Development and application of enzyme-linked immunosorbent assay for detecting Brucella antigen in vaginal discharge of cows

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GENERAL INTRODUCTION

Infectious diseases have traditionally been diagnosed by the cultivation of the infecting agent in an in vitro system or in laboratory animals. However, there are a number of limitations to this approach (106,110). For example, several viral agents that cause disease cannot be cultivated in generally available tissue culture or animal systems (57,81,110). In some cases, the infectious agents require a long period of time for cultivation and identification and results are not useful in the control of disease outbreaks (82). Similarly, the isolation of the causal organisms from infected animals for the diagnosis of brucellosis are not always successful due to various factors (6,12,15,18,31,48,49). Therefore, serologic tests are often used in the routine diagnosis of brucellosis (1,2,3,4,13, 21,22,26,36,40,41,46,58,61,62,73,74,76,78). These include serum agglutination test (SAT), complement fixation test (CFT), rivanol test, and other related tests. Diagnosis of brucellosis in cattle by skin test has not been successful since the test appears to lack specificity and sensitivity (44).

Brucella abortus, the causative agent of contagious abortion in cattle, also is infective to other animals and humans (59,66,107). The organisms usually gain entrance

orally and are ingested by polymorphonuclear cells, multiply within them, and are conveyed via the lymph nodes (16). In pregnant cows, the infection localizes in the placenta of the gravid uterus (67,71). Erythritol, a carbohydrate substance produced by the uterus, stimulates the growth of virulent strains of Brucella abortus. Erythritol occurs naturally in great concentration in the placenta and foetal fluids and is associated with the localization of the infection (68,88,89). When invasion of the gravid uterus occurs, the initial lesion is in the wall of the uterus; however, these organisms soon spread to the lumen of the uterus, causing severe ulcerative endometritis of the intercotyledonary spaces (97). The allantochorin, foetal fluid, and placental cotyledons are invaded by bacteria, the villi destroyed thereby causing abortion (88,97). Brucella usually localizes in the udder and adjacent lymph nodes of non-pregnant cows (24,56).

Brucella shed in the genital discharge at the time of calving or abortion are considered the most significant method of transmission (53). Although Brucella are shed in the milk and other secretions, these are not considered of major importance in the transmission of the disease in cattle (14,64,66). Many investigators have studied the genital shedding of Brucella abortus in pregnant and

nonpregnant cattle (8,16,24,25,34,35,56,70,71,86). In 1897, Bang first reported that a large number of organisms were shed in vaginal discharges and placenta at the time of abortion (8). Most early investigations reported on the occurrence of Brucella abortus in the reproductive tract at necropsy, rather than on the excretion of organisms by way of the vagina (24,86). Cotton reported that at necropsy, the maximum length of time Brucella abortus was found in the uterus was 52 days after abortion (24). It is generally accepted that the excretion of Brucella abortus in the vaginal discharge of cattle after normal or abnormal parturition is continuous for two weeks; shedding of Brucella abortus then diminishes in the next four-to-five weeks, and is intermittent for up to two years in 5-10% of the cows (16,35,56).

In pregnant cows before parturition, and in non-pregnant cows, the occurrence of Brucella abortus in the vaginal discharge was reported as slight and discontinuous; only a few reports have been made on the isolation of B. abortus from vaginal discharge of cows before parturition (34,56,71). The appearance of Brucella abortus in the vagina appears to be directly related to the freeing of the cervical plug, which allows the release of uterine contents (71).

Direct bacteriological isolation of Brucella may be made

from unpasteurized dairy products and from specimens such as blood, lymph nodes, placenta, aborted fetuses, and vaginal mucus of infected cows (1,59,62,96). However, these procedures are laborious, time-consuming and expensive; therefore, there has been a great deal of interest in the development of assays capable of detecting Brucella directly in clinical specimens.

Enzyme-linked immunosorbent assays (ELISA) have been developed and recognized as potentially useful procedures in the serodiagnosis of certain diseases (9,20,84,100,103, 104). The ELISA has also been used for the detection of hormones and toxins (60,83,84,102). Since ELISA is a simple, rapid, highly sensitive and reproducible test, it has been widely used for the detection of antibodies or antigens in certain infections (23,28,37,40,55,57,60,69,101,108, 110). Moreover, application of automated systems provide for the testing of a large number of samples simultaneously (94). The sensitivity of the ELISA is similar to radioimmunoassay, but radioactive materials are not required (69,84,110). Therefore, the objectives of this research were to develop and apply an ELISA system that had sufficient sensitivity to detect very low numbers of Brucella. This study consists of two separate, but related parts which were conducted in series:

1. Development of an enzyme-linked immunosorbent assay (ELISA) for detecting small numbers of *Brucella abortus* Strain 19: Assay parameters.

2. Application of the enzyme-linked immunosorbent assay (ELISA) for detection of *Brucella* antigens in vaginal discharge of cows.

SECTION I: DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTING *BRUCELLA ABORTUS* STRAIN 19: ASSAY PARAMETERS

SUMMARY

An enzyme-linked immunosorbent assay (ELISA) was developed for detecting heat-killed cells of *Brucella abortus* Strain 19. The concentration of the cell suspension was adjusted to McFarland Standard Tube No. 1 (OD = 0.337, measured at 480 nm), and serial dilutions were made from 1:512 to 1:65536. Carbodiimide (hydrogen cyanamide; cyanamide) was used as the antigen coupling agent to increase the covalent bonding, and ammonium chloride (NH_4Cl) was used as a blocking agent to neutralize residual active charges present on the cuvette surface. Block titrations revealed that a 1:640 dilution of guinea pig antibrucella serum incubated for 60 minutes, and a 1:600 dilution of goat antiguinea pig IgG conjugate incubated for 30 minutes provided optimal conditions for conducting the ELISA. A comparison of the results of ELISA with colony counts indicated that 250 colony forming units/well ($5x10^3$ CFU/ml) could be detected.

INTRODUCTION

Bovine brucellosis causes significant economic losses for livestock producers (66,86). It is also of public health concern because the disease can be transmitted from animals to man (66,107). Although routine serological tests are available for use in the detection of infected animals, the isolation of *Brucella* by bacteriologic techniques is often used to establish a definitive diagnosis of disease (1,59,62). Several problems are encountered in conducting bacteriologic examinations. These include: 1) the overgrowth of culture media by contaminating organisms, 2) the number of organisms present may be too low to be detected, 3) the interference of isolation by in vivo antimicrobial components (i.e., immunoglobulins, macrophages) in the tissues and body fluids (18), and 4) difficulty of maintaining viability while transporting specimens to the bacteriology laboratory. Therefore, there is a need for a rapid and reliable method to detect the presence of *Brucella*.

Enzyme-linked immunosorbent assay (ELISA) was first described by Engvall and Perlmann in 1971. Since the original description of this assay, there have been numerous reports describing the use of ELISA to detect either antigens or antibodies (14,17,20,23,28,30,37,39,52,55,57,60,

69,79,80,81,82,83,84,90,91,92,93,94,95,100,101,102,103, 104,108,109,110). Various reagents and systems have been developed in different laboratories to obtain optimal conditions for specific research purposes. It has been suggested that the ELISA procedure is superior to routinely used serologic methods (69,84). The sensitivity and specificity of ELISA have been demonstrated by application of ELISA for characterizing antigenic determinants of Brucella (90) and for quantitating various classes and subclasses of antibrucella immunoglobulins in cow milk (92).

A previous report indicated that different methods of coating antigens extracted from mycobacteria onto microcuvettes may influence the ELISA results (37); therefore, the first objective of this study was to compare four different methods of coating Brucella antigens onto polystyrene microcuvettes. Preliminary studies indicated that ELISA results varied when different concentrations of antiserum and/or conjugate were used; therefore, the second objective of this study was to evaluate the influence of antiserum concentration and/or conjugate dilution. Other parameters such as serum incubation time, conjugate incubation time and enzyme-substrate reaction time may also affect ELISA reactions; therefore, the third objective of this study was to compare and determine the satisfactory

incubation time intervals for serum and antigen, antispecies antibody and for enzyme and substrate for use for ELISA. The fourth objective of this investigation was to compare the results of ELISA and viable colony counts on culture to obtain information on the sensitivity of the ELISA in detecting Brucella.

MATERIALS AND METHODS

Antigen Preparation

Brucella abortus Strain 19 vaccine was reconstituted with diluent (National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, Iowa). Two hundred μl of the diluent was used to inoculate 5 ml quantities of tryptose broth, which were incubated at 37°C for 72 hours. A 2 ml amount of the 72-hour broth culture was used to inoculate Roux Flasks containing tryptose agar (130 ml), which were incubated at 37°C for 4 days. The cells were harvested by washing the surface with sterile phosphate-buffered saline. The cell suspension was autoclaved at 121°C for 15 minutes, and then washed two times with phosphate-buffered saline. The killed cell suspension density was adjusted to a McFarland Standard Tube No. 1. The optical density (OD=0.337) was measured at 480 nm on a Beckman Spectrophotometer Model No. 25. Serial two-fold dilutions of antigen (1:512 to 1:65,536) were made for ELISA using either 0.1 M Na_2CO_3 (pH 9.6) or 0.1 M phosphate-buffered saline (pH 7.2).

