.0015% phenol red in a 0.125 M solution of sodium dihydrogen phosphate (NaH_2PO_4) (pH 4.0) was dispensed in 1 ml amounts. Tubes were inoculated with a loopful each of test culture and incubated at 37±2°C. Initial color changes were recorded at 15, 30, and 60 minutes. Readings were recorded every 30 minutes for 4 hours and again at 24 hours. A yellow color was considered a negative result and a red color in 4 hours or less was considered a rapid urease positive while a red color in more than 4 hours was considered as a positive result. Positive and negative controls were inoculated for visual comparisons.

Oxidase test A small amount of test culture was placed on a strip of filter paper impregnated with 1% NNN'N'-tetramethyl-p-phenylene diamine dihydrochloride.[107] Lack of color development was considered a negative result and a purple color reaction was considered a positive result. Positive and negative controls were inoculated for visual comparisons.

Production of hydrogen sulfide (H_2S) The test cultures were inoculated onto a TSA slant and a strip of lead acetate paper was folded over the top of the tube so that the paper was suspended over the media. The slant was incubated at 37±2°C for 4 days. Papers were changed daily and results were recorded daily. Lack of color development on the lead acetate paper was considered a negative result and a black color reaction at any time was considered a positive result.

Lysis by bacteriophage A small amount of test culture was suspended in sterile saline and was inoculated onto a serum dextrose agar plate using sterile cotton swabs to produce a uniform lawn of the culture. Twenty μl of the routine test dilution (RTD) and 20 μl of 10^4 RTD of *Tbilisi* (Tb)[c] or rough canis specific (R/C)[c] bacteriophage was dropped onto the streaked plates. Plates were incubated at 37±2°C for 48 hours and lysis patterns were determined.

Agglutination in sera Cross absorbed polyclonal sera for the *abortus* (A) and *melitensis* (M) epitopes of smooth *Brucella* strains and rough (R) sera[c] were combined in a test tube with a cell suspension of the test culture (1:5 dilution).Two-fold dilutions were produced in 0.5 ml of phenolized saline to a final dilution of 1:320. Tubes were incubated at 37±2°C for 24 hours and agglutination was assessed.

Antigen storage studies

Three different storage temperatures (4±2°C, -20±2°C, and 25±4°C) were evaluated as were the effects of the presence of a preservative, and freeze/thaw cycles,. Aliquots of Triton X-100 extracted *B. canis* antigen with and without merthiolate (1:5000) as a preservative, were stored at 4±2°C and -20±2°C. The remaining aliquot without merthiolate

was stored at 25±4°C. Three vials of antigen were assayed at each of the 7 sampling times (0, 1, 4, 13, 26, 39, and 52 weeks). The influence of freezing and thawing the antigen was studied when the same 6 vials of antigen; 3 with and 3 without merthiolate were assayed at each of the sampling times. The reference ELISA was followed; antigen was diluted 1:200 in coating buffer, plates were blocked with 5% NFDM, and positive and negative serum pools were diluted 2-fold from 1:50 through 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

Production lot assessment

Three lots of Triton X-100 extracted *B. canis* antigen were produced and stored at 4±2°C. Three vials of each lot were assayed at 4 sampling times (0, 1, 4, and 13 weeks) following the ELISA described in antigen storage studies with the exception of using JIRL conjugate diluted 1:1000. The protein concentration of the production lots was determined using the Micro-Kjeldahl procedure.

Calculation of sensitivity, specificity, positive and negative predictive values, and accuracy of tests

The performance of the assay was determined by calculating sensitivity, specificity, positive and negative predictive values, and accuracy at various serum dilutions and with several minimum $OD_{450-650}$ values for interpretation as a positive result. Sensitivity was calculated by dividing the number of true positive samples that were test positive by the total number of true positives samples. Specificity was calculated by dividing the total number of true negative samples that were test negative by the total number of true negative samples.

The positive predictive value of the assay was determined by dividing the number of true positives that were test positive by the total number of test positive samples, while the negative predictive value was calculated by dividing the number of true negative samples that were test negative by the total number of test negative samples. The accuracy of the assay was calculated by dividing the sum of the true positive samples that were test positive and the true negative samples that were test negative, by the total number of samples.

Receiver-Operator-Characteristic (ROC) curves at several $OD_{450-650}$ were plotted to aid in the determination of optimal cutoff values. The true positive rate (sensitivity) was plotted on the vertical axis while false positive rate (1 - specificity) was plotted on the horizontal axis.[108,109]

Characterization of the Triton-X 100 extracted and 2-ME-TAT Antigens

To determine the nature of the epitope recognized by serum antibodies, the Triton X-100 extracted antigen was treated with 0.2% proteinase K at 37°C for 2 hours. Treated and untreated samples of Triton X-100 extracted antigen were subjected to SDS/PAGE and Western blot analyses using the *B. canis* positive serum pool as the primary antibody.

SDS polyacrylamide gel electrophoresis (SDS/PAGE). and Western blotting

Analyses were performed according to the method of Laemmli.[110,111] Extracted antigen (proteinase K treated and untreated) and whole-cell antigens were diluted 1:1 in 2X sample buffer (.12 M Tris-HCl, pH 6.8, 2.5% SDS, 0.005% bromophenol blue, 20% glycerol, 5% 2-Mercaptoethanol), boiled for 5 minutes in a 100°C water bath, and subjected to electrophoresis through a 2-D 12% acrylamide gel in running buffer (25 mM Tris, 193 mM glycine, 0.1 % SDS, pH 8.0) at 35 mA/gel for 2 hours or until the dye front reached the

bottom of the gel. Molecular weight standards were placed in the first well of the gel and used to determine the apparent molecular weight of the antigens. The separated proteins were electroblotted to polyvinylidene fluoride (PVDF) membranes[g] at 250 mA for 2 hours in transfer buffer (12 mM Tris, 96 mM glycine, pH8.3, with 20% methanol). The membranes were blocked at room temperature for 15 minutes in 5% NFDM. Serum pools from 10 dogs with no known exposure to *B. canis* (negative serum pool) and 10 dogs with known exposure to *B. canis* (positive serum pool) were diluted 1:100, were adsorbed to the PVDF for 2 hours at 37°C. Membranes were washed three times for 5 minutes each in 0.15 M PBS containing 0.5% Tween 20 (PBST) and reblocked in NFDM. Horseradish peroxidase (HRP)-labeled goat origin anti-canine IgG[f] diluted 1:1000 in NFDM was adsorbed to the membrane for 2 hours at 37°C. The membranes were washed three times for 5 minutes each in PBST. Freshly prepared TMB with TMB membrane enhancer (prepared according to manufacturer's instructions) was used as the color substrate.[f] After the color was allowed to develop for up to 30 minutes, membranes were washed 5 times in PBS and air dried.

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[g] Schleicher & Schuell, P.O. Box 2012, Keene, NH 03431.

RESULTS

Assay Development and Optimization

Antigen selection and optimization

Six antigens were evaluated including *B. canis* 2-ME-TAT, heat extracted *B. ovis*, heat extracted *B. canis*, ammonium sulfate precipitated *B. ovis*, ammonium sulfate precipitated *B. canis*, and Triton X-100 extracted *B. canis*. The reference ELISA was followed with the following exceptions: antigen preparations were diluted 1:50 in coating buffer, plates were blocked with 5% NFDM, KPL conjugate was diluted 1:1000, and TMB was used as a substrate. Because the Triton X-100 extracted *B. canis* antigen had the maximum variation between $OD_{450-650}$ values of the positive and negative pool and the least background, it was selected for optimization (Figure 1). The optimum dilution of the Triton X-100 antigen was determined by titration of the antigen at 1:50, 1:100, 1:200, 1:400, 1;1000, and 1:2000 dilutions following the procedure described above. A 1:200 dilution was the highest dilution at which antigen was in excess and was selected for use in the ELISA (Figure 2).

Serum dilution buffers

Five serum dilution buffers (SDB1, SDB2, SDB3, SDB4, and SDB5) were tested in an ELISA with no primary antibody to evaluate nonspecific reactivity of conjugates with the serum dilution buffers. Only SDB1 reacted in an ELISA with coated antigen but no primary antibody (Figure 3). Unacceptably high background values were observed at all dilutions using SDB1 and SDB2. High background values were observed using SDB3 at lower dilutions which were not apparent at higher dilutions (Figure 4). Background values

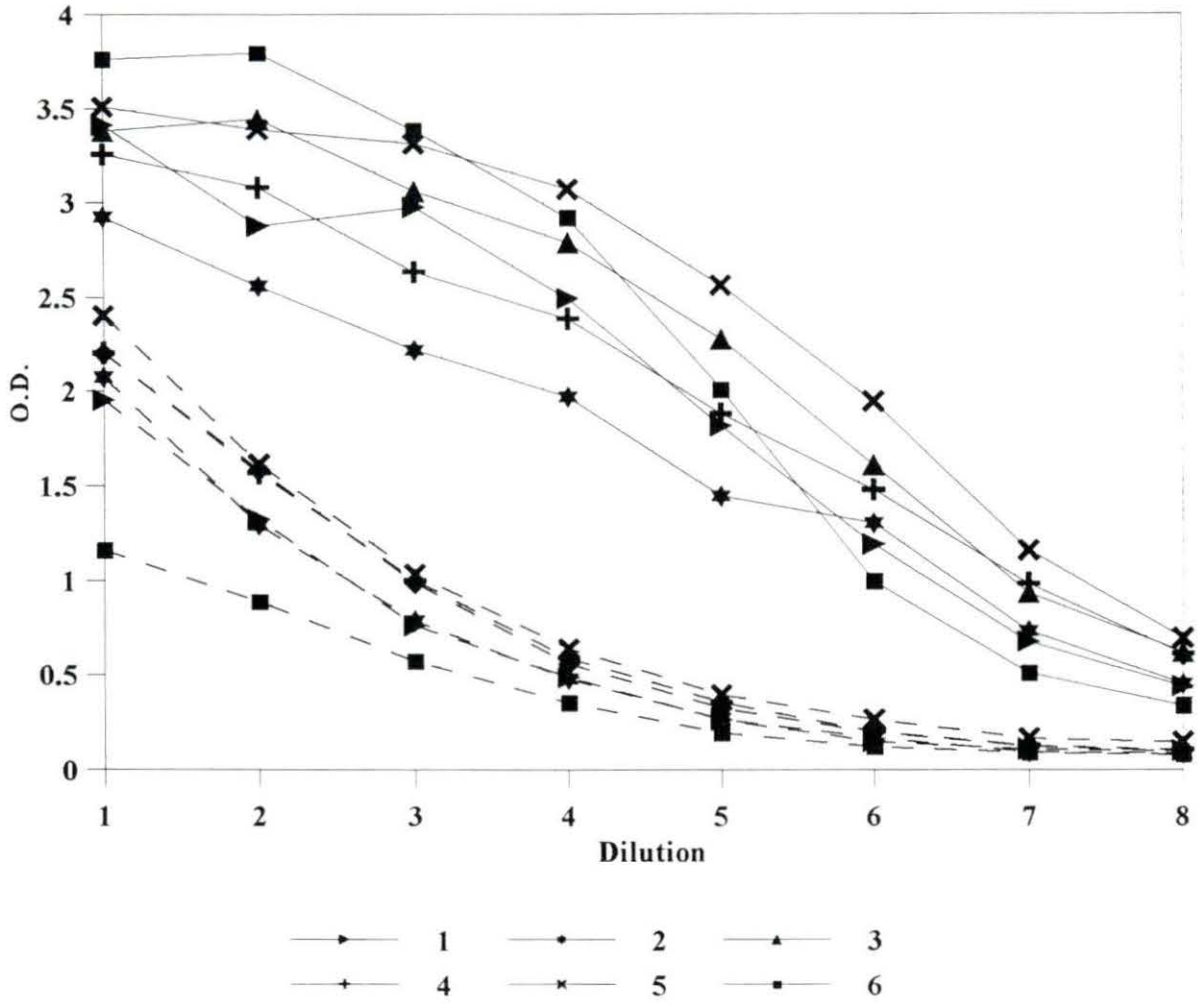

Figure 1. ELISA reactions observed using each of 6 antigen preparations.

The following antigens were assayed: (1) *B. canis* 2-ME-TAT antigen, (2) *B. canis* heat extracted antigen, (3) *B. ovis* heat extracted antigen, (4) *B. canis* ammonium sulfate precipitated antigen, (5) *B. ovis* ammonium sulfate precipitated antigen, and (6) *B. canis* Triton X-100 extracted antigen. Antigens were diluted 1:50 in antigen coating buffer and plates were blocked with 5% NFDM. ELISA antigens were compared using a negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines). Sera were diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and reactions and the $OD_{450-650}$ values were determined.

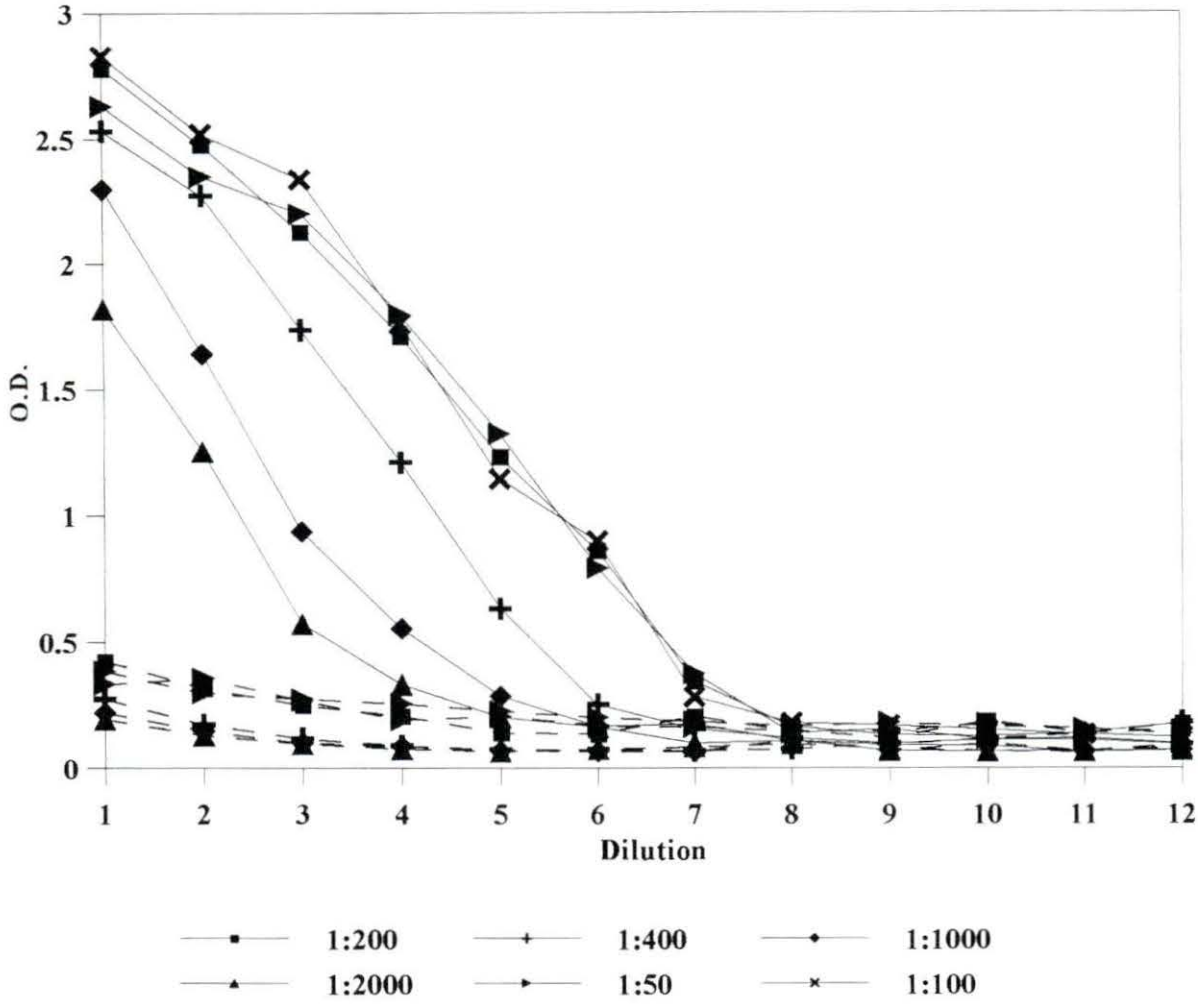

Figure 2. ELISA reactions observed using each of 6 Triton X-100 extracted antigen concentrations.

