

# Detection of Antibodies to *Anaplasma marginale* by an Improved Enzyme-Linked Immunosorbent Assay with Sodium Dodecyl Sulfate-Disrupted Antigen

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**Sensitivities of two enzyme-linked immunosorbent assays (ELISAs) with particulate and sodium dodecyl sulfate (SDS)-disrupted *Anaplasma marginale* antigen were compared. The quotient of positive reference sera divided by the absorbance quotient of a negative reference serum at identical dilution was termed the signal-to-noise ratio. Optimal signal-to-noise ratios were dependent on both pretreatment of antigen and antigen concentration. SDS disruption of anaplasma antigen resulted in a markedly improved signal-to-noise ratio of ELISA compared with ELISA with untreated antigen at identical antigen and serum dilutions. This represented higher sensitivity and lower background absorbance of the ELISA with disrupted antigen. SDS-disrupted *A. marginale* antigen was standardized by protein determination, and antigen, as well as precoated microtiter wells, was stored frozen without apparent loss of antigenic properties. ELISA results were in agreement with results of positive and negative control sera tested by the complement fixation test or by light microscopy *Anaplasma* diagnosis in Giemsa-stained blood films.**

Different intraerythrocytic rickettsia of the species *Anaplasma* are the cause of hemotropic disease in wild and domestic ruminants (6). *Anaplasma marginale* is the principal pathogenic agent in cattle. Worldwide distribution and significance in cattle production areas have been reported (8, 15). The disease is transmitted by a variety of biting flies and numerous ixodid tick species (5, 8, 14). Transmissibility by ixodid ticks may be isolate restricted (17). Transplacental and iatrogenic transmission have been reported (13, 19). Recently, attempts were made to develop an isolate-common surface protein subunit vaccine for cattle which appears promising (11).

Common diagnostic techniques for *A. marginale* include light microscopy of Giemsa-stained blood smears, card agglutination, and the complement fixation test (CFT). The indirect fluorescent-antibody test (microfluorometry) (3, 10), passive hemagglutination, capillary tube agglutination, the latex agglutination test (9), and the enzyme-linked immunosorbent assay (ELISA) (1, 12, 18) are other diagnostic assays. The ELISA is a simple, rapid, and sensitive assay that is appropriate for screening greater numbers of serum samples (18).

The present study was done to develop an ELISA with improved sensitivity and standardized solid face coating for *Anaplasma* serology. Optimal antigen pretreatment and antigen dilution for solid face coating were determined on the basis of absorbance of the final substrate reaction product. Absorbance of positive over negative reference sera at similar conjugate dilution was used as a descriptor of signal-to-noise ratio for comparison of optimal antigen concentration and immunosorbent characteristics.

## MATERIALS AND METHODS

**Antigen.** *A. marginale* CFT antigen prepared from infected bovine erythrocytes (National Veterinary Services

Laboratories, Ames, Iowa) was centrifuged at  $10,000 \times g$  for 10 min, and the sedimented cellular particles were suspended in 0.1 M NaHCO<sub>3</sub> buffer (pH 9.6) (packed cell volume, 5%). Aliquots of antigen suspension were either used directly, for solid face coating, or pretreated with 0.1% sodium dodecyl sulfate (SDS) in buffer for 20 min on a shaker (at room temperature). NaHCO<sub>3</sub> buffer without SDS was added to a final concentration of 0.005% SDS (7) (antigen dilution, 1:20; protein concentration, 140 µg/ml), and the antigen was stored at -70°C until use. Antigen dilutions (starting at 1:100; protein concentration, 28 µg/ml) were made with NaHCO<sub>3</sub> buffer with or without 0.005% SDS, on the basis of SDS content of the antigen solution.

**Antisera.** CFT-tested positive and negative reference sera to *A. marginale* and sera to other microbial agents were from National Veterinary Services Laboratories, Ames, Iowa. Sera from healthy cattle were from the serum collection at the Department of Veterinary Medicine, University of Zurich, Zurich, Switzerland. Sera were diluted with buffer A (0.05 M Tris hydrochloride [pH 7.4], 0.15 M NaCl, 1 mM EDTA, 0.1% bovine serum albumin, 0.05% Tween 20).

**Conjugate.** Horseradish peroxidase (HRPO)-conjugated anti-cow immunoglobulins from rabbits were from Dakopatts, Glostrup, Denmark). The working dilution was 1:1,000 with buffer A.

**Substrate.** Citric acid (0.15 M, pH 4) with 2.5 mM 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) (Boehringer GmbH, Mannheim, Federal Republic of Germany) and 0.008% H<sub>2</sub>O<sub>2</sub> was prepared freshly (16).