Methods of Antigen Coating

Four methods of coating antigens to polystyrene microcuvettes were evaluated. In the first method, the antigen was diluted in 0.1 M phosphate-buffered saline (pH 7.4) (PBS); 50 µl of each antigen dilution was added to separate wells of a Gilford microcuvette[1] and air-dried at 37°C for 16 hours. Each well was washed once with a wash solution containing 0.05 M NaCl and 0.5% Tween 80[2] adjusted to pH 7.5 with 1 N NaOH prior to the addition of antiserum.

In the second method, the antigen was diluted in 0.1 M Na_2CO_3 (pH 9.6); 50 µl of each antigen dilution was added to separate wells of a Gilford microcuvette and incubated for 16 hours at 4°C. The plates were washed 3 times with PBS (pH 7.4) and 3 times with wash solution prior to the addition of antiserum.

In the third method, the antigen was diluted in 0.1 M Na_2CO_3 (pH 9.6); 50 µl of each antigen dilution was added to separate wells of a Gilford microcuvette. Then 50 µl of carbodiimide[3] (hydrogen cyanamide) solution (1 mg/ml) in 0.1 M Na_2CO_3 (pH 9.6) was added. The cuvettes were incubated for 16 hours at 4°C and subsequently washed 3 times with

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[1] Gilford Instruments, Oberlin, OH.

[2] Fisher Scientific Company, Fairlawn, NJ.

[3] Sigma Chemical Co., St. Louis, Mo., Lot No. C2308.

PBS (pH 7.4) and 3 times with wash solution before the addition of antiserum.

In the fourth method, the antigen was diluted in 0.1 M Na_2CO_3 (pH 9.6); 50 μl of each antigen dilution was added to separate wells of a Gilford microcuvette. Then 50 μl of carbodiimide (1 mg/ml) in Na_2CO_3 (pH 9.6) was added. The microcuvettes were incubated for 16 hours at 4°C and subsequently washed three times with PBS; one hundred μl of 0.1 M NH_4Cl solution was then added to each well and incubated for 30 minutes at 22°C. The plates were washed 3 times with wash solution prior to the addition of antiserum.

Production of Antibrucella Serum

Guinea pig antibrucella serum was obtained from National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, Iowa. The procedure used was as follows: thirty guinea pigs were divided into 3 groups of 10 guinea pigs each. Group I was inoculated subcutaneously with 1.5×10^1 Colony Forming Units (CFU) *Brucella abortus* Strain 2308; group II was inoculated with 1.5×10^2 CFU *Brucella abortus* Strain 2308; group III was inoculated with 1.5×10^3 CFU *Brucella abortus* Strain 2308. Sera were collected from 2 guinea pigs in each group 7 weeks after challenge and at 1 week intervals thereafter for 4

[1]Sigma Chemical Company, St. Louis, MO.

weeks. Serum samples were tested and those with titers equal or greater than 1:800 were pooled. Positive and negative control sera were diluted to 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560 in 0.5 M NaCl containing 1% Tween 80 and 1% bovine serum albumin (BSA)[1] adjusted to pH 7.5 with 1 N NaOH for titration to determine a suitable dilution of antibrucella serum for use in ELISA.

Conjugate

An affinity purified goat antiguinea pig IgG(H+L) labeled with horseradish peroxidase by modification of the method of Nakane and Kawaoi (63) was also obtained commercially[2]. The conjugates were diluted to 1:300, 1:600, and 1:1200 in 0.5 M NaCl containing 1% Tween 80 and 1% bovine serum albumin (BSA) adjusted to pH 7.5 with 1.0 N NaOH to determine optimal conjugate concentration.

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[1]Sigma Chemical Company, St. Louis, MO.

[2]Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD.

Substrate

A working solution of substrate was prepared using a 3% hydrogen peroxide and 2,2'-azino-di-(3-ethylbenzthiozaline-6-sulfonate) (ABTS) in 0.05 M citric acid pH 4.0.[1]

ELISA Test Protocol

The ELISA was conducted by modification of procedures described previously (91,95). The methods of antigen binding to the polystyrene cuvettes have been described. Fifty µl of each dilution of antiserum (1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560) using 0.5 M NaCl buffered to pH 7.4 containing 1% Tween 80 and 1% bovine albumin was added to each cuvette and incubated for 15, 30, 60, or for 120 minutes at 22°C on a horizontal shaker[2], respectively. Following incubation, the microcuvettes were washed eight times with 0.5 M NaCl containing 0.5% Tween 80 adjusted to pH 7.5 with 1.0 N NaOH. Fifty µl of each conjugate dilution (1:300, 1:600, or 1:1200, was added to separate wells of the microcuvettes and incubated for 15 minutes, 30 minutes, 60 minutes, or 120 minutes, respectively; the cuvettes were washed eight times with the wash solution

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[1]The procedure for preparing ABTS was obtained from Dr. M. L. Bartlett, Los Alamos Scientific Laboratory, Los Alamos, NM.

[2]Arthur H. Thomas Co., Philadelphia, PA.

to remove excess conjugate. A working solution of ABTS and substrate was added to each well (0.1 ml/well) and incubated for 10, 20, 30, 40, 50, or for 60 minutes at 22°C. A Gilford PR-50 Processor Reader[1] was used at a wavelength of 405 nm to determine the color intensity of the ELISA reactions.

Viable Bacterial Count

A live Brucella abortus Strain 19 suspension was adjusted to a density comparable to a McFarland Standard Tube No. 1; the optical density (0.337) was measured at 480 nm on a Beckman Spectrophotometer. Serial two-fold dilutions from 1:512 to 1:65536 were made using peptone saline consisting of 1% peptone and 0.5% sodium chloride in distilled water. This solution was effective in maintaining the viability of Brucella suspensions at low concentrations (1); 0.1 ml of each dilution was plated on each of two tryptose agar plates. A uniform dispersion of inoculum on the agar plates was obtained by spreading with a nichrome wire. Three plates were made for each dilution to ensure accuracy. The inoculated plates were inverted and incubated for 4 days at 37°C, at which time the colonies were counted.

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[1]Gilford Instruments, Oberlin, OH.

RESULTS

Antigen Coating

The results of ELISA comparing different methods for coating of Brucella abortus Strain 19 whole cell antigen onto polystyrene cuvettes using a serum dilution of 1:640 and conjugate dilution of 1:600 are shown in Figures 1 and 2. When guinea pig antibrucella serum was used, the results indicated that cuvettes containing antigen suspended in Na_2CO_3 buffer incubated for 16 hours produced increased absorbance values (19% or more) for various antigen concentrations when compared with cuvettes containing antigen suspended in PBS and air-dried (Figure 1). Antigen suspended in Na_2CO_3 buffer and incubated with carbodiimide (50 ng/well) for 16 hours at 4°C produced increased absorbance values (49% or more) for various antigen concentrations when compared with cuvettes containing antigen suspended in PBS and air dried (Figure 1). Cuvettes containing antigen suspended in Na_2CO_3 and carbodiimide and subsequently treated with NH_4Cl produced increased absorbance values (57% or more) for various antigen concentrations when compared with cuvettes containing antigen suspended in PBS and air-dried (Figure 1) (Appendix Table A1).

Only minimal ELISA reactions (less than 0.06 absorbance value) were observed in cuvettes without antigen to which

serum, conjugate and substrate were added (Table A1).

Comparison between four antigen coating methods indicated that lower nonspecific absorbance values were observed in cuvettes containing antigen suspended in Na_2CO_3 buffer and carbodiimide, and subsequently treated with NH_4Cl when guinea pig negative control serum was used (Table A1). Greater decreases in nonspecific ELISA reactions were observed at higher antigen concentrations (i.e., 1:512, 1:1024) than at lower antigen concentrations (i.e., 1:32768, 1:65536). ELISA reactions observed at 60 minutes enzyme-substrate reaction time were similar to reactions obtained at 30 minutes enzyme-substrate reaction time for each of the procedures used for coating antigens onto polystyrene cuvettes (Figure 2) (Table A2). Therefore, antigen suspended in Na_2CO_3 buffer and incubated with carbodiimide (50 ng/well) for 16 hours at 4°C, and subsequently treated with NH_4Cl for 30 minutes was used in further studies.

Titration of Guinea Pig Antibrucella Serum

Serum dilutions of 1:40 and 1:80 produced nonspecific ELISA reactions (absorbance value greater than 0.25) in guinea pig negative control serum using a 1:300, 1:600 and 1:1200 dilution of conjugate. However, at higher serum dilutions, the nonspecific ELISA reactions were reduced

markedly (absorbance value less than 0.16) (Table A3-A5). Therefore, to minimize nonspecific background reactions, serum dilution of 1:160 or higher were used in ELISA.