Triton X-100 extracted antigen was diluted in antigen coating buffer (1:50, 1:100, 1:200, 1:400, 1;1000, and 1:2000) and plates were blocked with 5% NFDM. Dilutions were compared using a negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines). Two-fold dilutions of the serum pools were performed from 1:50 to 1:102,400 in SDB5. JIRL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

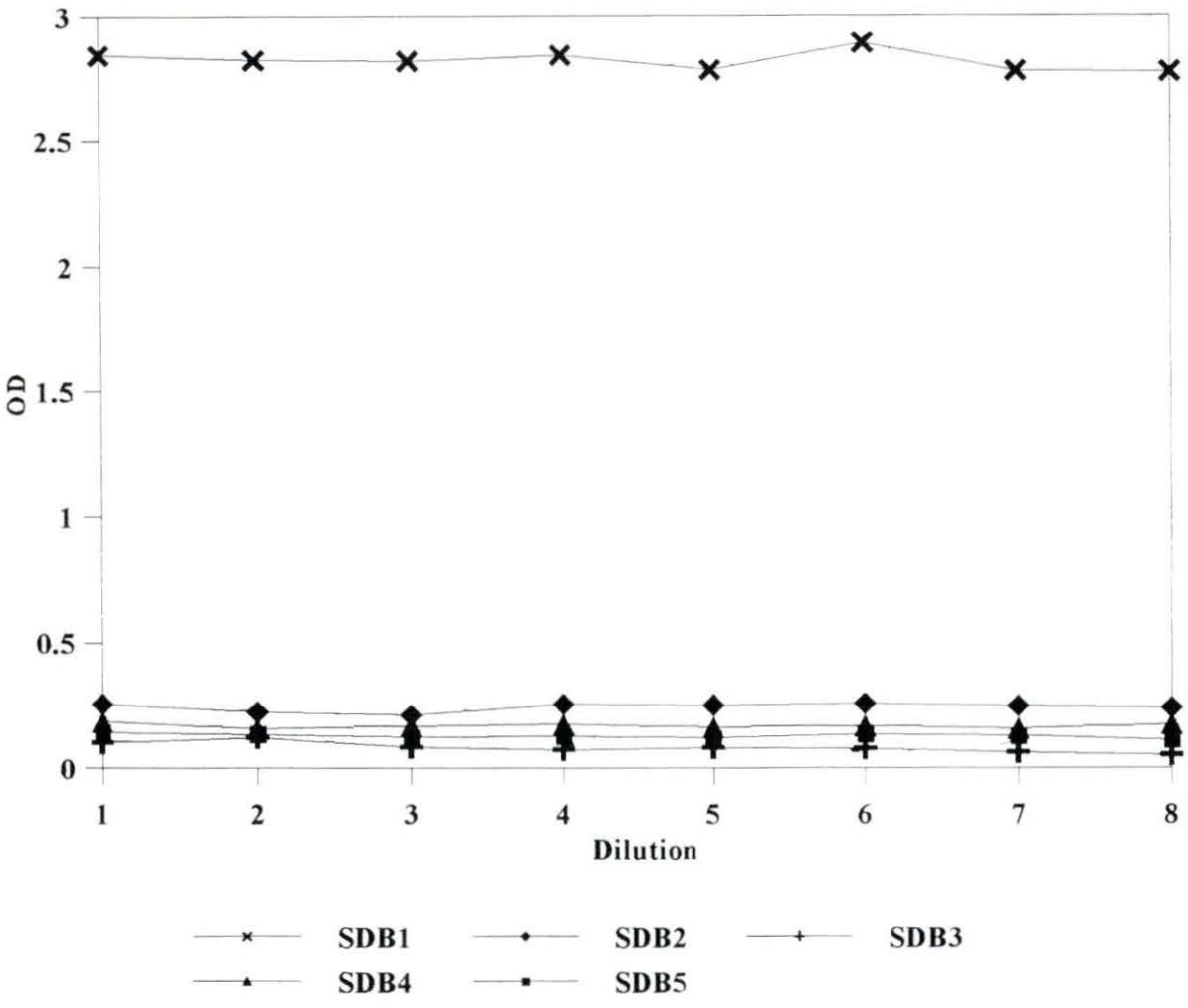

Figure 3 . ELISA reactions observed using each of five serum dilution buffers and no primary antibody.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer and plates were blocked with 5% NFDM. Serum dilutions buffers (200μl) were added to each well, and no primary antibody (canine serum) was added. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

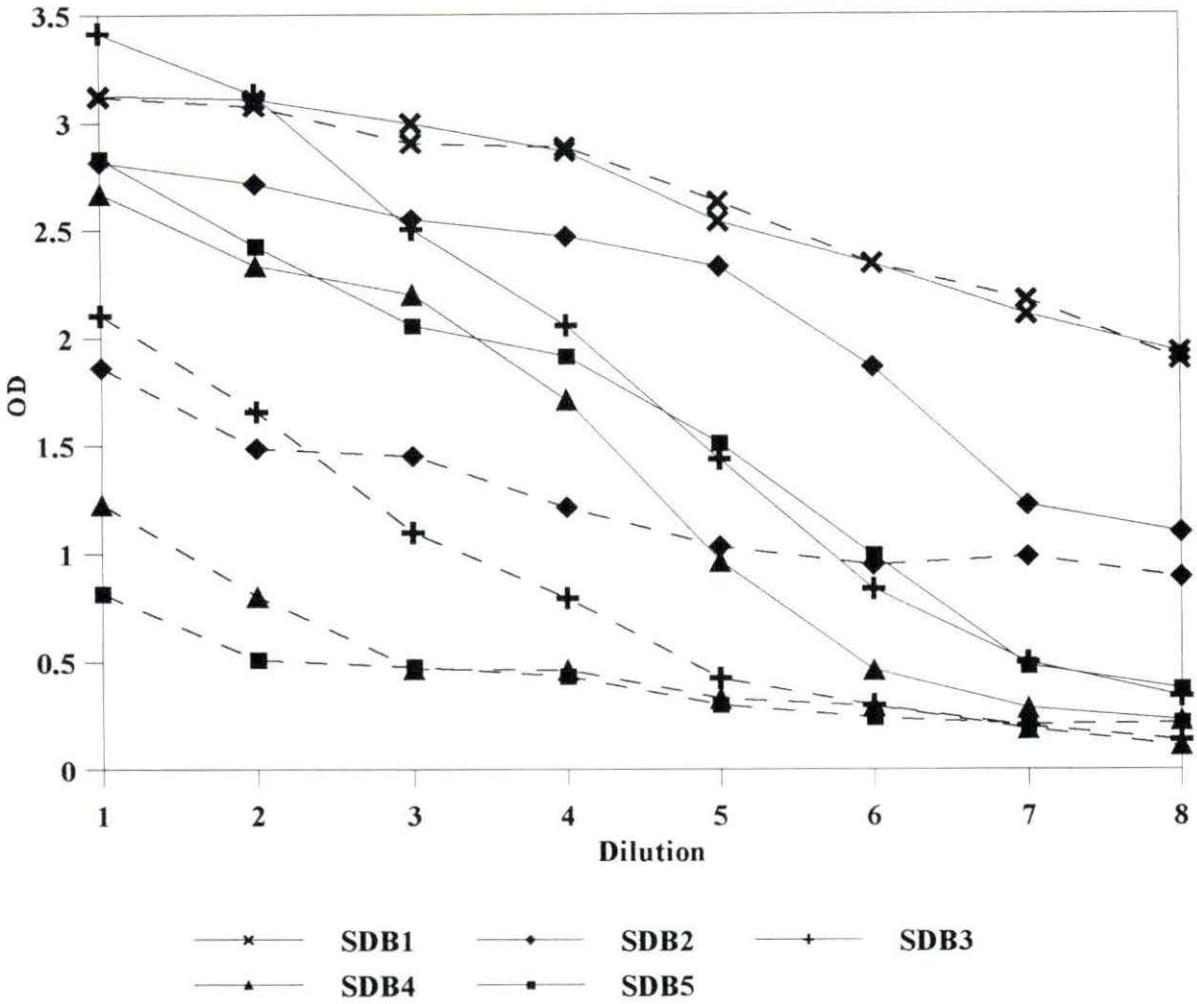

Figure 4. ELISA reactions observed using each of 5 serum dilution buffers.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer and the plates were blocked with 5% NFDM. Serum dilution buffers (SDB1, SDB2, SDB3, SDB4, and SDB5) were compared using a negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines). Serum pools were diluted 2-fold from 1:50 to 1:6400. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

observed with SDB4 and SDB5 were similar. Further evaluation of the serum dilution buffers was performed to determine if conjugate would bind non-specifically to microtiter wells in the presence of no antigen. The ELISA was followed with the exception of antigen coating. Serum dilution buffers (SDB1, SDB2, SDB3,SDB4, and SDB5) were used to serially dilute the negative and positive serum pools from 1:50 to 1:6400. PBS replaced serum dilution buffers and primary antibody (negative and positive serum pools) in a portion of the study to determine if conjugate would bind non-specifically to the polystyrene wells of the microtitration plate (Figure 5). Low background values were observed in the absence of serum dilution buffers and primary antibody (N-PAb). Low background values were also observed for SDB3, SDB4, and SDB5. Unacceptably high levels of background were observed with SDB1 and SDB2. SDB5 was selected for use in the ELISA because of the greater, more sustained differences in $OD_{450-650}$ readings between the positive and negative serum pools (Figure 4) .

Serum dilution range

Two-fold serial dilutions beginning with 4 different original serum dilutions (1:10, 1:20, 1:50, and 1:100) were evaluated using the reference ELISA, blocking with 5% NFDM, using KPL conjugate (1:1000), and using TMB as the substrate to determine appropriate serum dilution ranges for $OD_{450-650}$ readings (maximum difference between positive and negative serum pools with minimal background). At a 1:10 dilution, the background was unacceptably high. A 1:50 to 1:100 initial dilution followed by serial 2-fold dilutions provides an appropriate range of dilutions for serum assessment while avoiding unacceptable levels of non-specific reactivity (Figure 6).

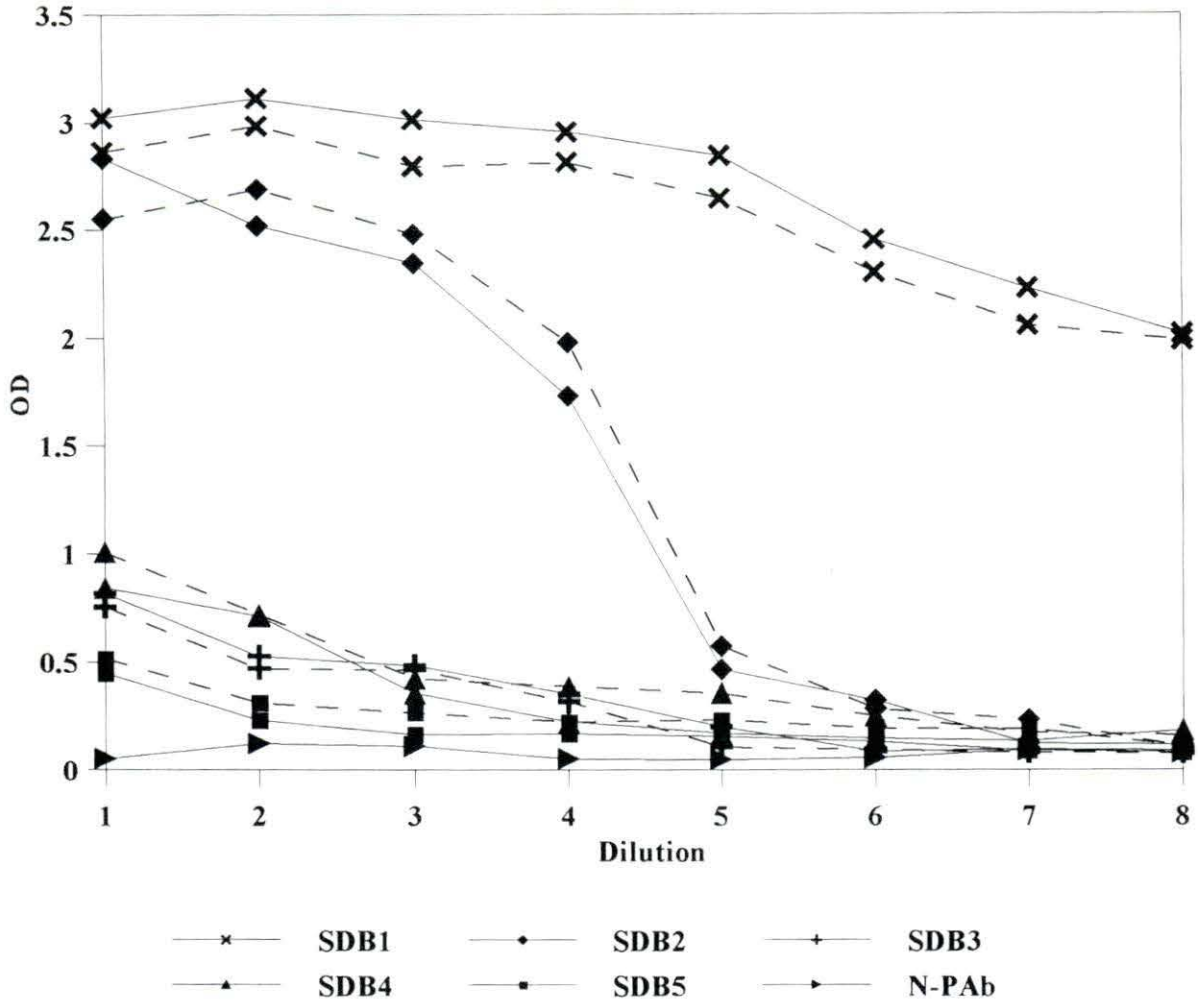

Figure 5. ELISA reactions observed using each of 5 serum dilutions buffers in the absence of antigen.

Uncoated plates (no Triton X-100 extracted antigen) were blocked in 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were diluted 2-fold from 1:50 to 1:6400 in 5 serum dilution buffers (SDB1, SDB2, SDB3, SDB4, and SBD5). No primary antibody (positive or negative serum pool) was added to a portion of the microtitration plate (NoPAb). KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

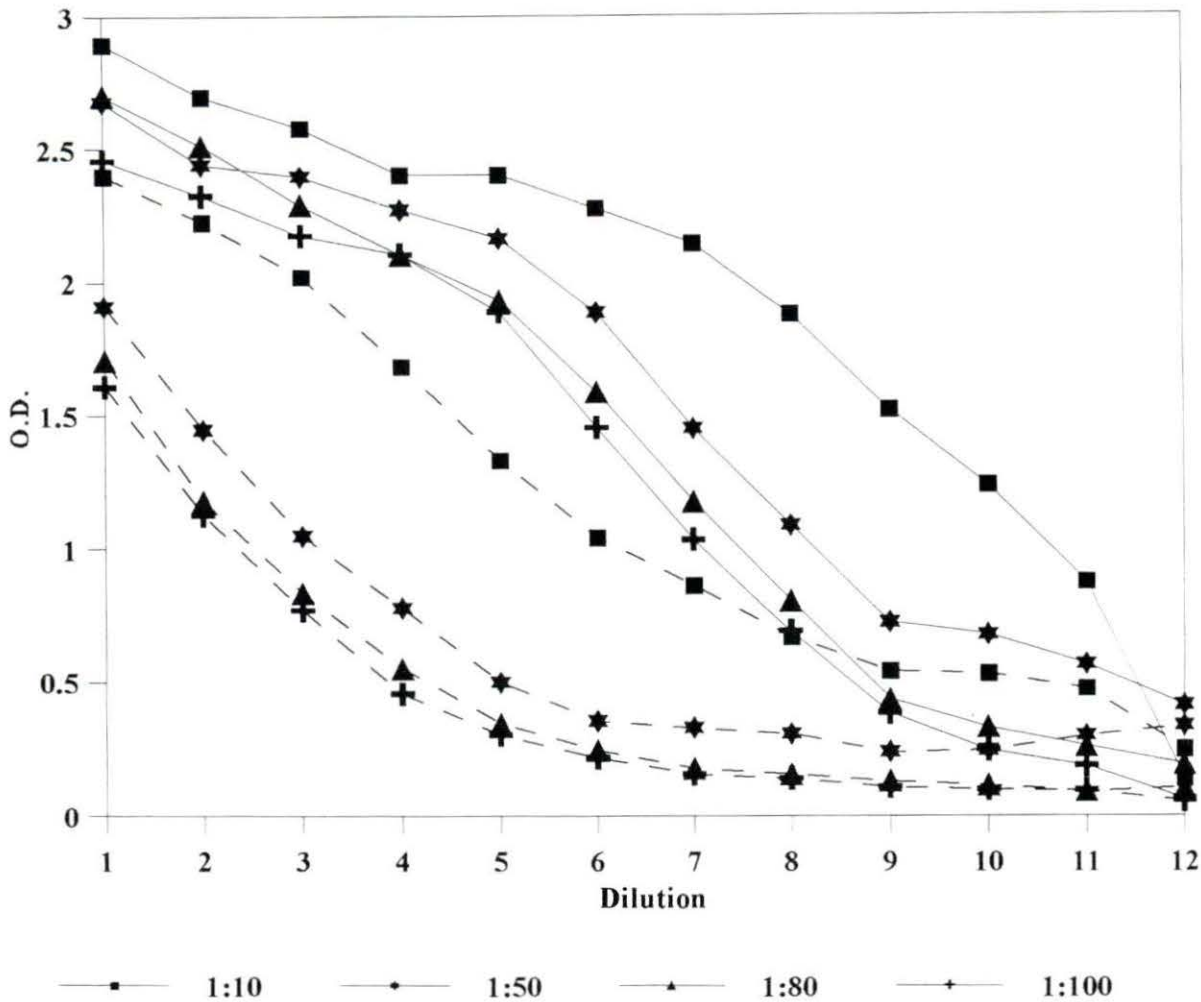

Figure 6 . ELISA reactions observed using each of 4 serum dilutions.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer and plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were diluted 2-fold starting at dilutions of 1:10, 1:50, 1:80, or 1:100. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

Blocker selection and concentration

Various blockers including 5% nonfat dry milk (NFDM), 1% polyvinyl alcohol (PVA), 1% bovine serum albumin (BSA), 5% fish gelatin (FG), PBST, and PBS were evaluated using the reference ELISA. Decreases in non-specific reactivity were observed, with the exception of BSA and PBS, without impairing detection of *B. canis* antibodies (Figure 7). Nonfat dry milk was selected for further evaluation because of the availability, low cost, ease of preparation, and biological degradability. Various concentrations (1, 2.5, 5, 7.5, and 10%) of NFDM were evaluated for use as a blocker using the above described procedure (Figure 8). Concentrations of 5% and above provided optimum blocking with least interference. A concentration of 5% NFDM was selected for use in the ELISA.