**ELISA protocol.** Microtiter wells (Immulon Removastrips; Dynatech Laboratories, Inc., Alexandria, Va.) were each incubated with 100 µl of antigen solution in NaHCO<sub>3</sub> buffer with and without 0.005% SDS at 37°C for 3 h (7). Wells provided for storage were precoated with SDS-disrupted antigen, sealed in plastic bags, and placed in a freezer at -20°C. Wells were washed three times with the aid of a squirt bottle for 3 min each time with 0.15 M NaCl solution

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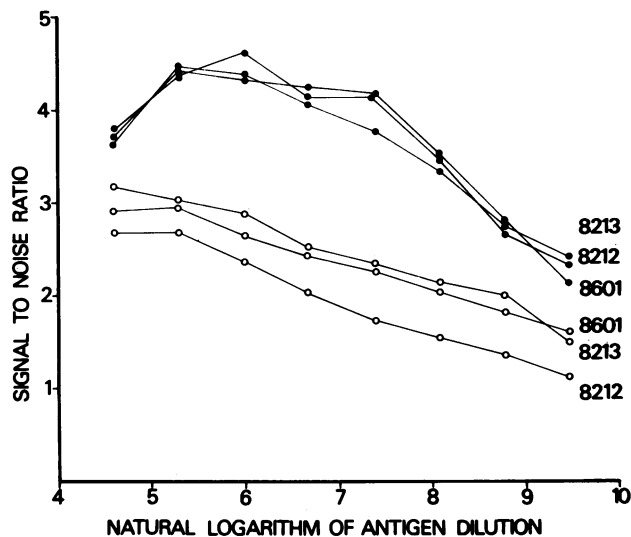


FIG. 1. Dependence of optimal signal-to-noise ratio on antigen pretreatment and dilution. The signal-to-noise ratio was obtained by dividing absorbance quotients (405 nm/570 nm) of three different positive reference sera (8212, 8213, and 8601 at a 1:100 dilution) by the absorbance quotient of negative control serum (8301). Symbols: ○, Cellular *A. marginale* antigen at different dilutions with NaHCO<sub>3</sub> buffer; ●, *A. marginale* antigen pretreated with SDS and diluted with NaHCO<sub>3</sub> buffer containing 0.005% SDS. Tests were run in triplicate.

containing 0.05% Tween 20. Diluted antiserum (100  $\mu$ l) was added, and wells were incubated at 37°C for 1 h. After subsequent washing of wells, HRPO-conjugated anti-cow immunoglobulins (100  $\mu$ l) were added, and wells were incubated for 1 h at 37°C. Wells were washed again, and 125  $\mu$ l of substrate solution was added. Reaction was allowed to proceed at room temperature for 15 min. Absorbance of the final product was measured in a model MR 580 ELISA reader (Dynatech) at 405 and 570 nm and expressed as the quotient 405 nm/570 nm. Each test was done in triplicate, and means were calculated with a connected IBM-XT personal computer by applying Immunosoft software (Dynatech). Each microtiter plate contained the following control wells: positive and negative reference sera, wells without coated antigen which were exposed to positive reference serum and conjugate to test unspecific adherence of immunoglobulins to solid face, wells not exposed to antiserum to test unspecific adsorption of conjugate to antigen, and wells not exposed to conjugate to measure background absorbance of substrate solution. Empty microtiter wells were used as blanks for the ELISA reader.

The absorbance quotient (405 nm/570 nm) of positive reference sera divided by the absorbance quotient of a negative reference serum at identical dilution was termed signal-to-noise ratio.

Signal-to-noise ratio of absorption coefficients of positive reference serum (8213) to negative reference serum (8301) (at 1:100 dilution) was set arbitrarily at 100%. Signal-to-noise ratios of test sera that were 35% or greater (compared with this value) were considered positive.

## RESULTS

Optimal *Anaplasma* antigen dilution for immunosorbent coating was dependent on pretreatment and concentration of antigen (Fig. 1). Disruption of cellular *Anaplasma* particles

with SDS resulted in an overall increased signal-to-noise ratio compared with untreated antigen at similar antigen and antiserum dilution. Measurements of pretreated antigen showed an optimal range between 1:200 and 1:2,000 dilutions. Antigen dilution of 1:2,000 with NaHCO<sub>3</sub> buffer (containing 0.005% SDS; protein concentration, 1.4  $\mu$ g/ml) was chosen for further experiments. Signal-to-noise ratios in the ELISA with untreated antigen decreased steadily from an initial antigen dilution of 1:100 up to the final investigated dilution of 1:12,800. Characteristically, triplicate absorbance measurements were in the range of the mean  $\pm$  10% (or less). The ELISA with different negative control sera and untreated anaplasma antigen had a higher background absorbance compared with the ELISA and SDS-pretreated antigen.