The titration results of guinea pig antibrucella serum using three different conjugate dilutions are shown in Figures 3-5. The results of ELISA using a 1:300 dilution of conjugate comparing seven dilutions of antibrucella serum are shown in Figure 3. At a 1:512 antigen dilution, consistent ELISA reactions were obtained using antibrucella serum dilutions of 1:160, 1:320, or 1:640. The percentage of ELISA absorbance value of these serum dilutions (based on a 100% value for a 1:40 serum dilution) was 75.4%, 75.3%, and 71.4%, respectively. However, the ELISA reactions decreased markedly at a serum dilution of 1:1280 (54.6%) and at a serum dilution of 1:2560 (55%). At a 1:4096 antigen dilution, the percentage of ELISA absorbance value for antiserum dilutions of 1:160, 1:320, and 1:640 was 79.4%, 74% and 70.8%, respectively (based on a 100% value for a 1:40 serum dilution); the ELISA reactions decreased markedly at a serum dilution of 1:1280 (55%) and at a serum dilution of 1:2560 (54.9%). At a 1:16384 antigen dilution, the percentage of ELISA absorbance value was 60.2% at a 1:160 dilution, 56.2% at a 1:320 dilution, 55.2% at a 1:640 dilution, 36.2% at a 1:1280 dilution, and 32.5% at a 1:2560 dilution, respectively. At a 1:65536 antigen dilution, the percentage of ELISA absorbance value for antiserum dilutions of 1:160,

1:320, 1:640, 1:1280, and 1:2560 was 56.4%, 52.1%, 51.4%, 34.8% and 31.8%, respectively. ELISA reactions decreased dramatically at a serum dilution of 1:1280 and 1:2560 for all antigen dilutions (Table A3).

The ELISA results using a 1:600 dilution of conjugate are shown in Figure 4. At a 1:512 antigen dilution, the percentage of ELISA absorbance value (based on a 100% value for 1:40 serum dilution) decreased to 75.3% at a 1:160 serum dilution, to 67.5% at a 1:320 serum dilution and 66.5% at a 1:640 serum dilution. However, a marked decrease was observed at a 1:1280 serum dilution (53%) (Table A4). Similar decreases were observed in ELISA results for other antigen dilutions using the same conjugate dilution (Figure 4) (Table A4).

The ELISA results using a 1:1200 dilution of conjugate, are shown in Figure 5. Results from two antigen dilutions (1:512 and 1:4096) produced similar curves. At a 1:512 antigen dilution, the percentage of ELISA absorbance value (based on a 100% value for a 1:40 serum dilution) was 60.2% at a 1:160 serum dilution, 48.3% at a 1:320 serum dilution, 44.8% at a 1:640 serum dilution, 27.8% at a 1:1280 serum dilution and 27.3% at a 1:2560 serum dilution. At a 1:4096 antigen dilution, the percentage of ELISA absorbance value was 65.1% at a 1:160 serum dilution, 60.2% at a 1:320 serum dilution, 56.9% at a 1:640 serum dilution, 44.9% at a 1:1280 serum dilution and 30.9% at a 1:2560 serum dilution, respectively.

ELISA reactions decreased markedly at serum dilution of 1:1280 and 1:2560 (Table A5). Similar decreases in ELISA reactions were also observed at other antigen dilutions (Table A5). These results indicated that a 1:640 serum dilution produced suitable ELISA reactions at each of the antigen and conjugate dilutions; therefore, it was used in further studies.

Titration of Goat Antiguinea Pig Conjugate

The results of ELISA comparing 3 different dilutions of goat antiguinea pig conjugate (1:300, 1:600, and 1:1200) are shown in Figure 6. At a 1:512 antigen dilution the absorbance value decreased slightly from 100% (based on the absorbance value obtained using a 1:300 conjugate dilution) to 84% using a 1:600 dilution of conjugate; however, the ELISA result decreased markedly to 21% using a 1:1200 conjugate dilution. At a 1:4096 antigen dilution, the absorbance value decreased from 100% (1:300 conjugate dilution) to 80% at a 1:600 conjugate dilution and to 26% at a 1:1200 conjugate dilution. At a 1:16384 antigen dilution, the absorbance value decreased from 100% (1:300 conjugate dilution) to 85% at a 1:600 conjugate dilution and to 41% at a 1:1200 conjugate dilution. At a 1:65536 antigen dilution, the absorbance value decreased from 100% to 92.7% at a 1:600 conjugate

dilution and to 43% at a 1:1200 conjugate dilution (Table A6). Significant decreases in ELISA reactions from 1:600 dilution of conjugate to 1:1200 dilution of conjugate were observed in all four antigen concentrations. Therefore, a 1:600 dilution of conjugate was selected for use in further studies.

Determination of Antigen-Antiserum Incubation Time

The results of ELISA, comparing various time intervals for antigen-antiserum incubation, are shown in Figures 7-9. The guinea pig antibrucella serum was used at a 1:640 dilution (as titrated previously). When a 1:600 dilution of goat antiguinea pig conjugate was incubated for 15 minutes, the results indicated that at a 1:512 antigen dilution, more than 95% of the reaction was completed following 60 minutes antigen-antiserum incubation (based on a 100% absorbance value obtained using a 120 minutes antigen-antiserum incubation time). At higher antigen dilutions, the ELISA reactions were reduced (Figure 7) (Table A7). Similar increases in absorbance value were observed with a 1:600 dilution of conjugate incubated for 30 minutes (Figure 8). At a 1:512 antigen dilution, following 60 minutes antigen-antiserum incubation, more than 90% of the reaction was completed (Table A8). When conjugate was

used at a 1:600 dilution for 60 minutes incubation, the results indicated that more than 88% of the reaction was completed following 60 minutes antigen-antiserum incubation time (Figure 9) (Table A9). Therefore, 60 minutes antigen-antiserum incubation time was used in conducting additional ELISA.

Determination of Conjugate Incubation Time

The results of ELISA, comparing various time intervals for conjugate incubation, are shown in Figure 10. At a 1:512 antigen dilution, greater than 94% of the ELISA reaction was completed after 30 minutes conjugate incubation time (based on a 100% absorbance value obtained using a 120 minutes conjugate incubation time). Similar reactions were observed for different antigen dilutions. Therefore, 30 minutes conjugate incubation time was used (Tables A7-A10).

Determination of Optimal Enzyme-Substrate Incubation Time

The development of ElISA reactions for different substrate incubation time intervals for four antigen concentrations using a 1:600 dilution of conjugate are shown in Figures 11-14. At a 1:512 antigen dilution, the ELISA reaction increased dramatically from 0.625 to 0.904 (45% increase) when the substrate incubation time was increased

from 10 minutes to 30 minutes. However, only a slight increase in ELISA reaction (less than 9%) was observed when the substrate incubation time was increased from 30 minutes to 60 minutes (Figure 11). At other antigen dilutions, similar increases in absorbance values were observed for different time intervals (Figures 12-14) (Tables A11-A12).

A graph showing the ratio of ELISA results for positive serum as compared to negative serum at different incubation time intervals for each of the four antigen dilutions was prepared (Figure 15) (Table A13).

$$\text{P/N ratio} = \frac{\text{ELISA result of guinea pig antibrucella serum (1:640)}}{\text{ELISA result of guinea pig negative control serum (1:640)}}$$

Maximum ratios were obtained at 30 minutes substrate incubation time. At a 1:512 antigen dilution, the difference observed was greater than at higher antigen dilutions (1:4096, 1:16384 or 1:65536). The results indicated that a 30 minute enzyme-substrate reaction time provided suitable ELISA results.

Viable Bacterial Count

The result (mean value of triplicate tests) of the viable counts for each of the dilutions of Brucella abortus Strain 19 are shown in Figure 16. Some variation was observed in the number of colonies obtained at dilutions of 1:512,

1:1024, 1:2048, 1:4096, 1:8192, 1:16384, 1:32768, and 1:65536 of a cell suspension comparable to a McFarland Standard Tube No. 1 (OD=0.337). The range at lower antigen dilutions was less than the range at higher antigen dilutions. The number of colonies (mean value) with corresponding range for each of the antigen dilutions were as follows: a 1:512 dilution contained

$57x10^4$ (55-58) colony-forming units/ml;
a 1:1024 dilution contained
$27x10^4$ (26-28) colony-forming units/ml;
a 1:2048 dilution contained
$17x10^4$ (15-18) colony-forming units/ml;
a 1:4096 dilution contained
$77x10^3$ (76-78) colony-forming units/ml;
a 1:8192 dilution contained
$36x10^3$ (34-39) colony-forming units/ml;
a 1:16384 dilution contained
$19x10^3$ (16-21) colony-forming units/ml;
a 1:32768 dilution contained
$10x10^3$ (7-11) colony-forming units/ml; and
a 1:65536 dilution contained
$5x10^3$ (4-7) colony-forming units/ml.