Conjugate selection and concentration

Individual lots of horse radish peroxidase conjugated, anti-canine heavy and light chain IgG from Jackson ImmunoResearch Laboratories (JIRL) (goat origin) and Kirkegaard Perry Laboratories (KPL) (rabbit origin) were compared using the reference ELISA. Plates were blocked with 5% NFDM and each conjugate was diluted 1:1000. The goat origin conjugate from JIRL had significantly lower backgrounds without lowering the $OD_{450-650}$ values of the positive reactions and was selected for the ELISA development (Figure 9). The optimum conjugate dilution was determined by comparing 6 JIRL conjugate dilutions (1:250, 1:500, 1:1000, 1:2500, 1:5000, and 1:10,000) using the procedure described above. A 1:1000 dilution was selected for further ELISA development and evaluation in order to ensure that the conjugate was in excess in all reactions (Figure 10).

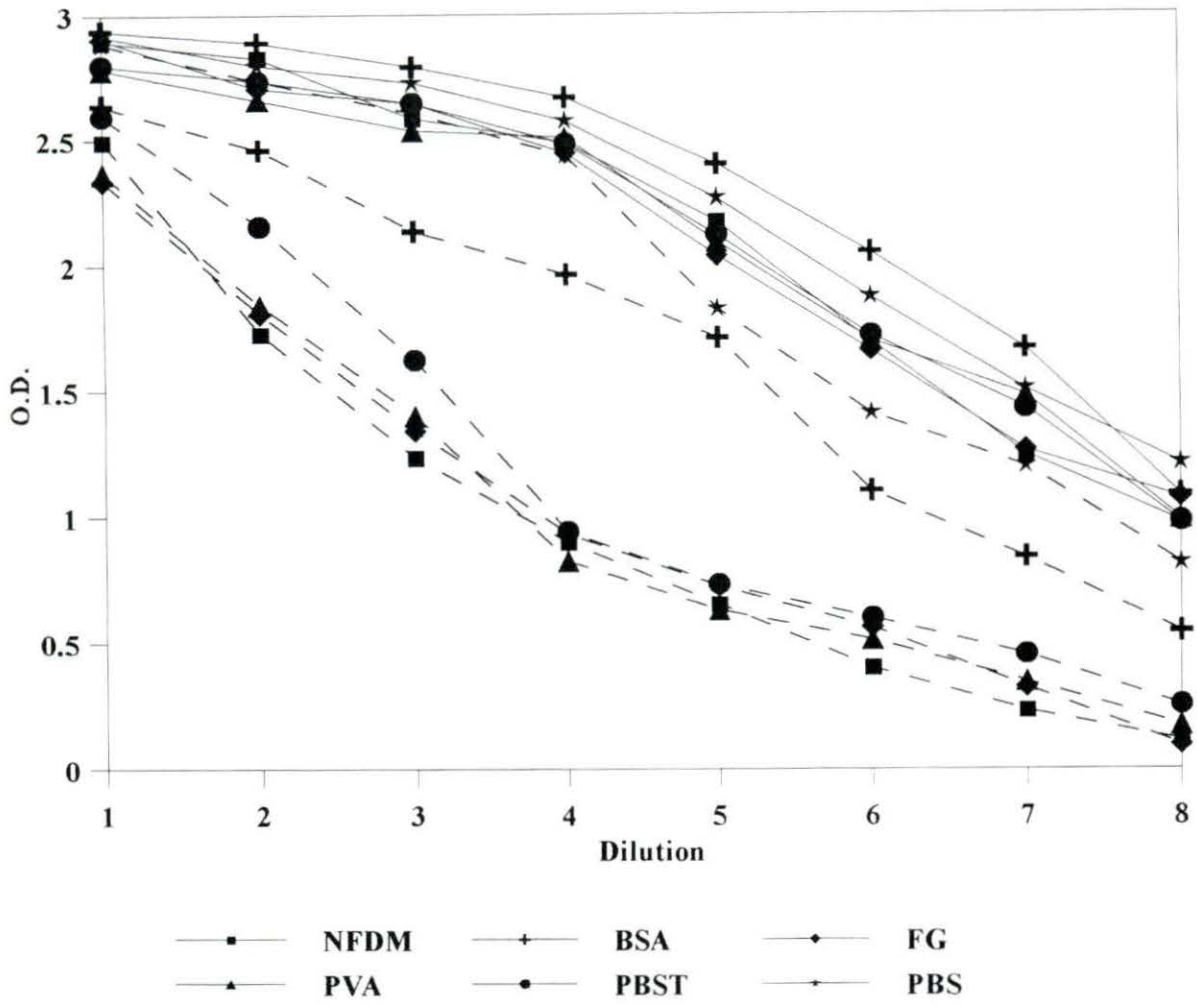

Figure 7. ELISA reactions observed using each of 6 blockers.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer. Plates were blocked with the following blockers: 5% nonfat dry milk (NFDM), 1% bovine serum albumin (BSA), 5% fish gelatin, 1% polyvinyl alcohol (PVA), phosphate buffered saline with 5% tween (PBST), and phosphate buffered saline (PBS). Blockers were compared using a negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines). Serum pools were diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

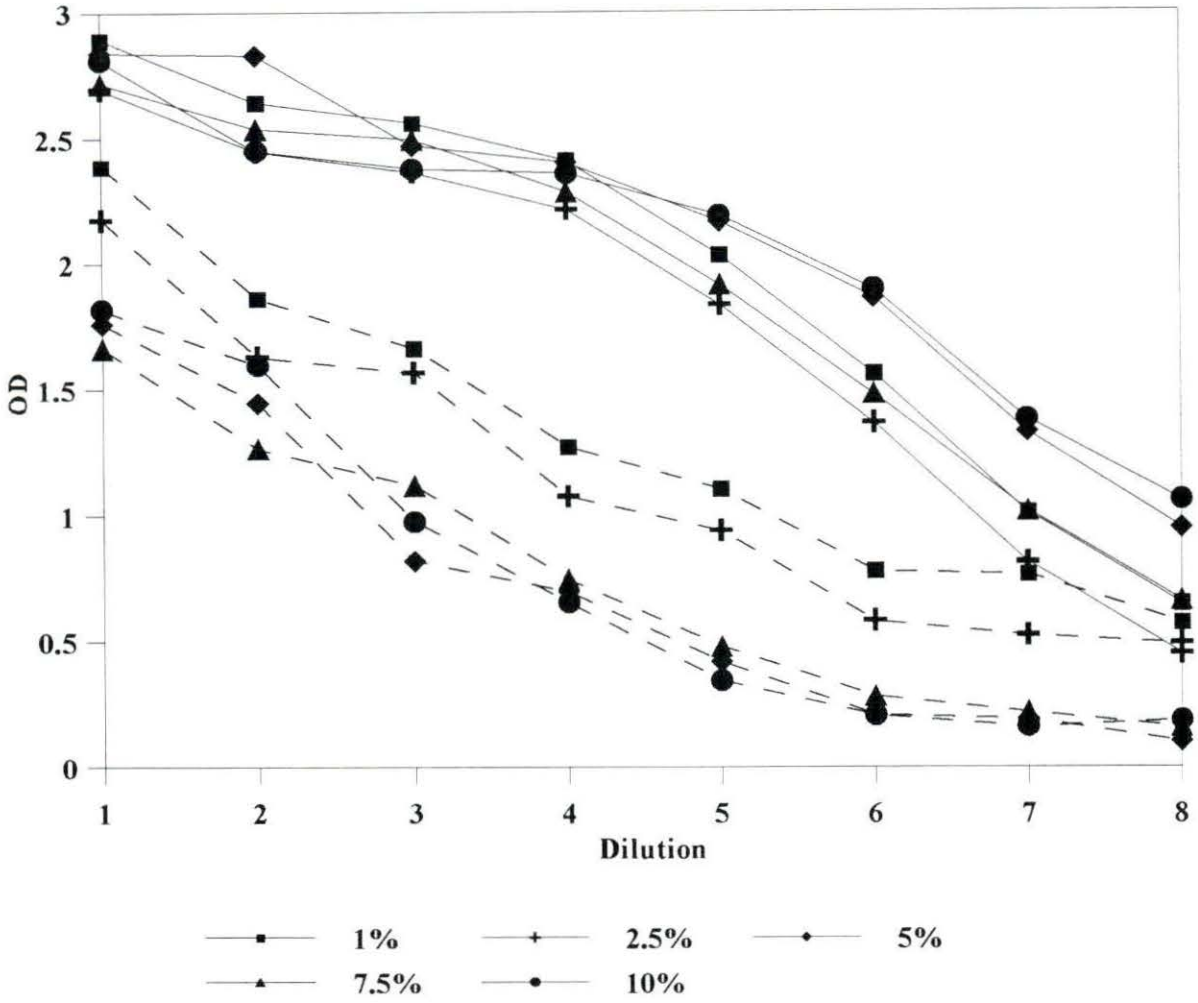

Figure 8. ELISA reactions observed using each of 5 concentrations of NFDM as blocker.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer and the plates were blocked with 1, 2.5, 5, 7.5, or 10% NFDM. Blocker concentrations were compared using a negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a serum pool from 10 dogs with known exposure to *B. canis* (solid lines). Serum pools were diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

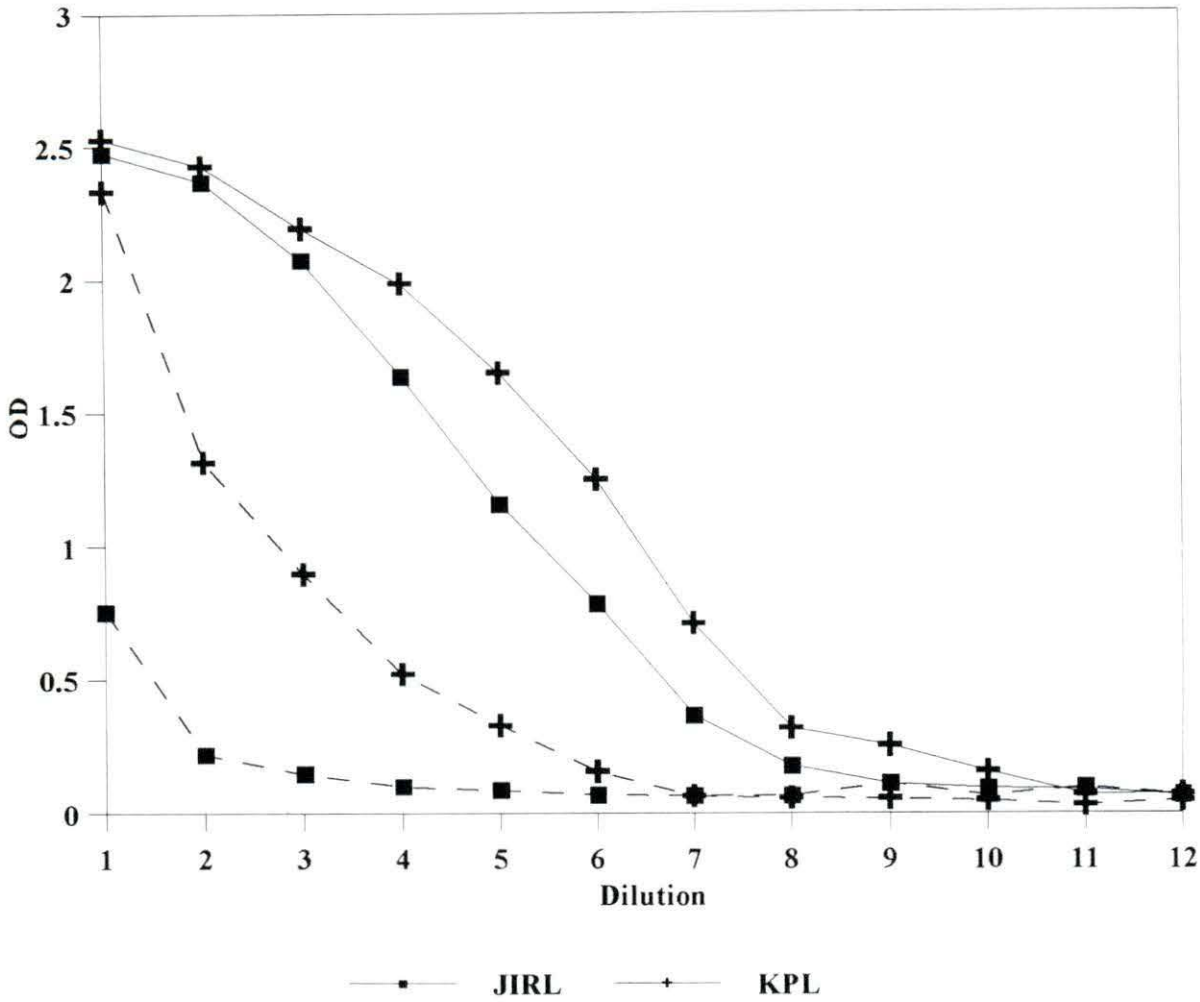

Figure 9 . ELISA reactions observed using each of 2 commercially available conjugates.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer and plates were blocked with 5% NFDM. Goat origin, horse radish peroxidase conjugated, anti-canine heavy and light chain IgG from Jackson ImmunoResearch Laboratories (JIRL) and rabbit origin, horse radish peroxidase conjugated, anti-canine heavy and light chain IgG from Kirkegaard Perry Laboratories (KPL) were compared using a negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines). Two-fold dilutions of the serum pools were performed from 1:50 to 1:102,400 in SDB5. JIRL and KPL conjugates were diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated the $OD_{450-650}$ values were determined.

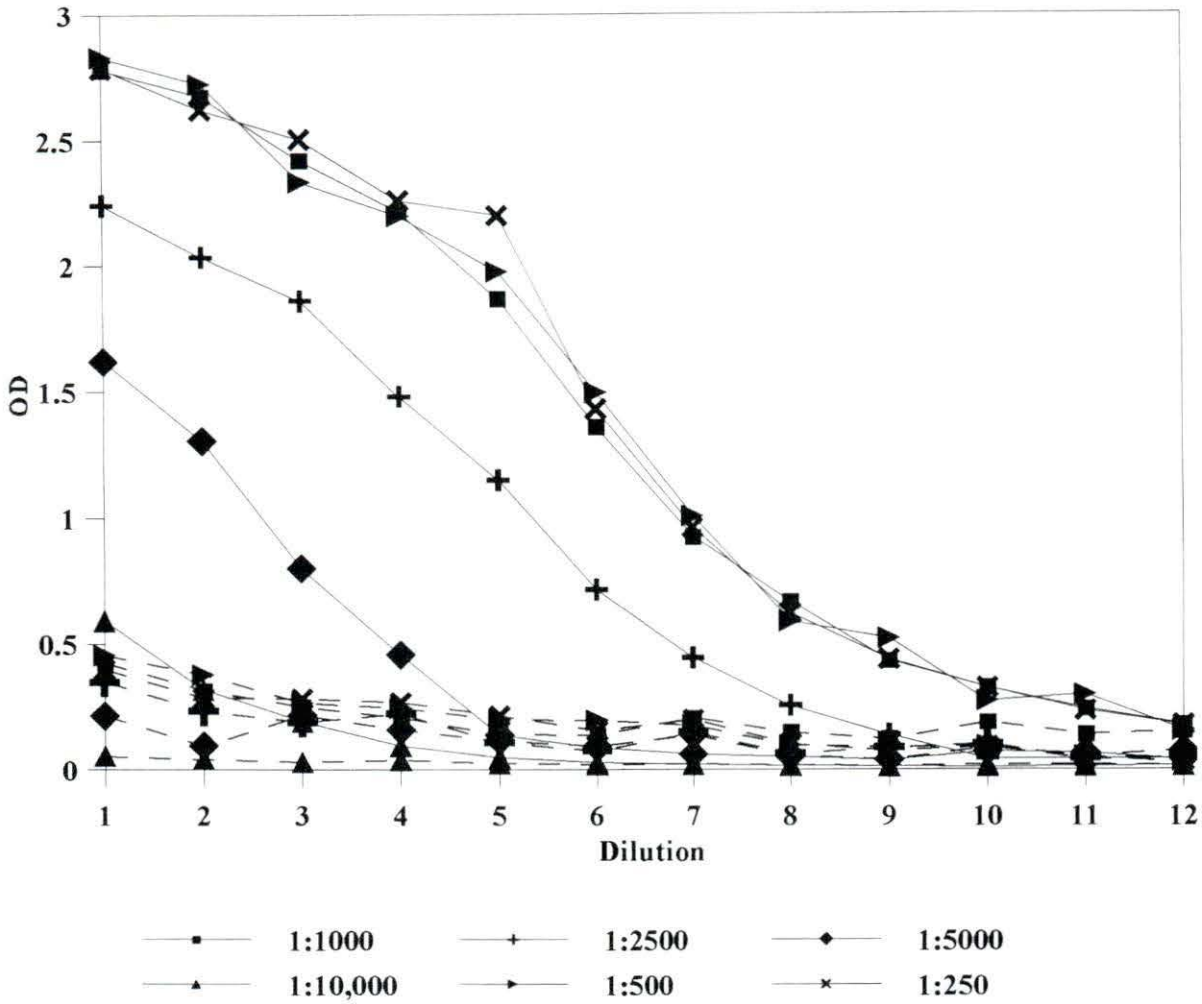

Figure 10. ELISA reactions observed using each of 6 goat origin conjugate concentrations.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer and plates were blocked with 5% NFDM. The optimum dilution of goat origin, horse radish peroxidase conjugated, anti-canine heavy and light chain IgG from JIRL was determined using a negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines). Two-fold dilutions of the serum pools were performed from 1:50 to 1:102,400 in SDB5. Conjugate was diluted 1:250, 1:500, 1:1000, 1:2500, 1:5000, or 1:10,000. TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

Substrate selection

ABTS and TMB substrates were compared using the reference ELISA (blocking with 5% NFDM, KPL conjugate diluted 1:1000, and 2-fold serum dilutions from 1:50 - 1:6400) to determine which substrate provided optimal color development. When using ABTS as a substrate, a higher background was detected in the lower dilutions of the negative serum pool. Although non-specific reactivity was not evident in higher dilutions of the negative serum pool, TMB was selected as a substrate (Figure 11).