Time-dependent development of absorbance of the reaction product by enzymatic substrate conversion was analyzed by using SDS-pretreated antigen. Measurements shown in Fig. 2 illustrate linear dependence of absorbance with time (between 5 and 50 min) after addition of substrate to wells. Absorbance of positive and negative reference sera over a range of different dilutions showed similar dependence but different slopes. Absorbance coefficients of negative reference serum at a particular dilution were smaller than those of positive reference serum. Consequently, with positive reference serum, more antibodies were specifically bound to antigen and greater amounts of conjugate were attached than with negative reference serum.

The optimal antiserum dilution for screening of serum samples was tested with standardized ELISA parameters as determined in preceding experiments (1:2,000 dilution of SDS-pretreated antigen, negative reference serum at dilutions corresponding to positive reference serum, 1:1,000 dilution of conjugate, and 15-min period between substrate addition and absorbance measurement). The signal-to-noise ratio was optimal in the range between 1:25 and 1:100 serum dilutions (Fig. 3). A decline of the signal-to-noise ratio with

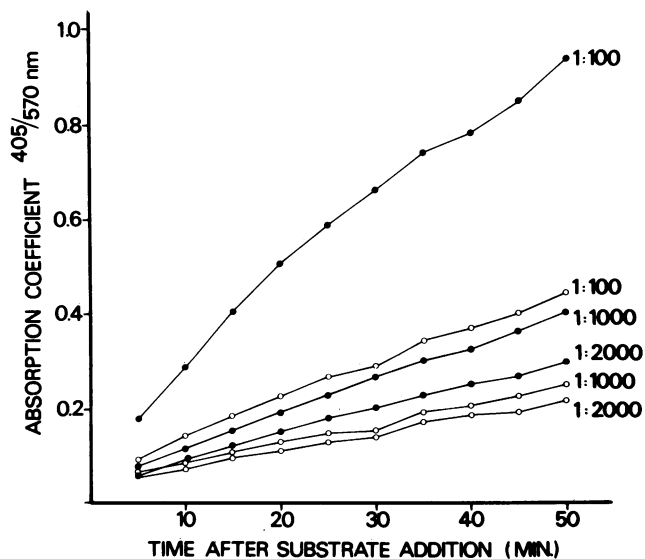


FIG. 2. Linear dependence of enzymatic substrate conversion over time. Symbols: ○, triplicate measurements with a negative reference serum (8301); ●, triplicate measurements with positive reference serum (8213) to *A. marginale*. Serum dilutions were 1:100, 1:1,000, and 1:2,000. SDS-pretreated antigen was diluted 1:2,000 with NaHCO<sub>3</sub> buffer containing 0.005% SDS.

increased serum dilutions was observed. Positive reference serum 8213 had optimal absorbance characteristics and was chosen at 1:100 dilution as a positive control in the following screening experiments.

A total of 66 serum samples were screened by ELISA at 1:100 dilution with buffer A; the results are presented in Table 1. CFT-positive antisera and sera from cattle with clinically diagnosed anaplasmosis (confirmed on Giemsa-stained blood films) had ELISA titers above the significance level: 50 to 190% of the signal-to-noise ratio of the positive control (8213). False-negative results were not observed. Sera of 12 healthy cattle showed no evidence of positive ELISA titers (signal-to-noise ratio of less than 18% of the positive control). A panel of 46 reference sera to microbial agents of various cattle diseases was screened by ELISA; positive titers to *A. marginale* were detected in 7 sera (15%).

Results were similar after retesting of sera with use of the same or other lots of SDS-pretreated antigen. Freezing of SDS-pretreated antigen solution at -70°C for up to 3 months, as well as the use of precoated, frozen wells, had no detectable effect on ELISA results. Absorbance of control wells without antigen, antiserum, or conjugate was at consistently low background levels.

DISCUSSION

An ELISA procedure with SDS-disrupted *A. marginale* antigen-coated wells had markedly improved features compared with a similar procedure with particulate antigen-coated microtiter wells. Signal-to-noise ratios were greater and represented higher sensitivity of the test at lower unspecific (background) absorbance. Consequently, use of disrupted antigen yielded greater numbers of coated microtiter wells compared with coating with a similar amount of cellular antigen. Accessibility of additional antigenic sites after SDS disruption of particulate antigen and partial denaturation of proteins used for solid face coating may be possible explanations for these results. In contrast to cellular

TABLE 1. ELISA on reference sera for detection of antibodies to *A. marginale*

Infection or immunization agent	ELISA serology		Specificity (%)
	No. positive	No. negative	
<i>A. marginale</i> <sup>a</sup>	8	0	100
Negative control sera <sup>b</sup>	0	12	100
Bovine viral diarrhea	0	1	100
Blue tongue virus	0	1	100
<i>Babesia bigemina</i>	1	4	80
<i>Babesia bovis</i>	0	2	100
<i>Mycobacterium bovis</i>	1	1	50
<i>Mycobacterium paratuberculosis</i>	0	2	100
<i>Campylobacter fetus</i>	2	8	80
<i>Leptospira interrogans</i> serovar hardjo	0	3	100
<i>Brucella abortus</i>	3	17	85

<sup>a</sup> Reference sera with positive CFT titers and sera from cattle with light microscopy-confirmed anaplasmosis diagnosis on blood smears.