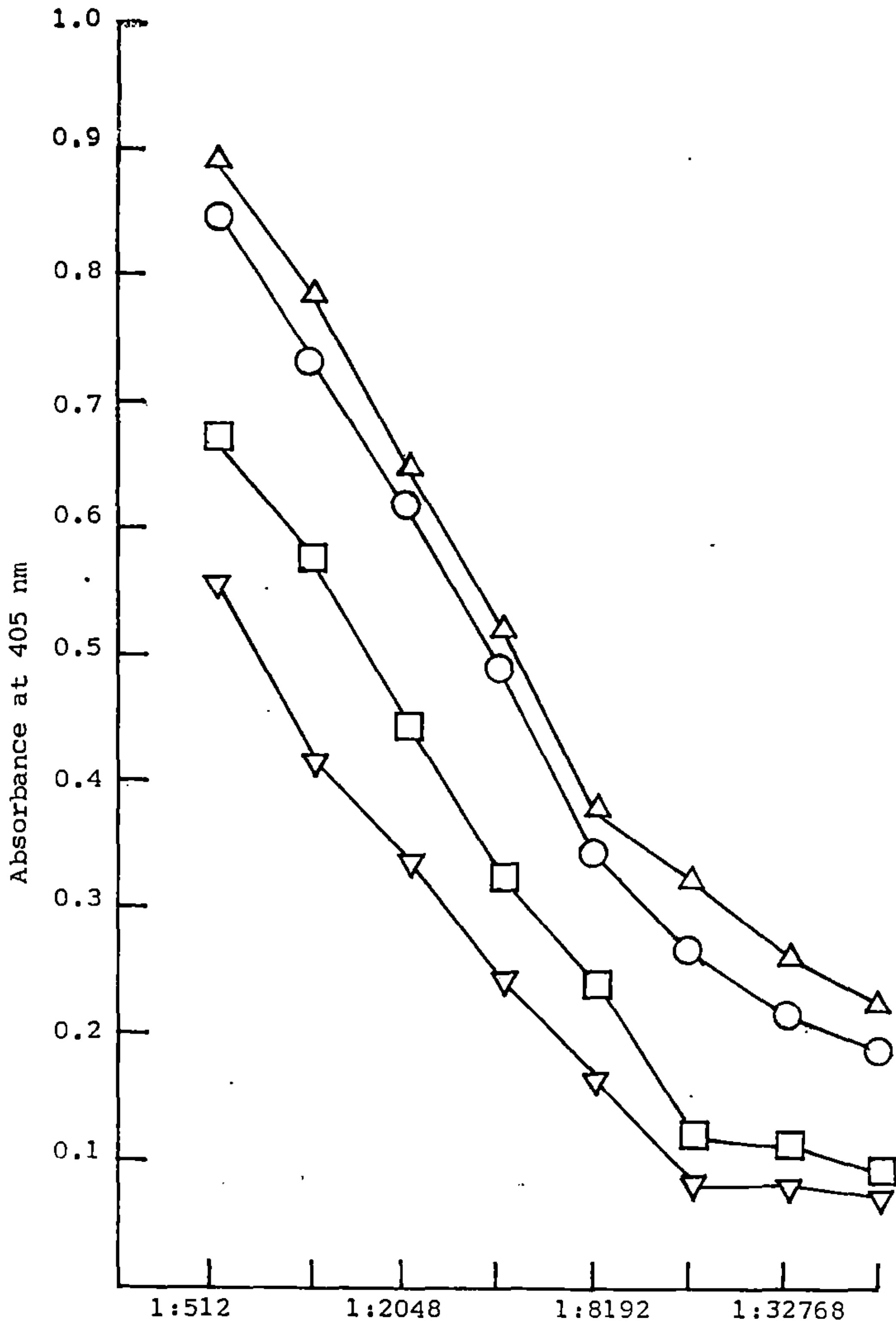
After the determination of optimal incubation time and appropriate concentration of antiserum, conjugate and of substrate, the ELISA results for different dilutions of Brucella abortus Strain 19 (antigen) were determined

(Figure 17).

A comparison of the ELISA results (absorbance value of 0.256), and viable count indicated that the ELISA developed had sufficient sensitivity to detect 250 colony-forming units of _Brucella_ cell per well (1:65536 dilution of McFarland Standard Tube No. 1). The ELISA absorbance values for wells containing no antigen was 0.051 or less (Table A1).

Figure 1. Results of ELISA, comparing different methods of coating of B. abortus Strain 19 whole cell antigen onto polystyrene cuvettes

(Cells (antigen) suspended in PBS (▽-▽), Na_2CO_3 (□-□), in Na_2CO_3 with carbodiimide (○-○) or suspended in Na_2CO_3 with carbodiimide and treated with NH_4Cl (△-△). Guinea pig anti-brucella serum used at a 1:640 dilution was incubated for 60 minutes; goat antiguinea pig conjugate labeled with horseradish peroxidase used at a 1:600 dilution was incubated for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)



Dilutions of Cell Suspension Comparable to McFarland Standard Tube No. 1

Figure 2. Results of ELISA, comparing different methods of coating of <u>B. abortus</u> Strain 19 whole cell antigen onto polystyrene cuvettes

(Cells (antigen) suspended in PBS (▽-▽), in Na_2CO_3 (□-□), in Na_2CO_3 with carbodiimide (○-○) or suspended in Na_2CO_2 with carbodiimide and treated with NH_4Cl (△-△). Guinea pig antibrucella serum used at a 1:640 dilution was incubated for 60 minutes; goat antiguinea pig conjugate labeled with horseradish peroxidase used at a 1:600 dilution was incubated for 30 minutes. The results were measured at 60 minutes enzyme-substrate reaction time)

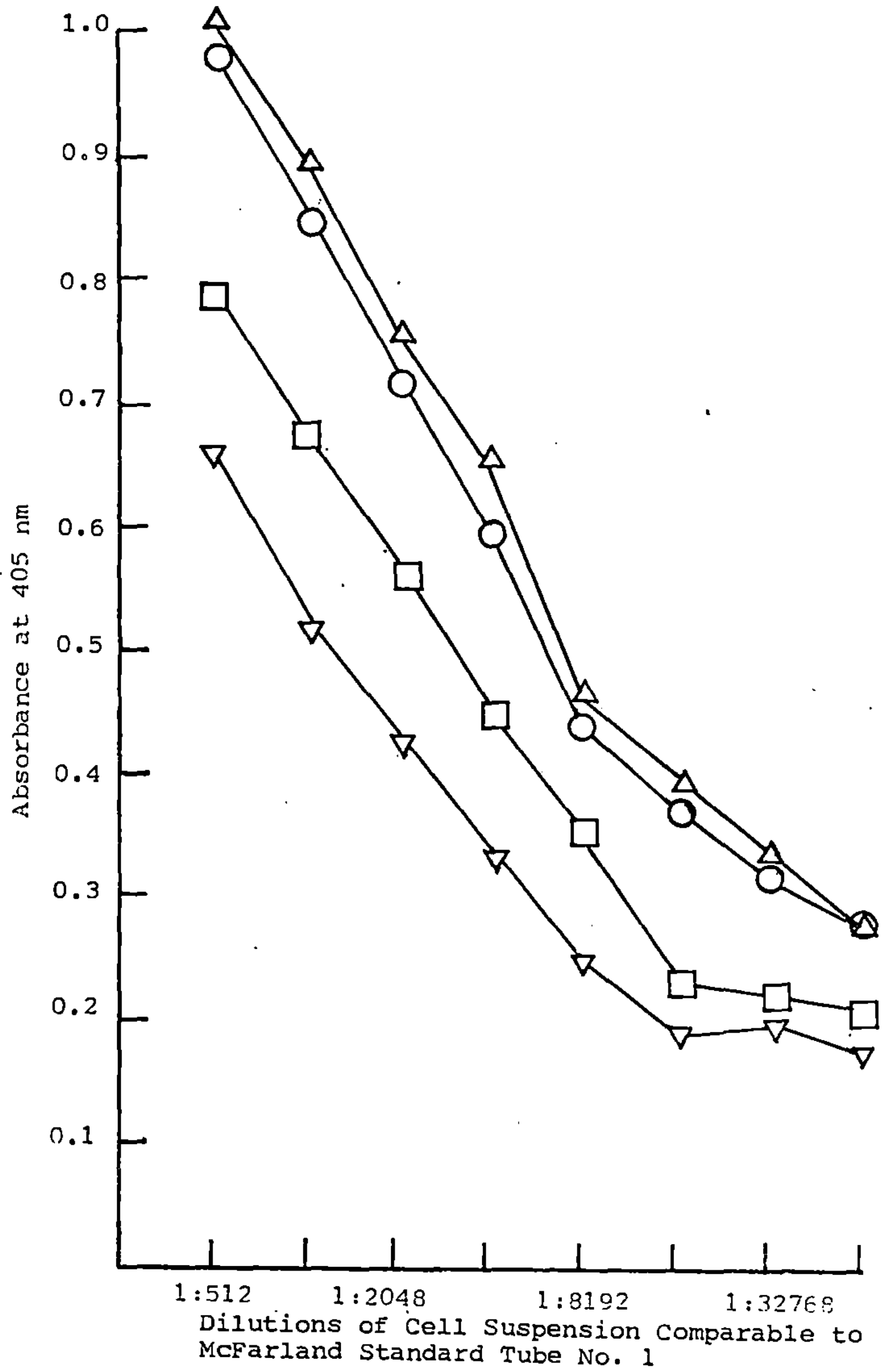

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance at 405 nm
1:512
1:2048
1:8192
1:32768
Dilutions of Cell Suspension Comparable to
McFarland Standard Tube No. 1