Antigen assessment

Studies to evaluate optimum storage temperatures, the effect of freeze thaw cycles, and the value of merthiolate as a preservative for antigen stability were performed using the reference ELISA. Triton X-100 extracted antigen was stored with or without merthiolate (1:5000) at 4±2°C and 20±4°C. Antigen without merthiolate was stored at 25±4°C. Assays were performed at 1 week, 4 weeks, 13 weeks, 26 weeks, 39 weeks, and 52 weeks post-preparation. Three vials of antigen from each treatment were assayed at each of the sampling dates.

Three vials of Triton X-100 antigen stored at 4±2°C with or without merthiolate (1:5000) were sampled as stated above using the procedure described for antigen selection. The $OD_{450-650}$ values of the positive and negative serum pools for the antigen stored without preservative were stable during the sampling period (Figure 12) although the antigen with added merthiolate had increased non-specific reactivity at 4 weeks which were observed during the remainder of the study (Figure 13).

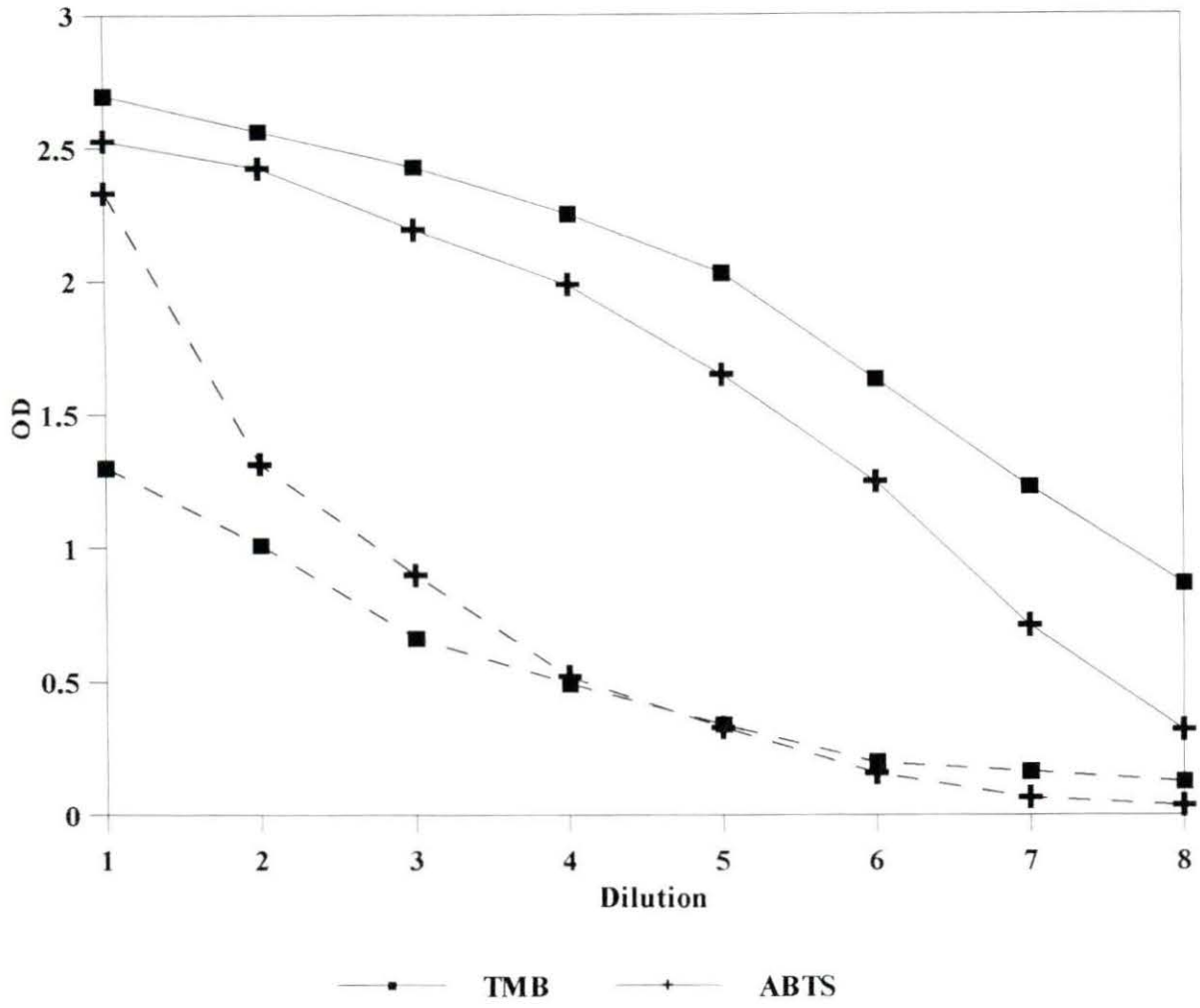

Figure 11. ELISA reactions observed using each TMB or ABTS as substrates.

Triton X-100 extracted antigen was diluted 1:200 in antigen coating buffer and plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were diluted 2-fold from 1:50 through 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB or ABTS were utilized according to manufacturer's instructions. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

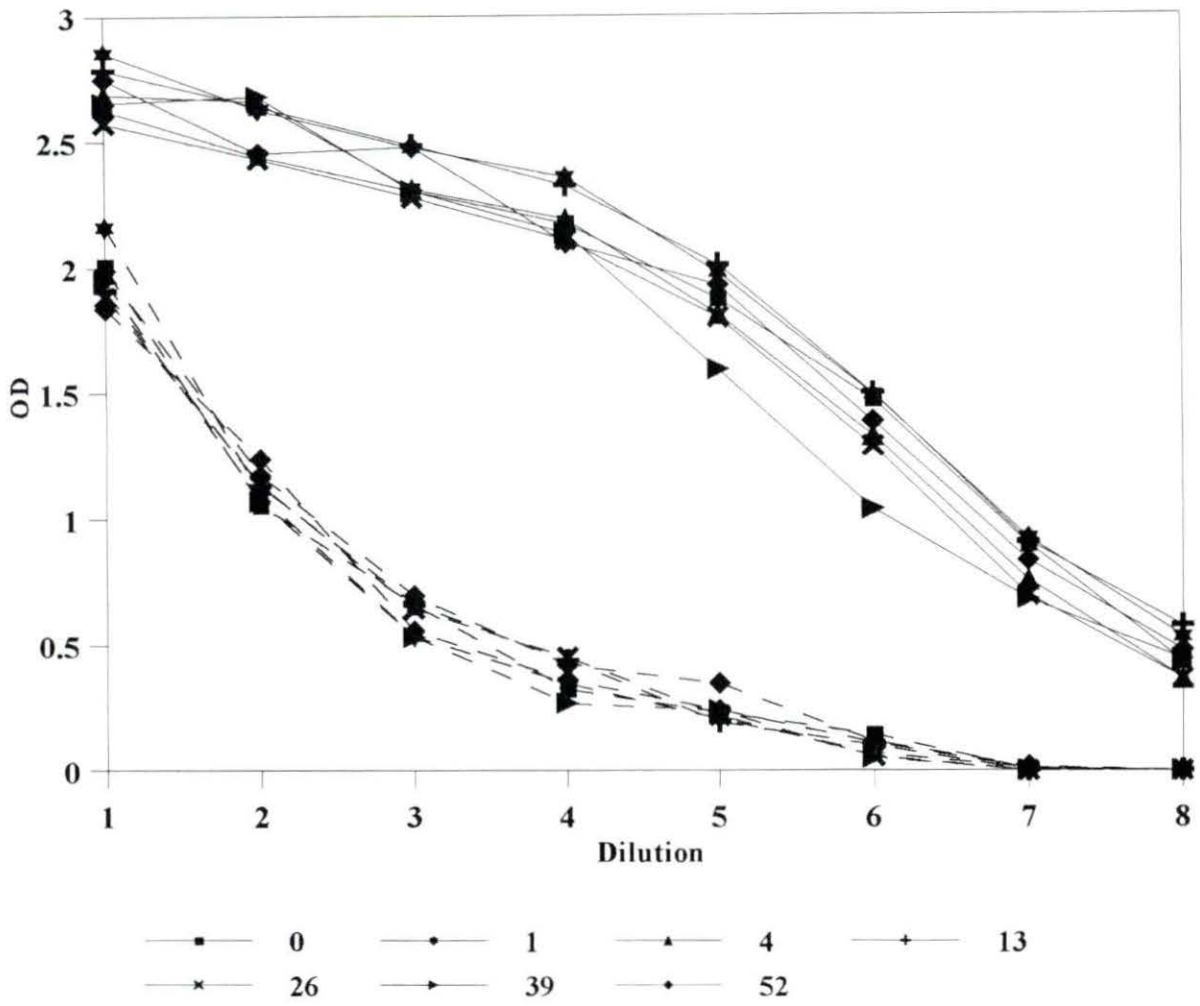

Figure 12. ELISA reactions observed using Triton X-100 extracted antigen (without merthiolate) stored at 4±2°C.

Antigen was stored at 4±2°C for 0, 1, 4, 13, 26, 39, or 52 weeks and then diluted 1:200 in antigen coating buffer. Plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were diluted 2-fold from 1:50 through 1:6400. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

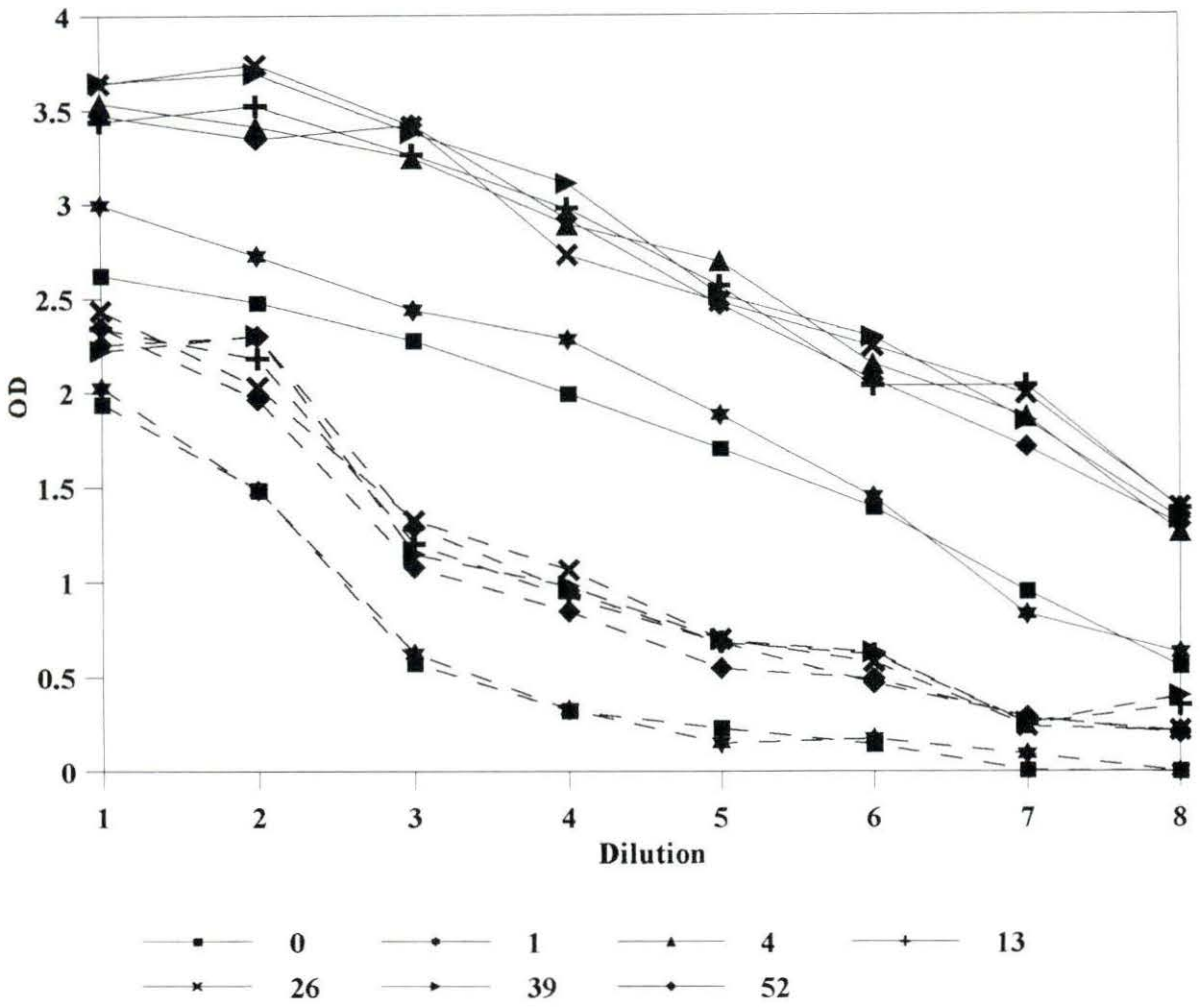

Figure 13. ELISA reactions observed using Triton X-100 extracted antigen (with merthiolate) stored at 4±2°C.

Antigen with merthiolate (1:5000) was stored at 4±2°C for 0, 1, 4, 13, 26, 39, or 52 weeks and then diluted 1:200 in antigen coating buffer. Plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 through 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

Three previously unthawed vials of Triton X-100 antigen stored at -20±4°C with or without merthiolate (1:5000) were sampled at the above listed times following the procedure described for antigen selection. The $OD_{450-650}$ readings of the dilutions of negative and positive serum pools were stable during the sampling period for antigens stored at -20±4°C with or without merthiolate (Figures 14 and 15).

The influence of repeated freezing and thawing of the Triton X-100 antigen was demonstrated when the same 6 vials of antigen (3 with merthiolate and 3 without merthiolate) stored at -20±4°C were assayed at each of the sampling times. The antigen remained relatively stable until week 13 (3 freeze/thaw cycles). Decreases in ELISA values were observed during subsequent assays (Figure 16 and 17).

When antigen was stored at 25±4°C and sampled at the times listed above using the procedure described for antigen selection, the antigen activity decreased rapidly and the antigen was minimally acceptable for use at 4 weeks and unacceptable for use by 13 weeks (Figure 18). Antigen storage at 4±2°C without preservative provided optimum reactivity.

Production lot assessment

Three lots of Triton X-100 extracted antigen were produced and stored at 4±2°C without merthiolate. The antigens were compared using the positive and negative serum pools and the procedure described for antigen selection. No significant differences were detected between the 3 lots of antigen at the time of production or after 13 weeks of storage (Figure 19). The protein concentrations of the 3 lots of Triton X-100 extracted antigen were 8.66 mg/ml, 8.43 mg/ml, and 8.79 mg/ml for a mean concentration of 8.63 mg/ml.