<sup>b</sup> Sera from healthy cattle.

*Anaplasma* particles, disrupted antigen was readily standardized by protein determination and was stored frozen either as a stock solution or with precoated microtiter wells, without apparent loss of antigenic characteristics. Cellular *Anaplasma* antigen must be stored at 4°C. SDS pretreatment of particulate antigens (viruses, bacteria) (7) is a simple, efficient alternative to disruption by ultrasonication (1, 4). At a final concentration of 0.005%, SDS did not interfere with coating properties of microtiter wells in this ELISA.

Linearity of development of reaction product absorbance up to 50 min after substrate addition indicated that consistent results were obtained when a reaction termination step was omitted.

General reliability of the test was confirmed by 100% agreement of ELISA results with CFT-tested positive and negative sera. Sera from cattle that had anaplasmosis (as confirmed on Giemsa-stained blood films) reacted positively in the ELISA, but reactions of sera from healthy cattle were always negative. Simultaneous reaction of a small percentage of reference sera to various microbial agents with *Anaplasma* antigen was attributed to one or more of the following reasons. A coincident infection with *A. marginale* or previous vaccination of animals with *A. marginale* could explain a simultaneous antibody titer to multiple microbial agents, particularly since ELISA reaction was observed only in sera that originated in the United States. A reaction of normal bovine sera with common erythrocytic antigens has been described (9, 10), and erythrocyte stromatal impurities often contaminate cellular *Anaplasma* antigen preparations (2). Finally, *A. marginale* shares common antigenic sites with other hemotropic rickettsia (14); similarly, antisera to other bacterial agents could cross-react with *A. marginale* antigens. Binding of antibodies to common epitopes may be enhanced by SDS disruption of particulate antigen and subsequent exposure of internal antigenic sites.

Properties of the *A. marginale* ELISA with particulate antigen were investigated earlier (18). Advantages of ELISA over CFT were pointed out. The ELISA is sensitive, rapid, and reliable. Because it is less complex than CFT and can be fully automated, it is suitable for the screening of large numbers of serum samples. Lack of specificity was attributed to anti-species conjugates (18). Subsequently, improved results were obtained with protein A-HRPO conjugates.

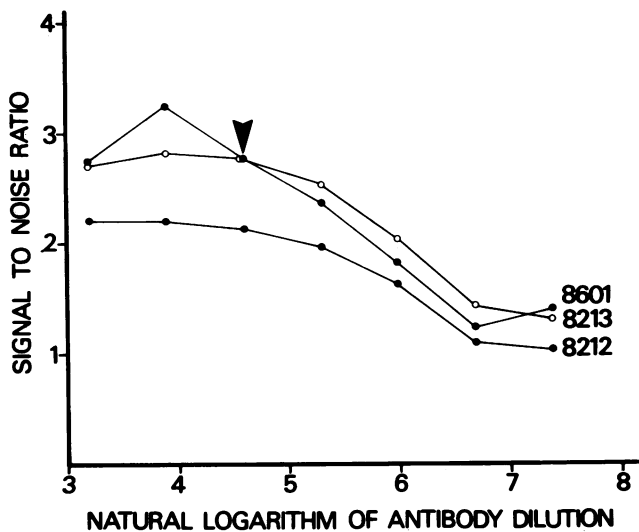


FIG. 3. Determination of optimal serum dilution for detection of antibodies to *A. marginale*. Triplicate measurements were obtained with three different positive reference sera to *A. marginale*. ○, Positive reference serum (8213) used for screening of serum samples in Table 1; ●, remaining positive sera. Arrow indicates optimal dilution point at 1:100. Dilution of SDS-pretreated antigen was 1:2,000 with NaHCO<sub>3</sub> buffer containing 0.005% SDS.

Results were limited to detection of specific antibodies of the immunoglobulin G subclass 2 (IgG2) class, owing to preferential binding of protein A to bovine IgG2 (M. R. Hall, C. O. Thoen, and B. Blackburn, Proc. 3rd Int. Symp. World Assoc. Vet. Lab. Diag. 1983, p. 673–684). The present study provides evidence that, in addition to suitable anti-species conjugate, properties of the coated antigen are essential for ELISA specificity. This is in agreement with results of a recent report that provided laboratory and field evidence for accuracy and specificity of an *A. marginale* ELISA procedure with comparable experimental conditions (1).

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