Figure 3. Comparison of ELISA reactions for different dilutions of guinea pig antibrucella serum

(Various dilutions of guinea pig antibrucella serum were incubated with different concentrations of _B. abortus_ Strain 19 (antigen) for 60 minutes. Affinity purified goat antiguinea pig IgG labeled with horseradish peroxidase (conjugate) was used at a 1:300 dilution for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

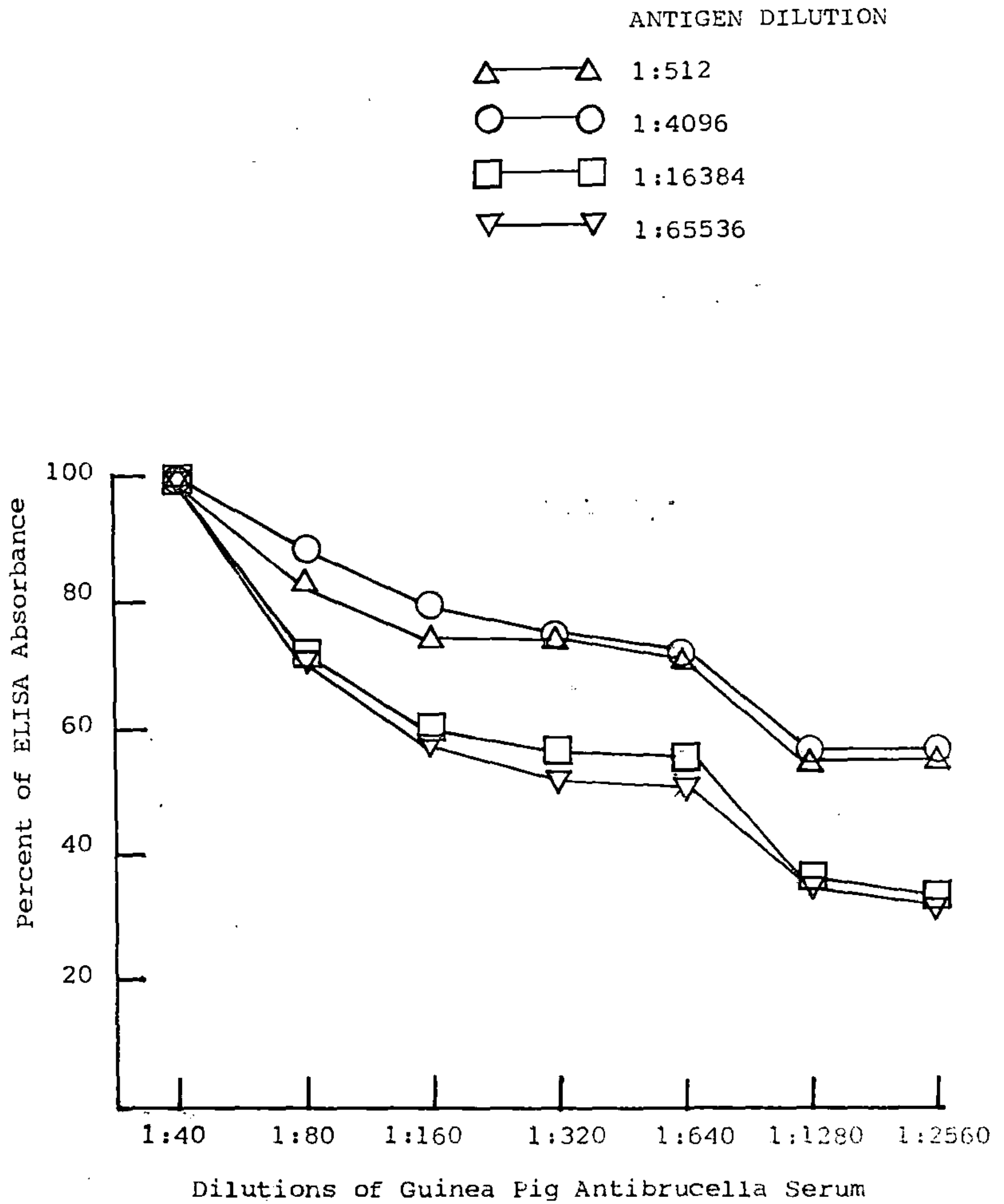

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
100
80
60
40
20
Percent of ELISA Absorbance
1:40
1:80
1:160
1:320
1:640
1:1280
1:2560
Dilutions of Guinea Pig Antibrucella Serum

Figure 4. Comparison of ELISA reactions for different dilutions of guinea pig antibrucella serum

(Various dilutions of guinea pig antibrucella serum were incubated with different concentrations of B. abortus Strain 19 (antigen) for 60 minutes. Affinity purified goat antiguinea pig IgG labeled with horseradish peroxidase (conjugate) was used at a 1:600 dilution for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

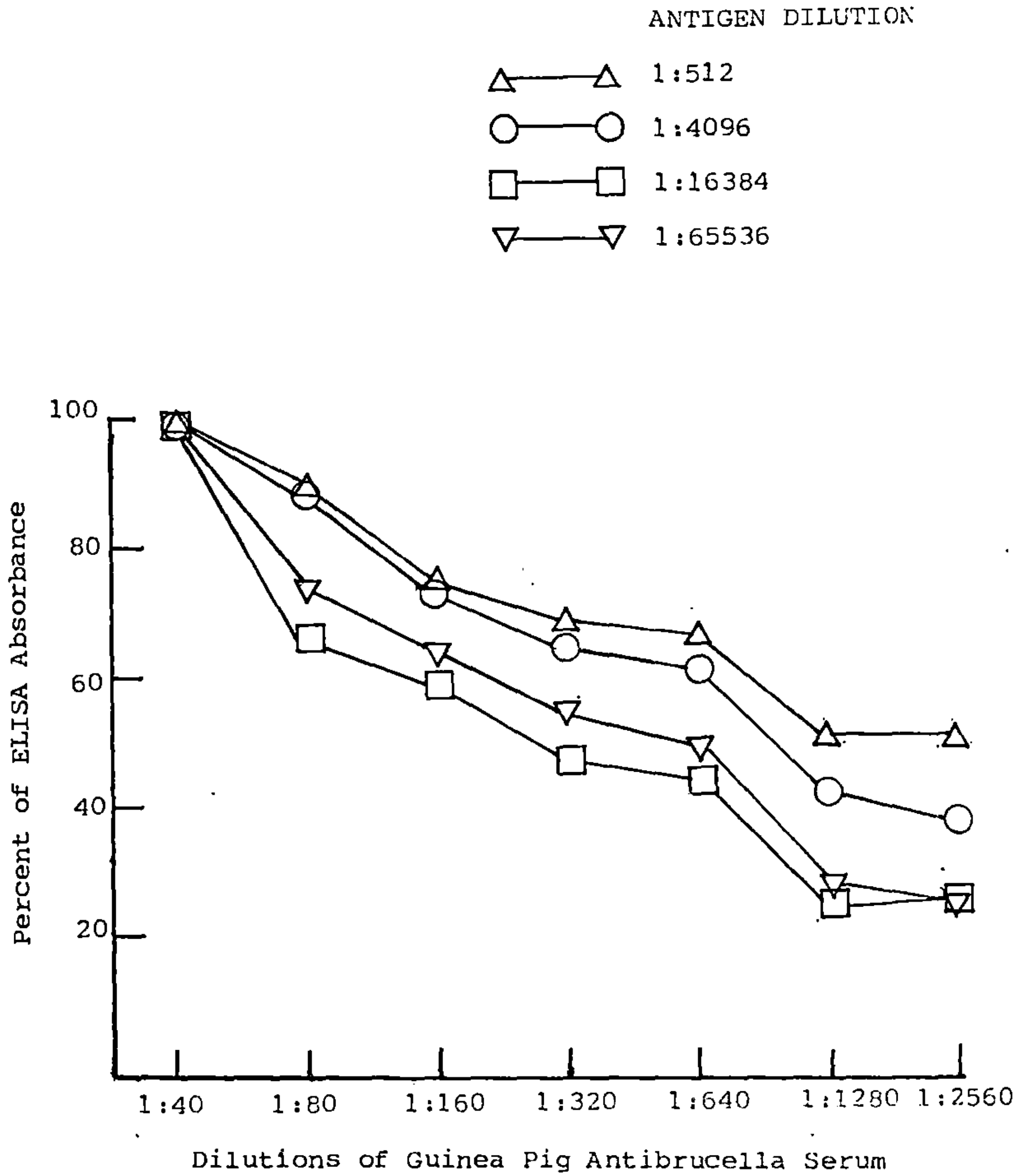

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
100
80
60
40
20
Percent of ELISA Absorbance
1:40
1:80
1:160
1:320
1:640
1:1280
1:2560
Dilutions of Guinea Pig Antibrucella Serum