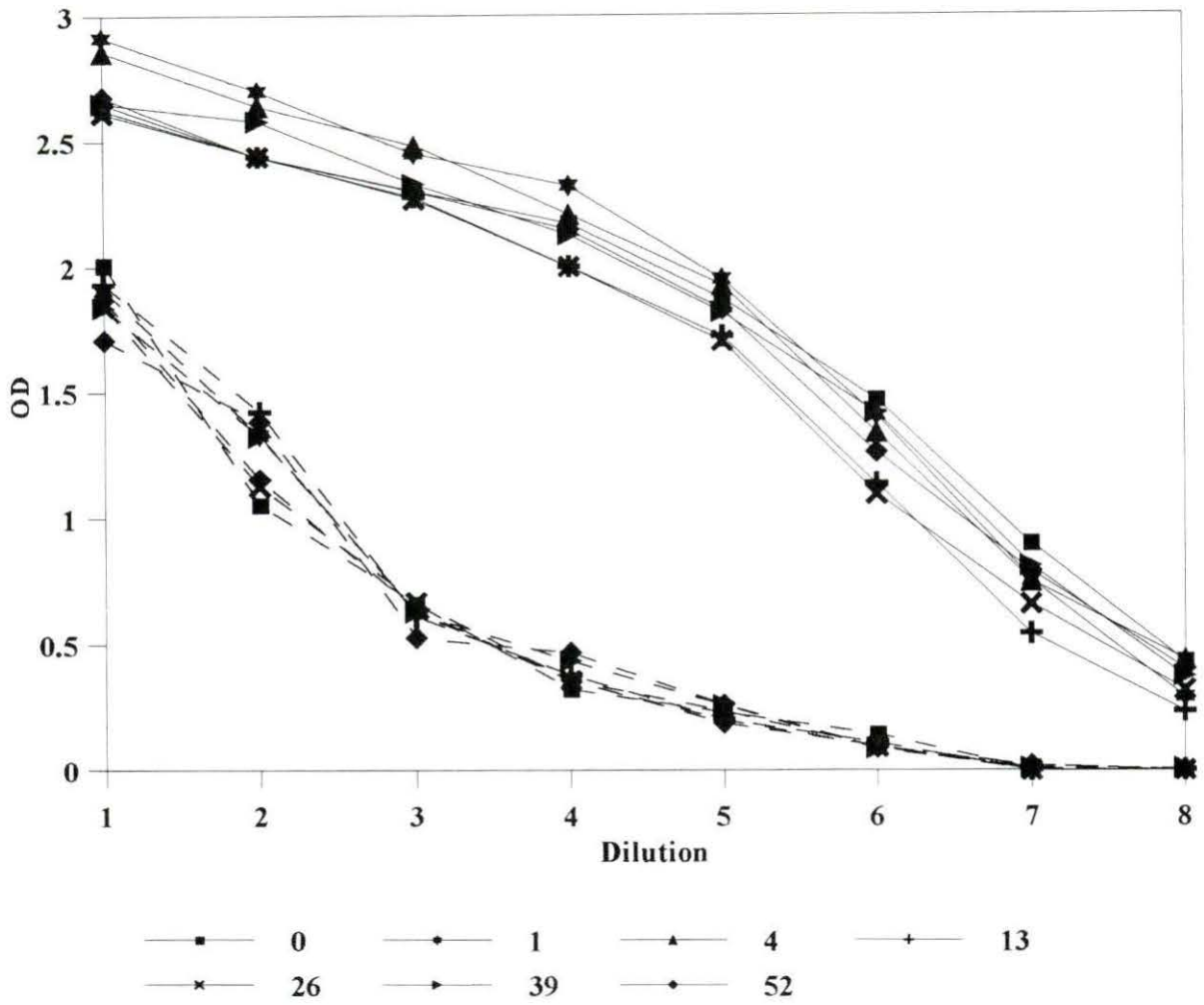

Figure 14. ELISA reactions observed using Triton X-100 antigen (without merthiolate) stored at -20±2°C.

Antigen was stored at -20±2°C for 0, 1, 4, 13, 26, 39, or 52 weeks and then diluted 1:200 in antigen coating buffer. A previously unthawed vial of antigen was selected for use at each sampling time. Plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

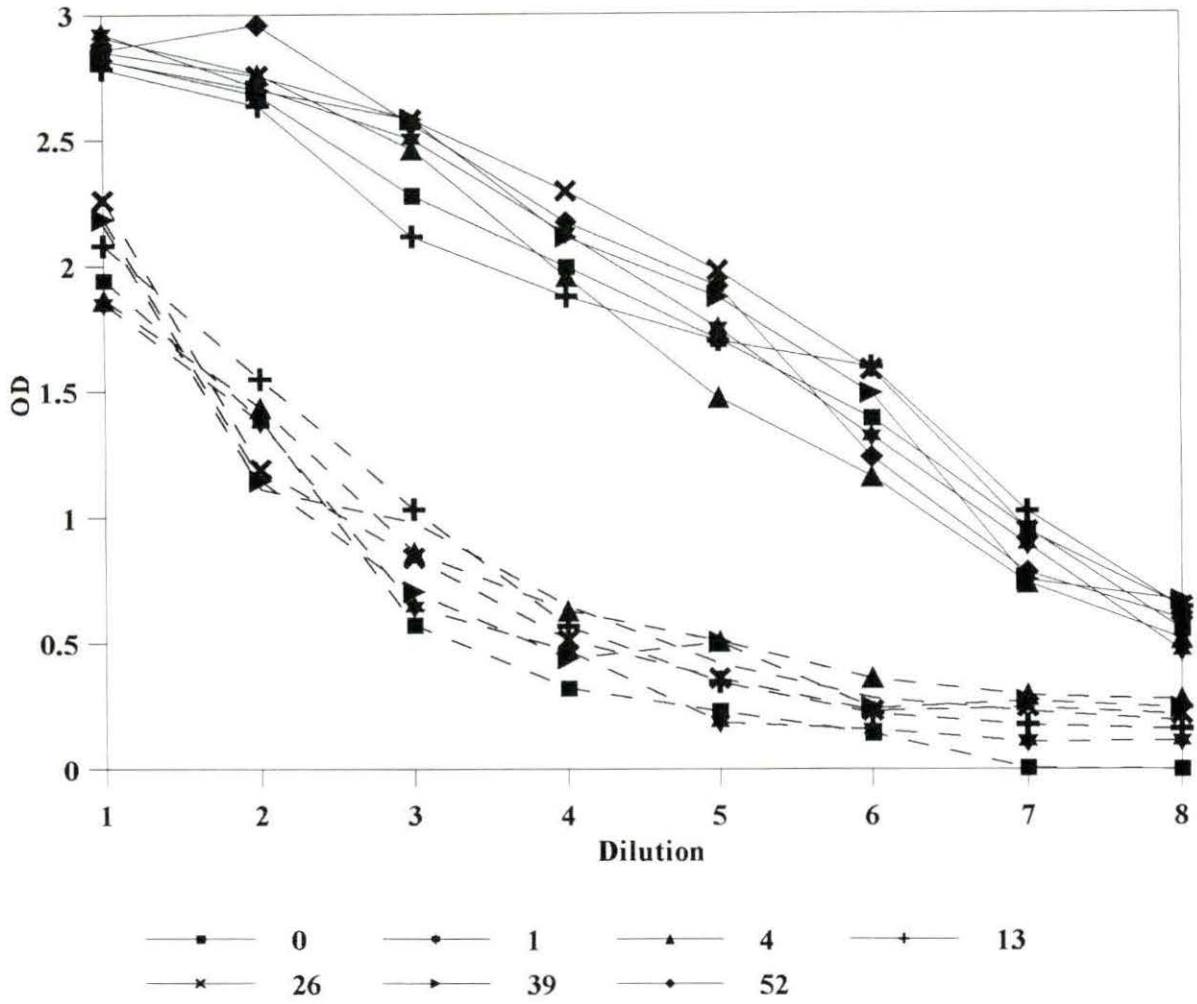

Figure 15. ELISA reactions observed using Triton X-100 extracted antigen (with merthiolate) stored at -20±2°C.

The antigen with merthiolate (1:5000) was stored at -20±2°C for 0, 1, 4, 13, 26, 39, or 52 weeks and then diluted 1:200 in antigen coating buffer. A previously unthawed vial of antigen was selected for use at each sampling time. Plates were blocked with 5% NFDM. A negative serum pools from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

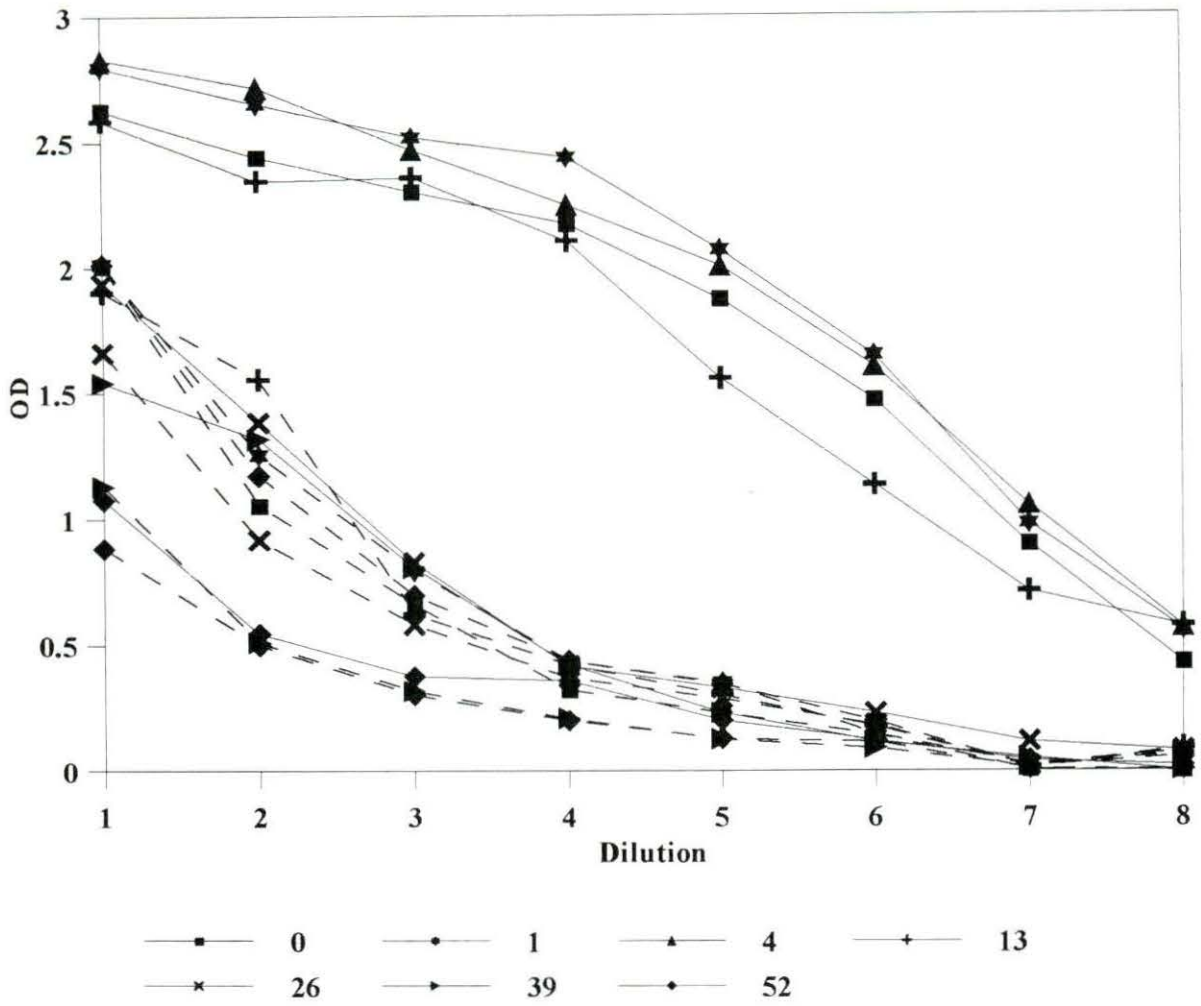

Figure 16. ELISA reactions observed using Triton X-100 extracted antigen (without merthiolate) stored at -20±2°C and subjected to repeated freeze/thaw cycles.

Antigen was stored at -20±2°C for 0, 1, 4, 13, 26, 39, or 52 weeks and then diluted 1:200 in antigen coating buffer. The same vials were assayed at each sampling time. Plates were blocked with 5% NFDM. A negative serum pools from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

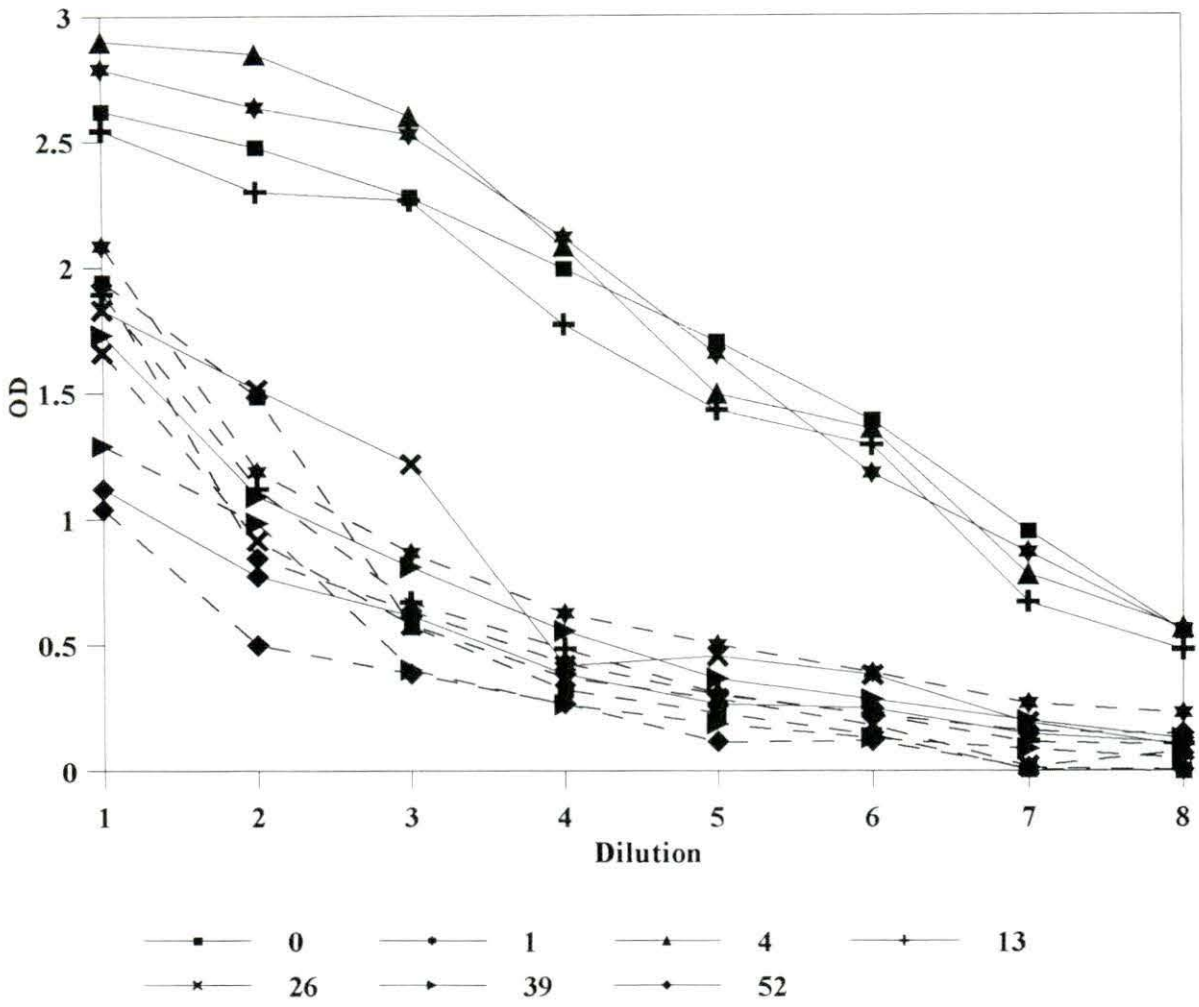

Figure 17. ELISA reactions observed using Triton X-100 extracted antigen (with merthiolate) stored at -20±2°C and subjected to repeated freeze/thaw cycles.

Antigen with merthiolate (1:5000) was stored at -20±2°C for 0, 1, 4, 13, 26, 39, or 52 weeks and then diluted 1:200 in antigen coating buffer. A previously unthawed vial of antigen was selected for use at each sampling time. Plates were blocked with 5% NFDM. A negative serum pools from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

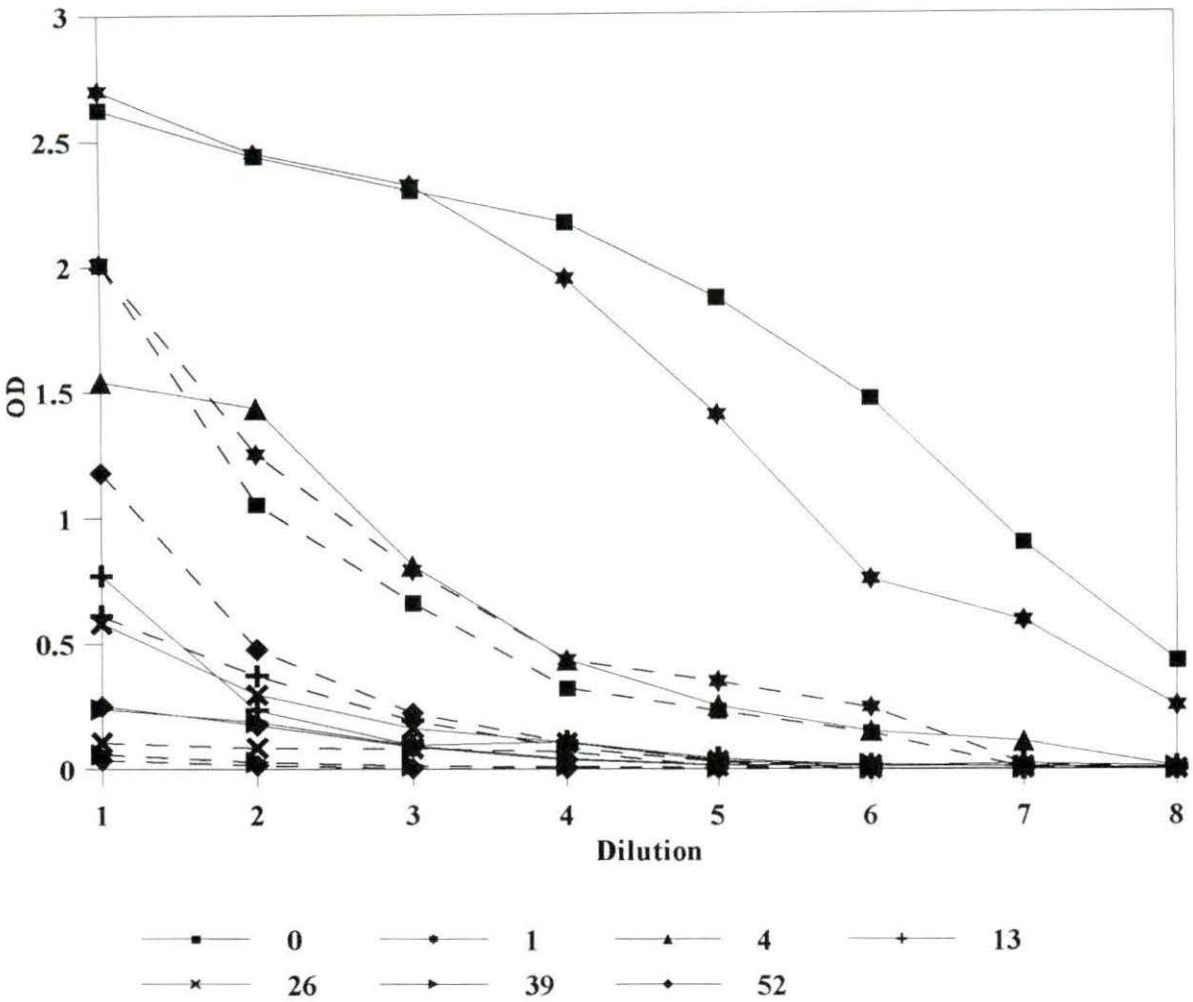

Figure 18. ELISA reactions observedusing Triton X-100 extracted antigen (without merthiolate) was stored at 25±4°C.

Antigen was stored at 25±4°C for 0, 1, 4, 13, 26, 39, or 52 weeks and then diluted 1:200 in antigen coating buffer. Plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 to 1:6400 in SDB5. KPL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

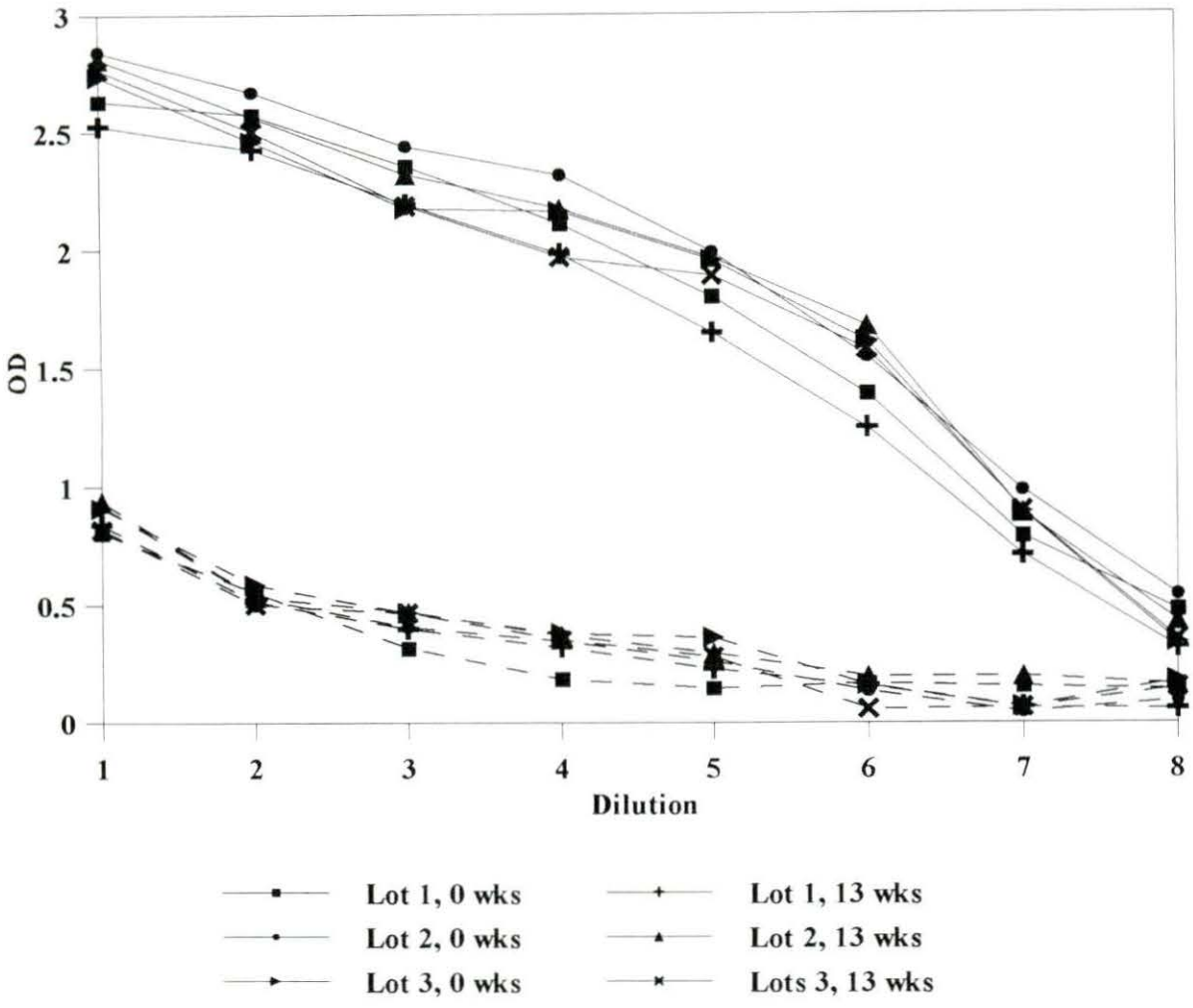

Figure 19. ELISA reactions observed using each of 3 lots of Triton X-100 extracted antigen.

Three individual lots of Triton X-100 extracted antigen without merthiolate were assayed and then stored at 4±2°C for 13 weeks. Antigen was diluted 1:200 in antigen coating buffer and plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 to 1:6400 in SDB5. JIRL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

Assay Evaluation

Serum panel criteria

Positive and negative serum panels were established for the determination of the sensitivity, specificity, positive and negative predictive values, and accuracy of the assay. Serum samples approved for use in the positive serum panel were *B. canis* culture positive and 2-ME-TAT positive. Negative samples were required to be culture negative and 2-ME-TAT negative with no known exposure to *B. canis*.

Brucella canis was isolated and identified from whole blood samples of 69 of 304 dogs sampled from a single kennel during a period in which reproductive failure and other signs of *B. canis* infection were present. None of the isolates required CO_2 for growth, produced H_2S, or grew in the presence of basic fuschin. All isolates grew in the presence of thionin, were rapidly urease positive and, were oxidase positive. Agglutination occurred with the rough *Brucella* antisera and not with antisera to smooth *Brucella*. None of the isolates were lysed by either dilution of *Tbilisi* bacteriophage, however all isolates were lysed by the R/C bacteriophage. Of the 69 hemoculture positive dogs, 66 were found to be positive by the 2-ME-TAT and were accepted for use in the positive serum control panel. An additional 12 serum samples from dogs with positive hemoculture and 2-ME-TAT results were collected from throughout the United States.

Negative sera for the serum panel were collected from 32 specific pathogen free dogs. An additional 43 sera were collected from 2-ME-TAT negative dogs with no known exposure to *B. canis*, but with possible exposure to other common canine pathogens. The resulting serum panel contained 78 positive samples and 75 negative samples which were considered as

the true positive and true negative samples for the evaluation of the ELISA.

Evaluation of sensitivity, specificity, positive and negative predictive values, and accuracy

In order to establish parameters for interpretation of ELISA results, sensitivity and specificity were calculated at various serum dilutions and with several minimum $OD_{450-650}$ readings for interpretation as a positive result. The $OD_{450-650}$ readings at 1:160 dilution are represented in Figure 20. If an $OD_{450-650}$ value of ≥ 1.3 is interpreted as a positive test result, the sensitivity and specificity of the assay were 95% and 85%, the positive predictive value was 87%, the negative predictive value was 94%, and the accuracy was 90% (Table 1). At the same serum dilution, if an $OD_{450-650}$ of ≥ 1.5 was interpreted as a positive result, the sensitivity was 92% and the specificity was 93%, the positive predictive value was 94%, the negative predictive value was 92%, and the accuracy was 93% (Table 2).

The $OD_{450-650}$ readings at 1:320 dilution are represented in Figure 21. If an $OD_{450-650}$ value of $\geq .1.1$ is interpreted as a positive test result, the sensitivity and specificity of the assay were 96% and 97%, the positive predictive value was 97%, the negative predictive value was 96%, the accuracy was 97% (Table 3). At the same serum dilution, if an $OD_{450-650}$ of ≥ 0.9 was interpreted as a positive result, the sensitivity was 97%, the specificity was 88%, the positive predictive value was 89%, the negative predictive value was 97%, and the accuracy was 93% (Table 4).

The $OD_{450-650}$ readings at 1:640 dilution are represented in Figure 22. If an $OD_{450-650}$ value of $\geq .0.7$ is interpreted as a positive test result, both the sensitivity and specificity of the assay were 96%, the positive predictive value was 95%, the negative predictive value was

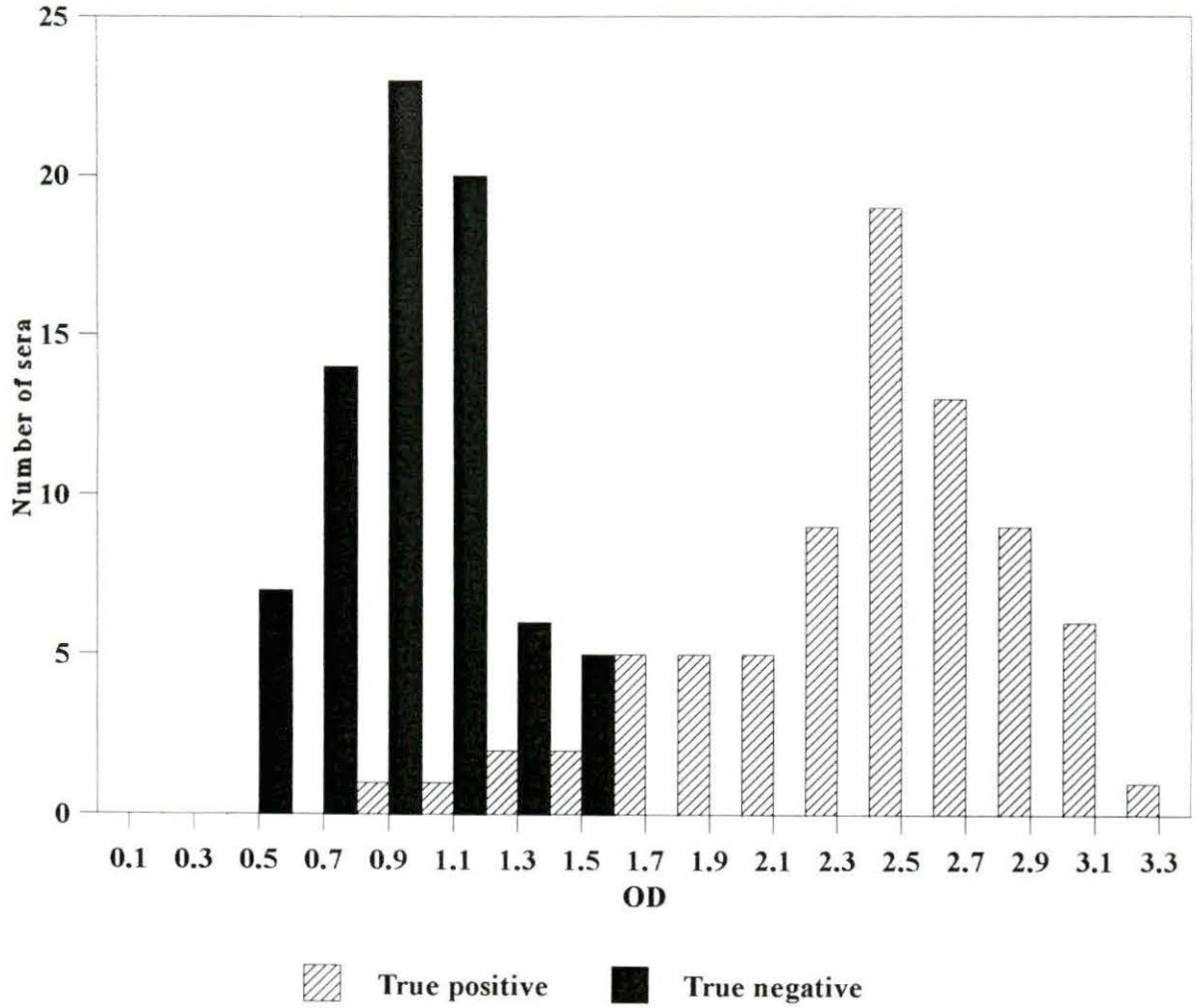

Figure 20. ELISA reactions observed at a 1:160 dilution of the positive and negative serum panels using the reference ELISA with Triton X-100 extracted antigen (lot 1).

Table 1. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:160 and a minimum positive $OD_{450-650}$ value of ≥1.3.

	True Positive	True Negative	
Test Positive	74	11	85
Test Negative	4	64	68
	78	75	

Calculations:

Sensitivity = 95%
Specificity = 85%
Positive predictive value = 87%
Negative predictive value =94%
Accuracy = 90%

Table 2. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:160 and a minimum positive $OD_{450-650}$ value of ≥1.5.

	True Positive	True Negative	
Test Positive	72	5	77
Test Negative	6	70	76
	78	75	

Calculations:

Sensitivity = 92%
Specificity = 93%
Positive predictive value = 94%
Negative predictive value =92%
Accuracy = 93%

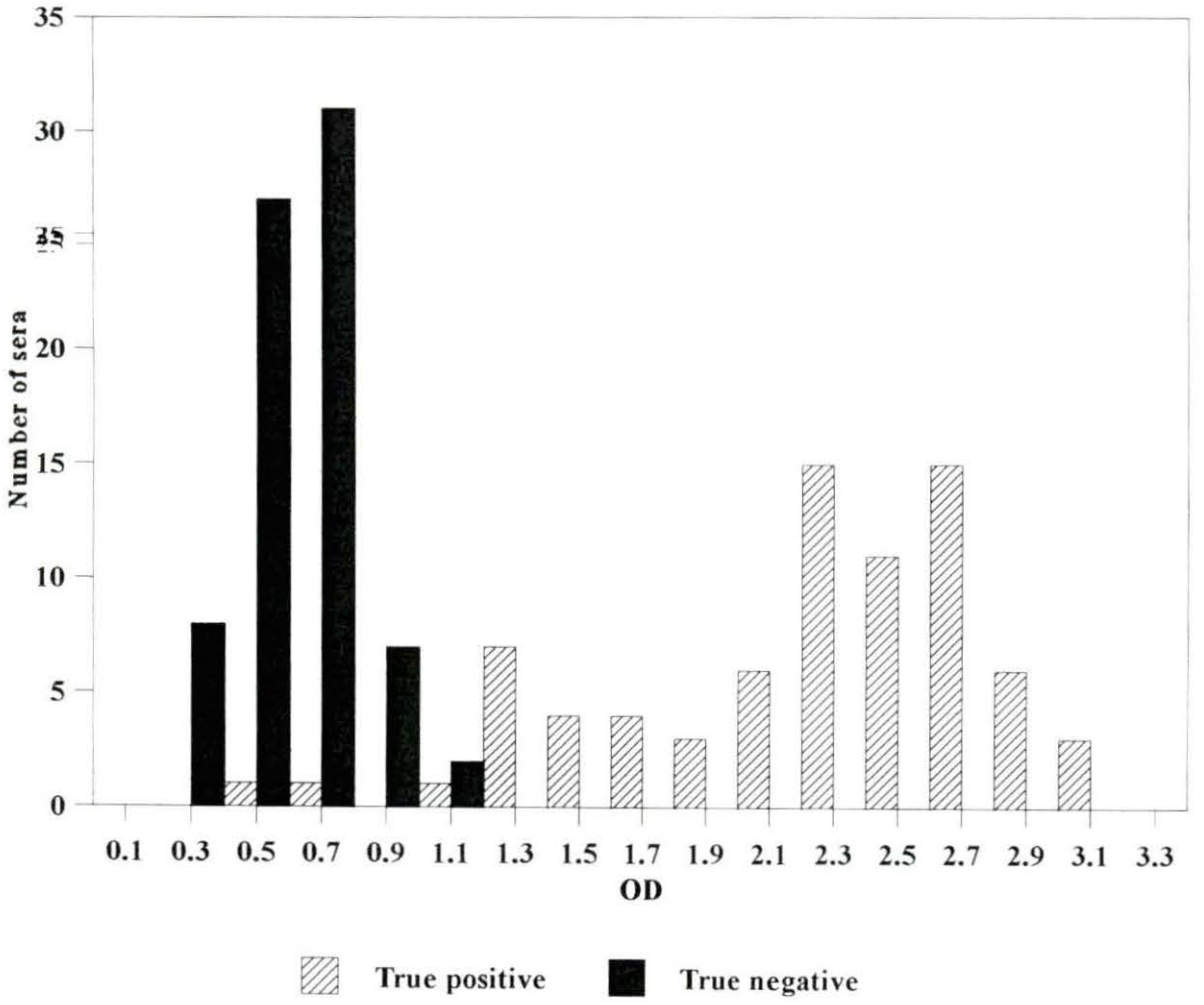

Figure 21. ELISA reactions observed at a 1:320 dilution of the positive and negative serum panels using the reference ELISA with Triton X-100 extracted antigen (lot 1).