Figure 5. Comparison of ELISA reactions for different dilutions of guinea pig antibrucella serum

(Various dilutions of guinea pig antibrucella serum were incubated with different concentrations of *B. abortus* Strain 19 (antigen) for 60 minutes. Affinity purified goat anti-guinea pig IgG labeled with horseradish peroxidase (conjugate) was used at a 1:1200 dilution for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

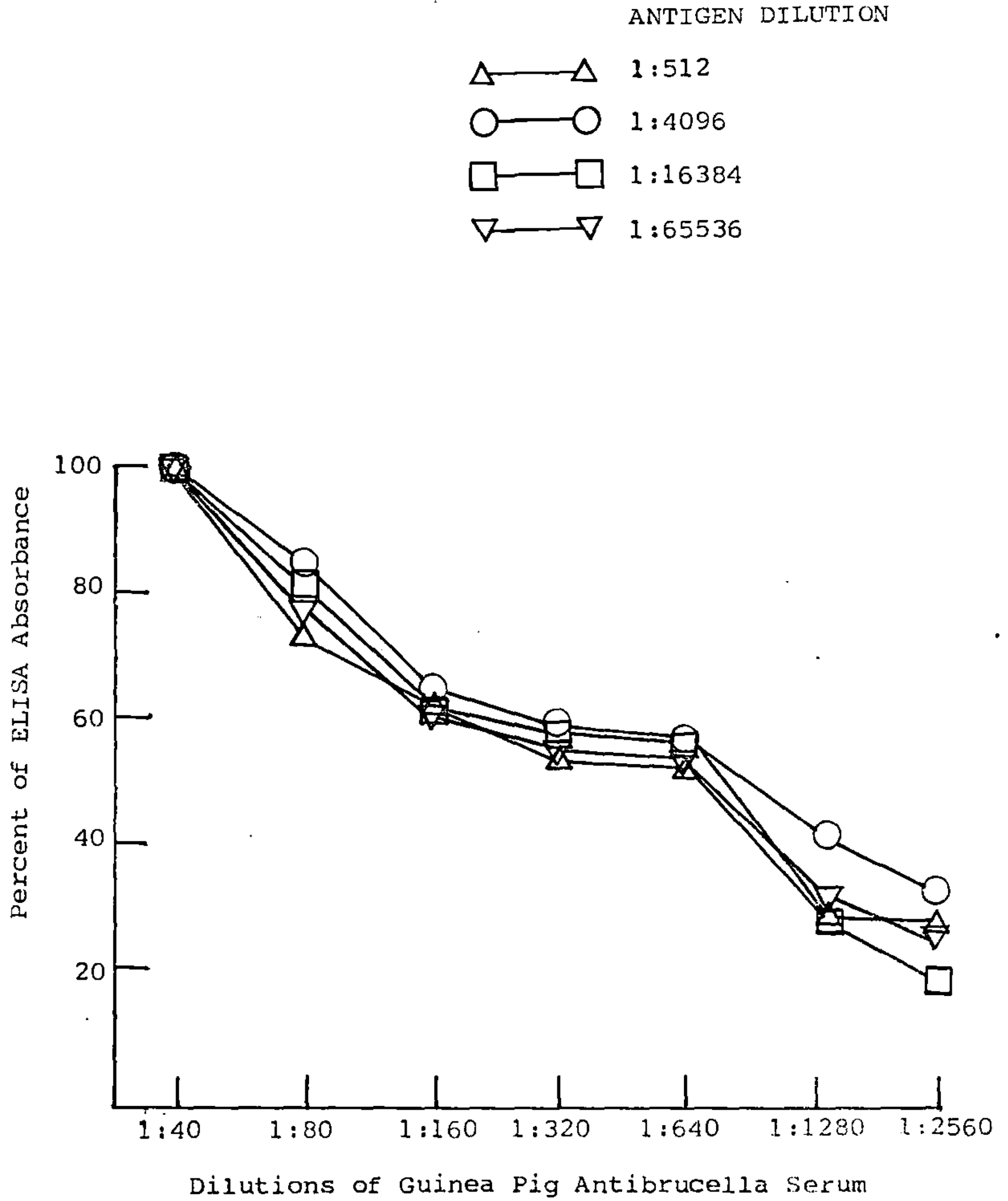

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
100
80
60
40
20
Percent of ELISA Absorbance
1:40
1:80
1:160
1:320
1:640
1:1280
1:2560
Dilutions of Guinea Pig Antibrucella Serum

Figure 6. Comparison of ELISA reactions for three different dilutions of goat antiguinea pig IgG labeled with horseradish peroxidase (conjugate)

(Guinea pig antibrucella serum (1:640 dilution) was incubated with different concentrations of *B. abortus* Strain 19 (antigen) for 60 minutes. Conjugate dilutions used were incubated for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

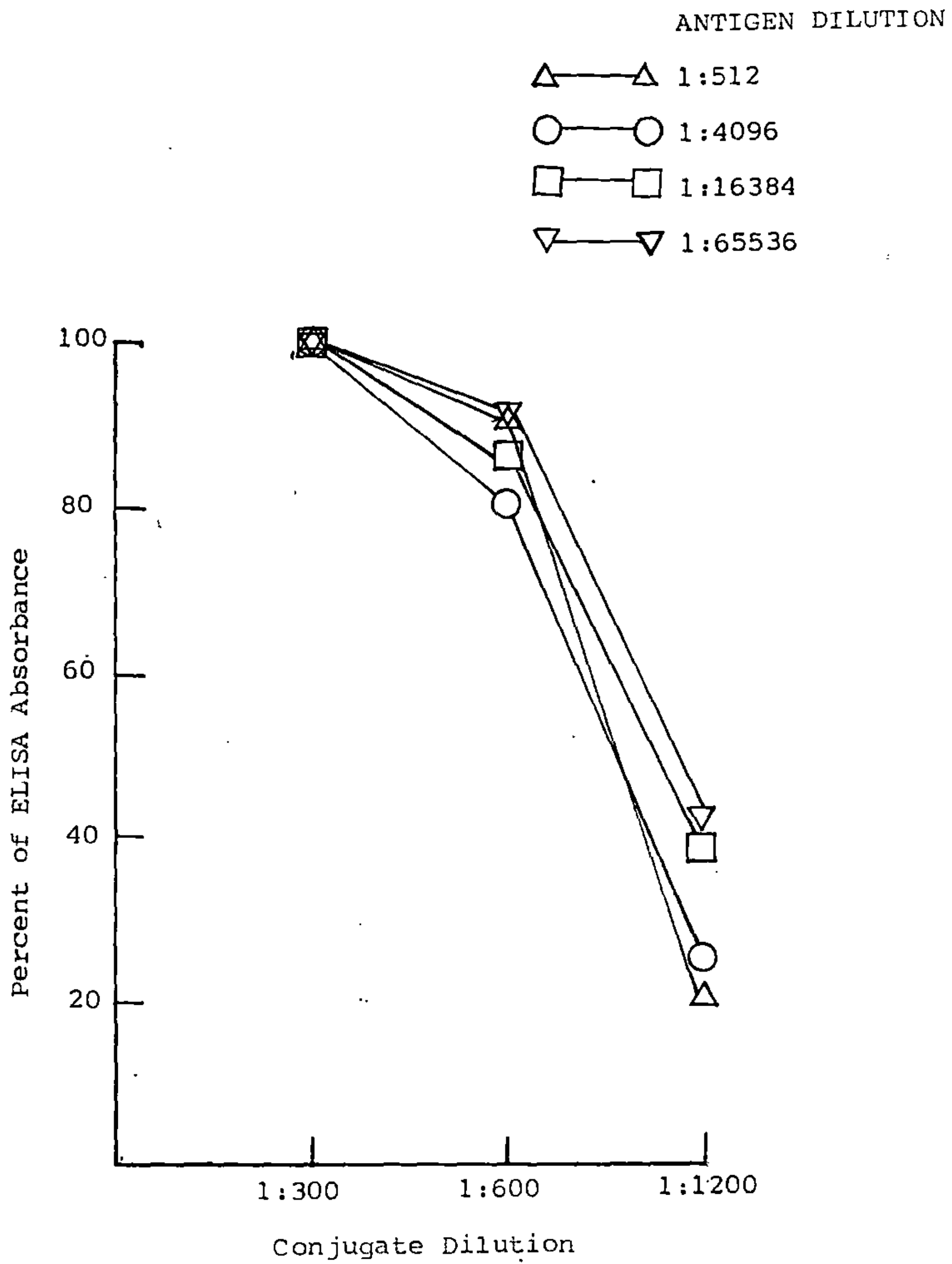

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
100
80
60
40
20
Percent of ELISA Absorbance
1:300
1:600
1:1200
Conjugate Dilution