Table 3. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:320 and a minimum positive $OD_{450-650}$ value of ≥ 1.1.

	True Positive	True Negative	
Test Positive	75	2	77
Test Negative	3	73	76
	78	75	

Calculations:

Sensitivity = 96%
Specificity = 97%
Positive predictive value = 97%
Negative predictive value =96%
Accuracy = 97%

Table 4. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:320 and a minimum positive $OD_{450-650}$ value of ≥0.9.

	True Positive	True Negative	
Test Positive	76	9	85
Test Negative	2	66	68
	78	75	

Calculations:

Sensitivity = 97%
Specificity = 88%
Positive predictive value = 89%
Negative predictive value =97%
Accuracy = 93%

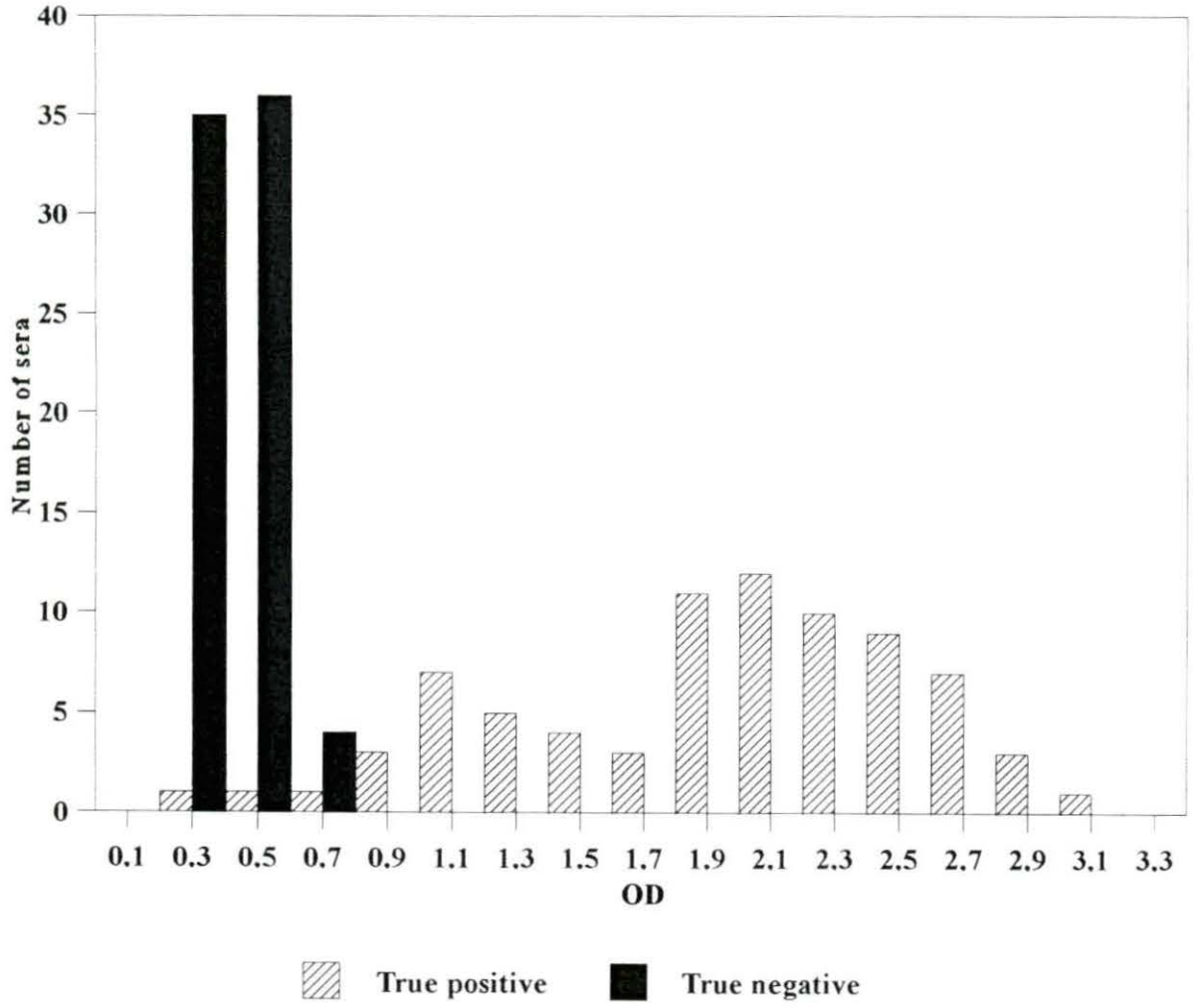

Figure 22. ELISA reactions observed at a 1:640 dilution of the positive and negative serum panels using the reference ELISA with Triton X-100 extracted antigen (lot 1).

96%, and the accuracy was 95% (Table 5). At the same serum dilution, if an $OD_{450-650}$ of ≥0.9 was interpreted as a positive result, the sensitivity was 92%, the specificity was 100%, the positive predictive value was 100%, the negative predictive value was 93%, and the accuracy was 96% (Table 6).

Selection of $OD_{450-650}$ cut-off values for the optimization of sensitivity and specificity were also determined using computerized Receiver-Operator Characteristic (ROC) plots. The true-positive rate (sensitivity) was plotted on the y-axis against false-positive rates (1-specificity) on the x-axis at a several serum dilutions of 1:160 (Figure 23) and 1:320 (Figure 24). The parametric estimate is shown by the solid ROC curve. At a serum dilution of 1:160 and an $OD_{450-650}$ of 1.290 the estimated sensitivity was 99% and estimated specificity was 90%, while an $OD_{450-650}$ of 1.445 the estimated sensitivity was 98% and the estimated specificity was 97%, . (Figure 23). Using a serum dilution of 1:320 and an $OD_{450-650}$ of 0.850 the estimated sensitivity was 97% and the estimated specificity was 91%, while at an $OD_{450-650}$ of 1.000 the estimated sensitivity was 95% and estimated specificity was 98%. (Figure 24).

ELISA results for 3 hemoculture positive and 2-ME-TAT negative sera

Three serum samples from hemoculture positive dogs which were 2-ME-TAT negative were also evaluated by ELISA (Figure 25). At serum dilutions a 1:160, 1:320, and 1:640, serum samples 2 and 3 would be considered positive using both cut-off values chosen at each serum dilution.

Table 5. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:640 and a minimum positive $OD_{450-650}$ value of ≥0.7.

	True Positive	True Negative	
Test Positive	75	4	79
Test Negative	3	71	74
	78	75	

Calculations:

Sensitivity = 96%
Specificity = 96%
Positive predictive value = 95%
Negative predictive value =96%
Accuracy = 95%

Table 6. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:640 and a minimum positive $OD_{450-650}$ value of ≥0.9.

	True Positive	True Negative	
Test Positive	72	0	72
Test Negative	6	75	81
	78	75	

Calculations:

Sensitivity = 92%
Specificity = 100%
Positive predictive value = 100%
Negative predictive value =93%
Accuracy = 96%

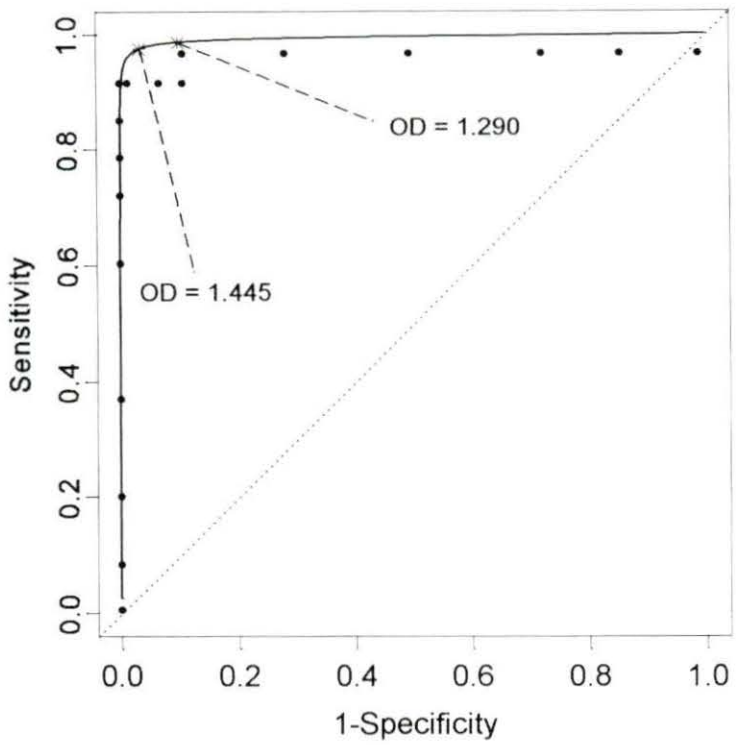

Figure 23 . Random-Operating Characteristics (ROC) analysis of sensitivity and specificity at a serum dilution of 1:160.

The true positive rate (sensitivity) was plotted on the y-axis against the false positive rates (1-specificity) at a serum dilution of 1:160. The empirical sensitivity and specificity at selected $OD_{450-650}$ cutoffs are shown by points. The parametric estimate is shown by the solid ROC curve. At $OD_{450-650}$ of 1.290, the estimated sensitivity is 99% and the estimated specificity is 90%. At an $OD_{450-650}$ of 1.445, the estimated sensitivity was 98% and the estimated specificity was 97%.

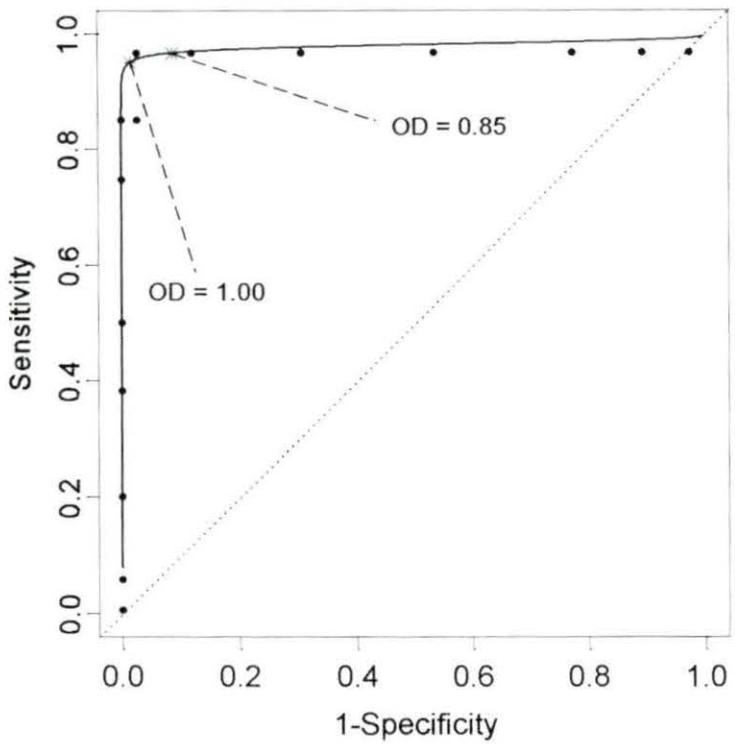

Figure 24 . Random-Operating Characteristics (ROC) analysis of sensitivity and specificity at a serum dilution of 1:320.

The true positive rate (sensitivity) was plotted on the y-axis against the false positive rates (1-specificity) at a serum dilution of 1:320. The empirical sensitivity and specificity at selected $OD_{450-650}$ cutoffs are shown by points. The parametric estimate is shown by the solid ROC curve. At $OD_{450-650}$ of 0.850, the estimated sensitivity is 97% and the estimated specificity is 91%. At an $OD_{450-650}$ of 1.000, the estimated sensitivity was 95% and the estimated specificity was 98%.

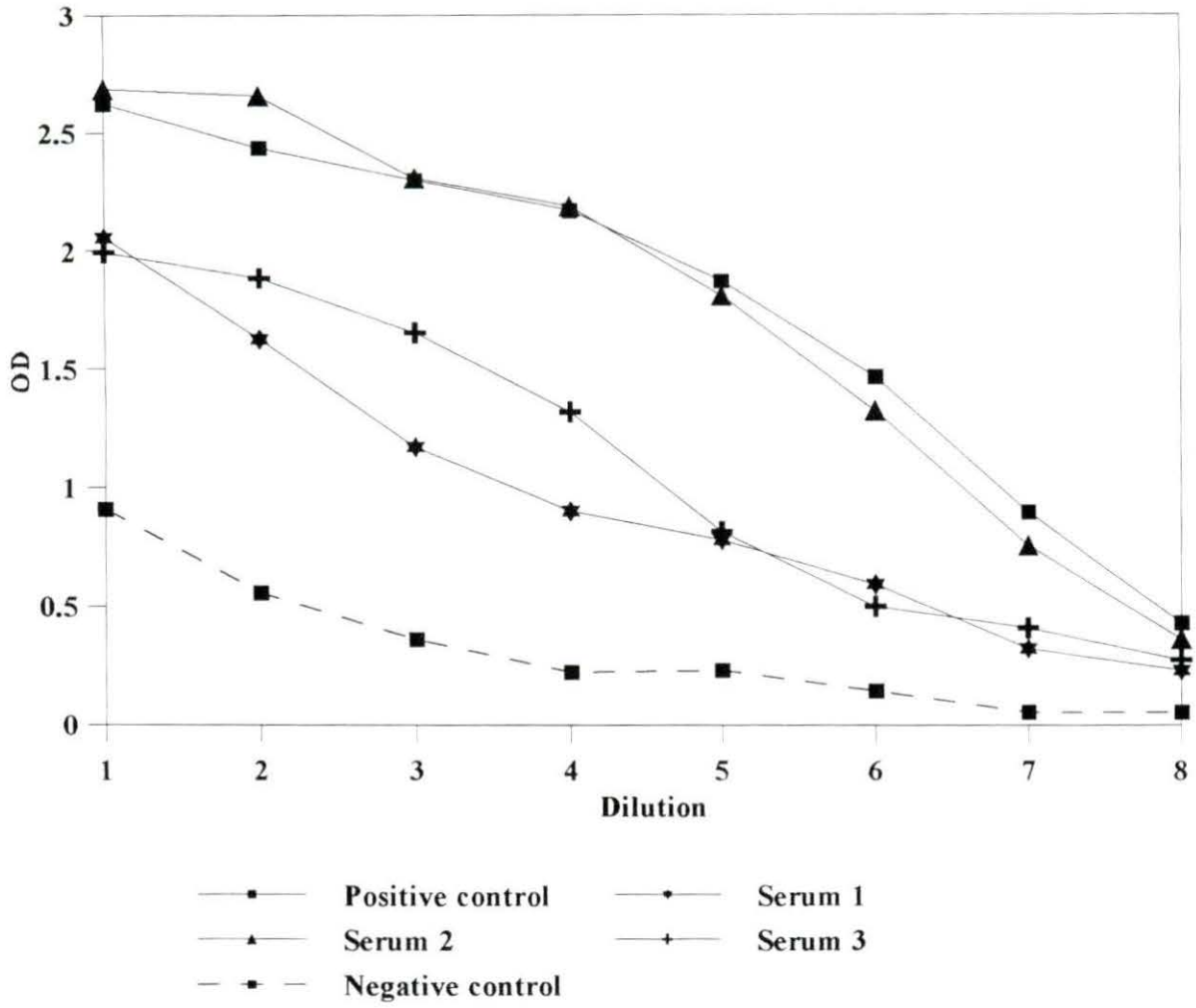

Figure 25. ELISA reactions observed for 3 hemoculture positive and 2-ME-TAT negative sera.

Antigen was 1:200 in antigen coating buffer. Plates were blocked with 5% NFDM. A negative serum pool from 10 dogs with no known exposure to *B. canis* (dashed lines) and a positive serum pool from 10 dogs with known exposure to *B. canis* (solid lines) were added to microtitration wells and diluted 2-fold from 1:50 to 1:6400 in SDB5. JIRL conjugate was diluted 1:1000 and TMB was utilized as a substrate. Reactions were chemically terminated and the $OD_{450-650}$ values were determined.

Characterization of the Triton X-100 extracted and 2-ME-TAT antigens

Western blot analysis of *B. canis* Triton X-100 extracted antigen demonstrated far fewer bands recognized by serum from dogs exposed to *B. canis* compared to whole cell 2-ME-TAT antigen (Figure 26). The apparent molecular weights (molecular mass) of the primary bands recognized in the Triton X-100 extracted antigen are 20 and 31 kDa. Numerous bands over a wide range of molecular weights (7.1 to >208 kDa) were recognized. When serum from dogs with no known exposure to *B. canis* was used as primary antibody, no bands were recognized in either extract, demonstrating the specificity of the western blot (Figure 27).

To determine the nature of the epitopes recognized in the western blot analysis, the Triton X-100 extracted antigen was treated with 0.2% proteinase K at 37°C for 2 hours. Treated and untreated samples of Triton X-100 extracted antigen were subjected to SDS/PAGE and Western blot analyses using positive or negative serum pools as the primary antibody. These results demonstrate a predominant protein component of the epitopes recognized by the positive serum pool.