Figure 7. Results of ELISA using various time intervals for antigen-antiserum incubation

(Guinea pig antibrucella serum (1:640 dilution) was incubated with different concentrations of *B. abortus* Strain 19 antigen for 15, 30, 60, or for 120 minutes. Goat antiguinea pig conjugate used at a 1:600 dilution was incubated for 15 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

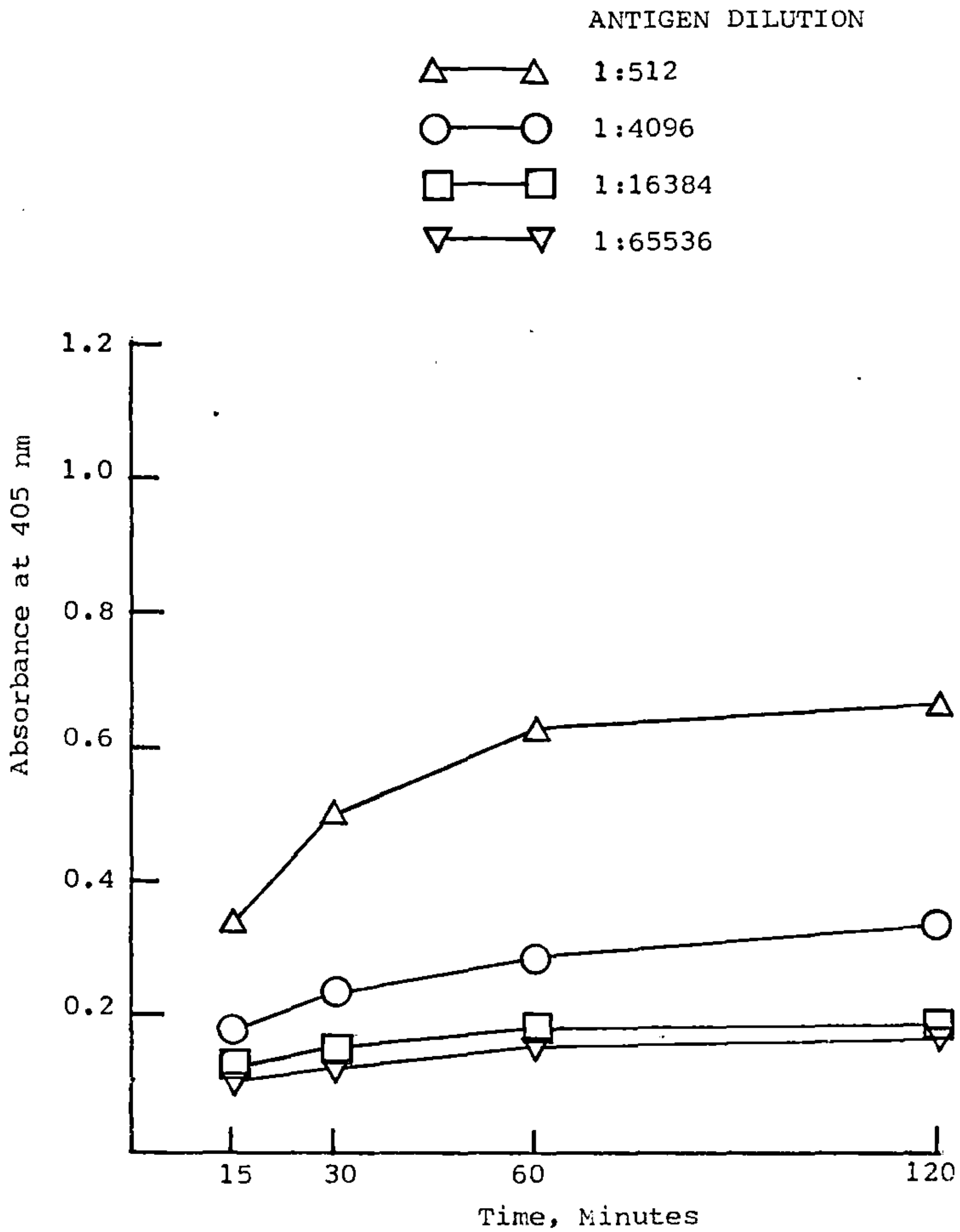

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance at 405 nm
15
30
60
120
Time, Minutes

Figure 8. Results of ELISA using various time intervals for antigen-antiserum incubation

(Guinea pig antibrucella serum (1:640 dilution) was incubated with different concentrations of *B. abortus* Strain 19 antigen for 15, 30, 60, or for 120 minutes. Goat antiguinea pig conjugate used at a 1:600 dilution was incubated for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

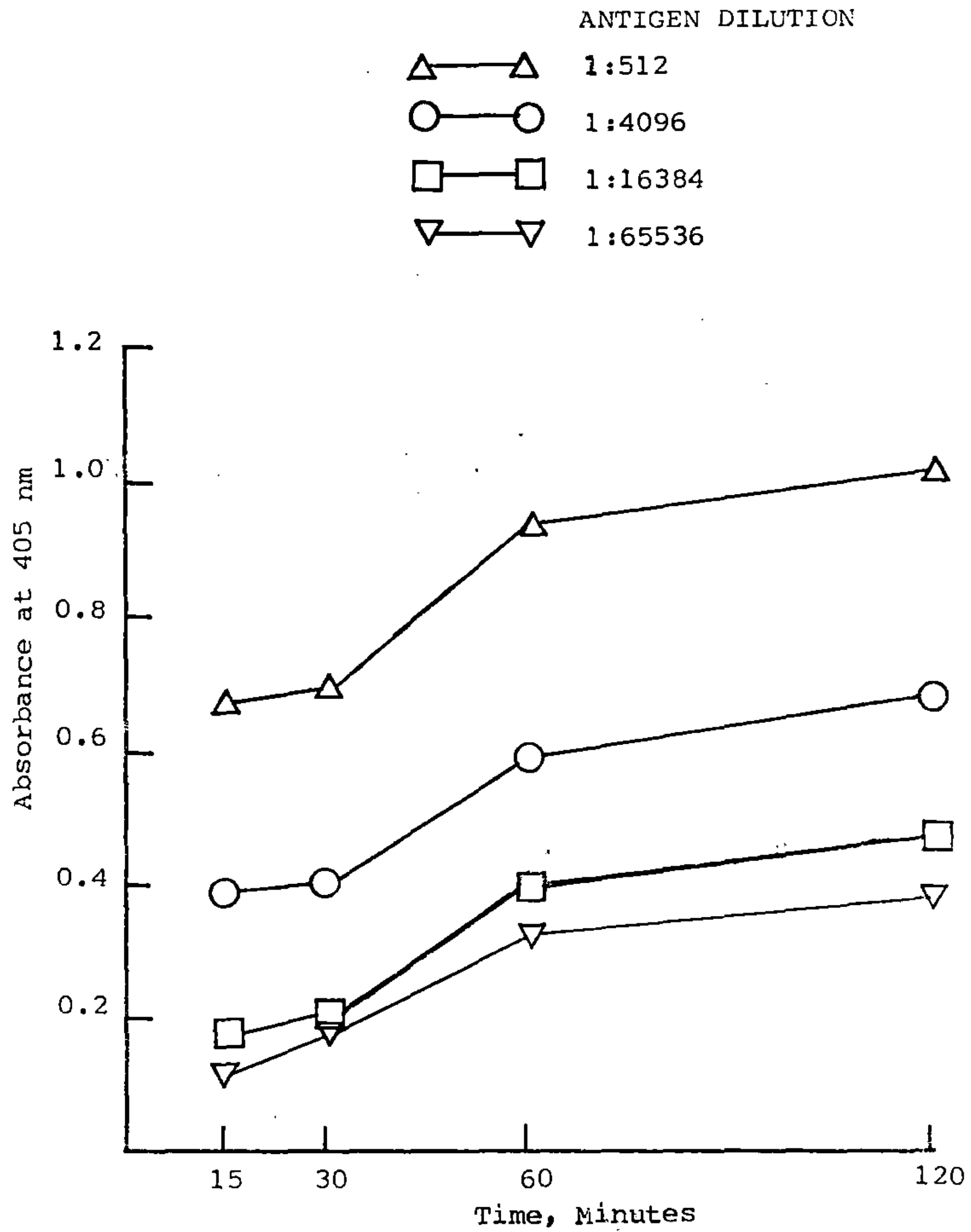

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance at 405 nm
15
30
60
120
Time, Minutes