Field Evaluation

In order to access the accuracy of the assay under field conditions, results of samples from the kennel previously described were compared by culture, 2-ME-TAT, and ELISA. Since the kennel was known to harbor dogs infected with *B. canis*, the most sensitive evaluation criteria were selected to decrease the number of false negative results. A minimum positive $OD_{450-650}$ value of ≥ 0.9 at a serum dilution of 1:320 was interpreted as a positive result (Table 4). Sixty-four samples were culture, 2-ME-TAT, and ELISA positive. Two samples were culture and 2-ME-TAT positive but ELISA negative; an additional 2 samples

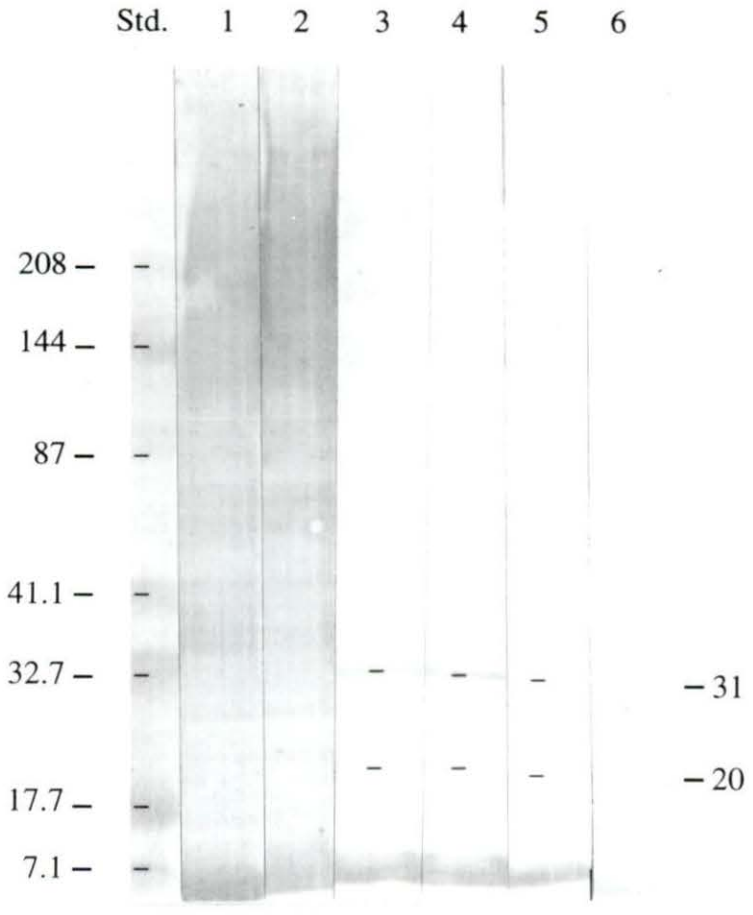

Figure 26. Western blot demonstration of epitope recognition and the proteinacious nature of the epitope by serum from dogs with known exposure to *B canis* (primary antibody).

Lane 1 was *B. canis* cells, lane 2 was *B. canis* 2-ME-TAT antigen, lanes 3-5 were Triton X-100 extracted *B. canis* antigen lots 1, 2, and 3, respectively, and lane 6 was proteinase K treated Triton X-100 extracted *B. canis* antigen lot 1.

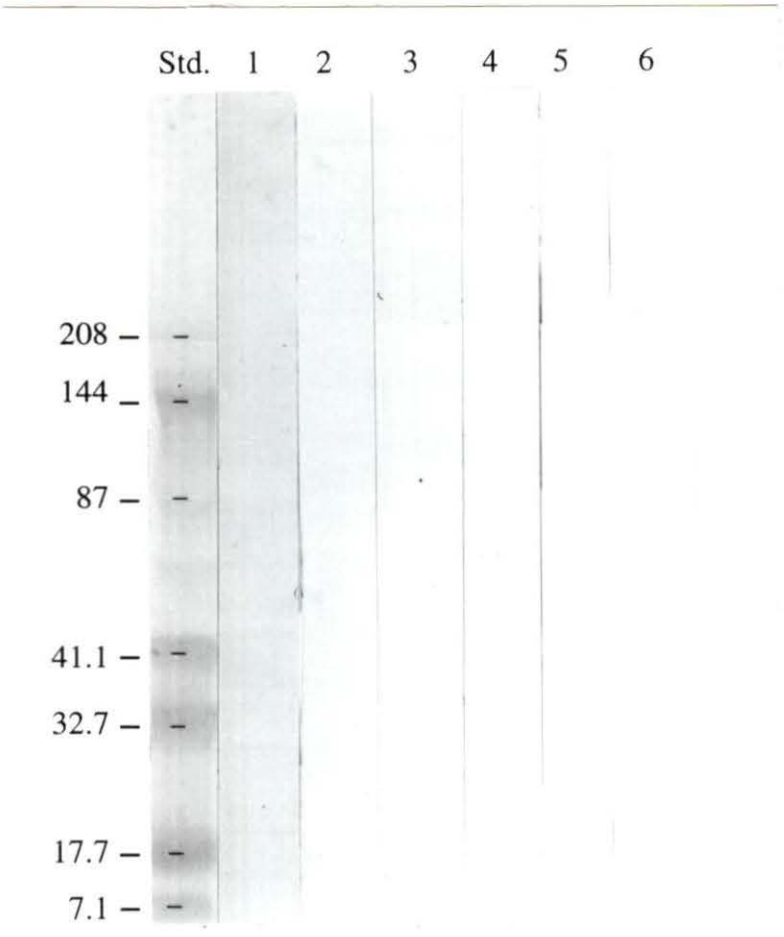

Figure 27. Western blot demonstration of epitope recognition and the proteinacious nature of the epitope by serum from dogs with no known exposure to *B canis* (primary antibody).

Lane 1 was *B. canis* cells, lane 2 was *B. canis* 2-ME-TAT antigen, lanes 3-5 were Triton X-100 extracted *B. canis* antigen lots 1, 2, and 3, respectively, lane 6 was proteinase K treated Triton X-100 extracted *B. canis* antigen lot 1.

were culture and ELISA positive but 2-ME-TAT negative. Twenty-eight samples were positive on both serologic tests but were culture negative. Six samples were positive only with the 2-ME-TAT, while 26 were positive only with the ELISA. A total of 175 samples were culture, 2-ME-TAT, and ELISA negative (Table 7). Using these criteria, if hemoculture positive results were considered true positives and hemoculture negative results were considered true negatives, the sensitivity of the ELISA was 96%, the specificity of the ELISA was 77%, the positive predictive value of the ELISA was 55%, the negative predictive value of the ELISA was 98%, and the accuracy of the ELISA was 81% (Table 8). However, if the criteria for true positives were changed to include hemoculture and/or 2-ME-TAT positive results as true positives, the sensitivity of the ELISA was 91%, the specificity of the ELISA was 87%, the positive predictive value of the ELISA was 78%, the negative predictive value of the ELISA was 97%, and the accuracy of the ELISA was 88% (Table 9).

Based on the sensitivity of the 2-ME-TAT, testing of 304 samples would be expected to result in 10 -20 false positives. This would be likely to reflect the most accurate infective status of the dogs from which the samples were hemoculture and ELISA negative but 2-ME-TAT positive. If the six samples that were positive only with the 2-ME-TAT were considered false positive results on the 2-ME-TAT and the 6 samples were considered negative, the sensitivity of the ELISA was 97 %,the specificity of the ELISA was 87%, the positive predictive value of the ELISA was 78%, the negative predictive value of the ELISA was 98%, and the accuracy of the ELISA was 90% (Table 10).

Table 7. Comparison of culture, 2-ME-TAT, and ELISA results from 304 dogs in a kennel with known exposure to *B. canis*. Sensitivity and specificity of the ELISA were calculated at a serum dilution of 1:320 and a minimum positive $OD_{450-650}$ value of ≥0.9.

Culture	2-ME-TAT	ELISA	Number of dogs
+	+	+	64
+	+	-	2
+	-	+	2
+	-	-	1
-	+	+	28
-	+	-	6
-	-	+	26
-	-	-	175

Table 8. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:320 and a minimum positive $OD_{450-650}$ value of ≥0.9 when hemoculture positive samples were considered true positives.

	True Positive	True Negative	
ELISAt Positive	66	54	120
ELISA Negative	3	181	184
	69	235	

Calculations:

Sensitivity = 96%
Specificity = 77%
Positive predictive value = 55%
Negative predictive value =98%
Accuracy = 81%

Table 9. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:320 and a minimum positive $OD_{450-650}$ value of ≥0.9 when hemoculture and/or 2-ME-TAT positive results were considered true positives.

	True Positive	True Negative	
ELISA Positive	94	26	120
ELISA Negative	9	175	181
	103	201	

Calculations:

Sensitivity = 91%
Specificity = 87%
Positive predictive value = 78%
Negative predictive value =97%
Accuracy = 88%

Table 10. Sensitivity, specificity, predictive values, and accuracy of the ELISA at a serum dilution of 1:320 and a minimum positive $OD_{450-650}$ value of ≥0.9 when hemoculture and/or 2-ME-TAT positive results were considered true positives. The 6 samples that were positive only with the 2-ME-TAT were considered to be false positives on the 2-ME-TAT, and were therefore considered as true negatives.

	True Positive	True Negative	
Test Positive	94	26	120
Test Negative	3	181	184
	97	207	

Calculations:

Sensitivity = 97%
Specificity = 87%
Positive predictive value = 78%
Negative predictive value =98%
Accuracy = 90%

DISCUSSION

An ELISA was developed for the detection of antibodies specific for *B. canis* in canine serum samples. In the optimized procedure, the Triton X-100 extracted *B. canis* antigen is diluted 1:200 in coating buffer and 100 μl are adsorbed to each well of an Immulon II 96-well microtitration plate. Plates are incubated for 60 ± 15 minutes at 37±2°C and then stored at 4±2°C overnight or longer. Plates are washed 3 times in PBST, blocked in 5% NFDM, and incubated at 37±2°C for 60 ± 15 minutes. After washing in PBST 3 times, positive and negative control sera and test serum samples are added and two-fold dilutions in PBST are made by serial transfer. The plates are sealed, incubated at 37±2°C for 30 ±5 minutes, and washed 3 times in PBST. Goat origin anti-canine horse radish peroxidase labeled IgG diluted 1:1000 is adsorbed to detect bound immunoglobulin, and plates are sealed and incubated at 37±2°C for 30 ± 5 minutes. Plates are then washed 3 times in PBST and the TMB substrate is added. Plates are rotated until color develops and the reactions are chemically terminated with 2.5 M H_2SO_4 and the $OD_{450-650}$ values are determined. The OD_{450} is graphed against the serum dilution. Validity requirements for positive and negative serum samples are dependant upon assay applications, as are criteria for categorization of unknown samples.

Based on results of the various studies, a number of recommendations and parameters for the Triton X-100 extracted *B. canis* ELISA have been determined. Unacceptable levels of background were observed when BSA was utilized in the assay in serum dilution buffers and blockers. Bovine serum albumin, therefore, should not be used in the assay. Differences in nonspecific reactivity of conjugates were observed and may be due to species origin, lot to lot variation, or production methods. Therefore, individual lots of conjugate must be evaluated

prior to use to determine the optimum dilution of conjugate and ensure lack of nonspecific reactivity. Further information gathered during additional field evaluations may aid in determination of the acceptability of different species origin conjugates in the assay.

Antigen storage studies demonstrated the Triton X-100 extracted *B. canis* antigen held at 25±4°C or stored at -20±2° and subjected to repeated freezing and thawing degraded rapidly. However, initial studies indicated the antigen is stable for 1 year at 4±2°C without the use of preservative. Stability will continue to be monitored to validate end of dating requirements; until real time studies are completed, antigen lots should not be used more than one year after production.

The assay has been shown to be a suitable as a replacement for, or alternative to, the currently available serologic assays which involve subjective interpretation and have suboptimal sensitivity and specificity. Using various serum dilutions and minimum OD_{450} values for interpretation of positive test results, sensitivity, specificity, and accuracy can be as high as 99%, 100%, and 97% respectively. This demonstrates that the assay is applicable for routine diagnostic use,providing increased sensitivity, specificity, and accuracy as well as reducing the subjectivity of the results and providing titer determination capabilities. For many applications, the ELISA provides improved testing at a lower cost. Because of the significant economic impact of *B. canis* infection, especially on kennels, and because of the zoonotic nature of the disease, improved testing could result in decreased costs and decreased human exposure to a zoonotic disease.

The sensitivity and specificity of the semi-quantitative ELISA can be adapted by selection of minimum positive $OD_{450-650}$ values based on the dynamics of the test population.

For example, in a kennel with known exposure to *B. canis*, maximum sensitivity is desired to ensure elimination of all infected animals. For such use, a low minimum $OD_{450-650}$ value will increase sensitivity but will decrease specificity. However, when the status of individual animals is of primary importance, especially in situations in which valuable breeder dogs have unknown exposure histories, it is desirable to increase the minimum $OD_{450-650}$ necessary for interpretation as positive to ensure maximum accuracy of the assay.

Sequential titers may also aid in the determination of the infective status of individual dogs. If serum from an animal of unknown status has a low or questionable titer, an early response to infection may be present. Failure to increase titers over the next two to three weeks might be due to an immunologic response to a cross reactive agent or due to previously cleared infection, since titers would be expected to increase over time during an active infective state. Further characterization of the serologic response of experimentally infected dogs is expected to provide additional data for evaluation of sequential titers.

Western blot analyses of the Triton X-100 extracted *B. canis* antigen demonstrated predominant immunologically recognized components of the antigen with apparent molecular weights of 20 and 31 kDa. Research with other *Brucella* species has demonstrated immunologically reactive outer membrane proteins with the similar apparent molecular weights.[112,113,114,115,116] These may represent conserved proteins important to the development of protective immune responses in infected host animals. Further testing would be required to determine the relationship of the immunogenic proteins within the genus *Brucella*. The proteinacious nature of the antigen was demonstrated by treatment of the antigen with proteinase K which destroyed the epitopes recognized by immune sera (from dogs with

known exposure to *B. canis*).

The field evaluation demonstrated the effective use of the ELISA in a diagnostic setting. Since *B. canis* had been recently isolated from dogs in the kennel, the evaluation criteria were selected to optimize the sensitivity of the assay and thereby decrease the number of false negative results. Discrepancies in hemoculture and serologic results were oberved for 65 of the 304 samples. Of the 69 hemoculture positive samples, a total of 3 were culture positive but were negative with the 2-ME-TAT while 1 sample was negative with the ELISA. According to unpublished data from our laboratory, the ELISA has been shown to detect a serologic response as early as 10 days post exposure. It is possible that the 2 ELISA and culture positive samples that were 2-ME-TAT negative, were samples from dogs recently infected with *B. canis*. Evaluation of the ELISA is difficult because of the lack of serum samples available that are known true positives. Exposure in an infected kennel is not known to result in 100% prevalence of infection. The low sensitivity and specificity of the 2-ME-TAT make it difficult to determine the infection status of dogs when serologic results of the 2-ME-TAT do not agree with those of the ELISA. For example, Table 10 is likely to be representative of the true field evaluation. Testing of 304 samples by the 2-ME-TAT would be expected to result in 10-20 false positives. Therefore, considering the 6 samples that were culture negative, ELISA negative, and 2-ME-TAT positive as false positives (true negatives) is expected to more accurately reflect the actual infective status of those dogs. Future work with experimentally infected dogs will clarify discrepancies between hemoculture and serologic results.

As with any serologic assay, it is also important to determine at what point in the

infective process an immunologic response can be detected. Eight SPF dogs have been experimentally infected with a field isolate of *B. canis* and the serologic response is being monitored over a period of several months using the 2-ME-TAT and the ELISA for characterization of the serologic response. Preliminary results demonstate the ability of the ELISA to detect a serologic response 2 to 4 weeks earlier than the 2-ME-TAT. This suggests the use of the ELSIA in routine diagnostic testing would be a significant improvement over the use of the 2-ME-TAT as well as a substantial savings of resour ces.

Future Studies

The efficiency of the assay will be determined in field studies by laboratories which will be provided with the assay protocol, ELISA antigen, and blind serum panels of known positive and known negative sera. Upon satisfactory completion of the field studies, the *B. canis* ELISA will be proposed as a replacement test for, or alternative to, the 2-ME-TAT to the Diagnostic and Interpretive Serology Committee of the American Association of Veterinary Laboratory Diagnosticians. At that time, determination of appropriate minimum OD_{450} for positive results will be addressed using data from experimentally infected animals as well as data from other laboratories.

Improved serologic assays are also needed for other members of the genus *Brucella*. The elimination of brucellosis caused by *B. abortus* or *B. suis* is the focus of USDA Eradication Programs. These programs are nearing completion and new assays with increased specificity are especially useful in decreasing the number of false positives seen when monitoring populations with a low prevalence of disease. Because these agents share many of the same phenotypic characteristics which impact the assay design, it is likely that similar

ELISAs may provide improved diagnostic capabilities. Assays similar to the *B. canis* ELISA are currently being evaluated using extractions from *B. abortus*, *B. suis*, and non-program *Brucella* species (*B. melitensis* and *B. ovis*) and are expected to contribute to improved diagnostic testing for those agents.

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