Figure 9. Results of ELISA using various time intervals for antigen-antiserum incubation

(Guinea pig antibrucella serum (1:640 dilution) was incubated with different concentrations of *B. abortus* Strain 19 antigen for 15, 30, 60, or 120 minutes. Goat antiguinea pig conjugate used at a 1:600 dilution was incubated for 60 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

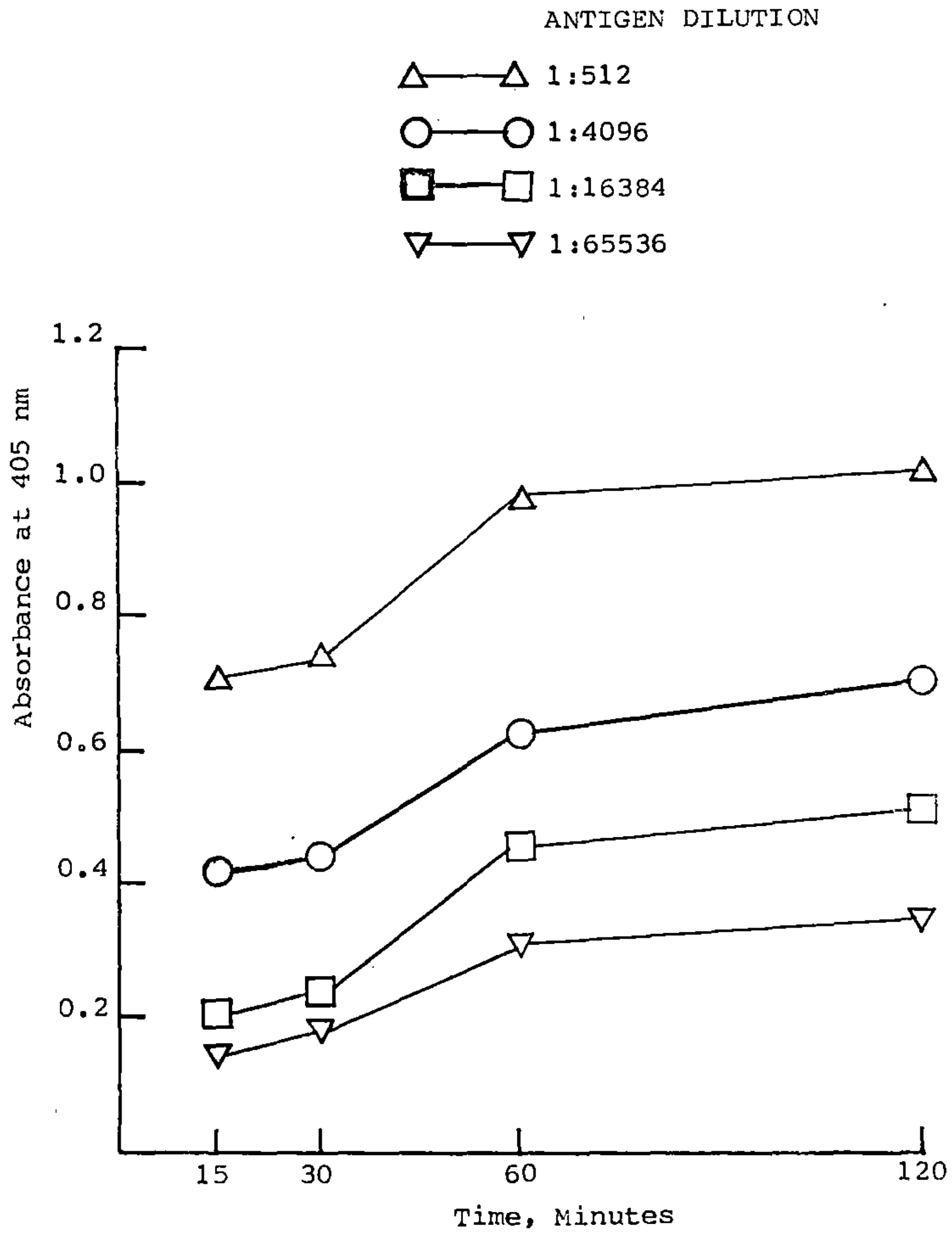

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance at 405 nm
15
30
60
120
Time, Minutes

Figure 10. Results of ELISA using various time intervals for incubation of conjugate

(Guinea pig antibrucella serum (1:640 dilution) was incubated with different concentrations of *B. abortus* Strain 19 antigen for 60 minutes. Goat antiguinea pig conjugate was used at a 1:600 dilution; the conjugate was incubated for 15, 30, 60, or for 120 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

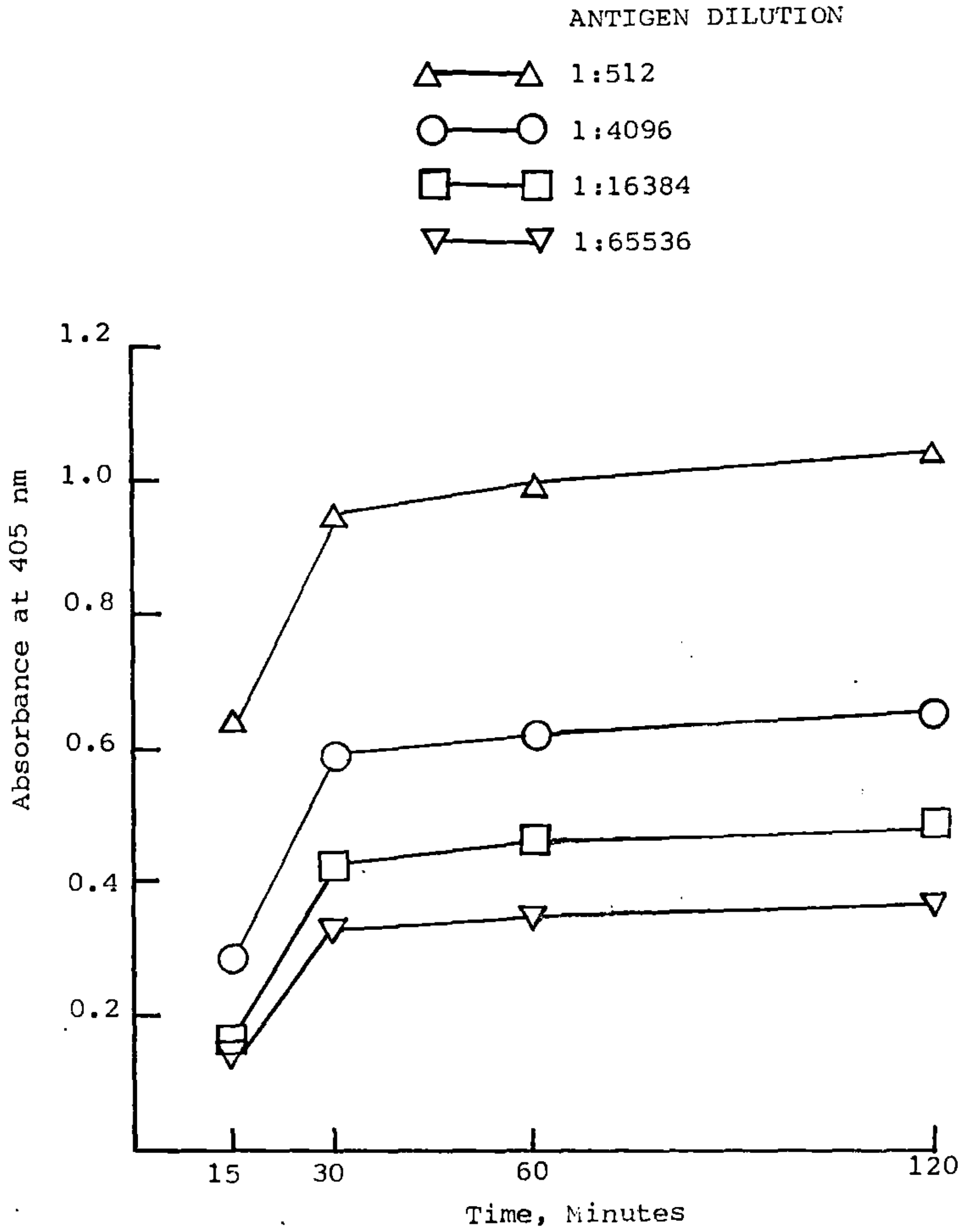

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance at 405 nm
15
30
60
120
Time, Minutes

Figure 11. Results of ELISA reactions for different enzyme-substrate incubation time intervals

(Guinea pig antibrucella serum (1:640 dilution) was incubated with a 1:512 dilution of *B. abortus* Strain 19 cell suspension comparable to a McFarland Standard Tube No. 1 for 60 minutes. Goat antiguinea pig conjugate used at a 1:600 dilution was incubated for 30 minutes. The results (absorbance values) following enzyme-substrate incubation at 10, 20, 30, 40, 50, and 60 minutes were measured)

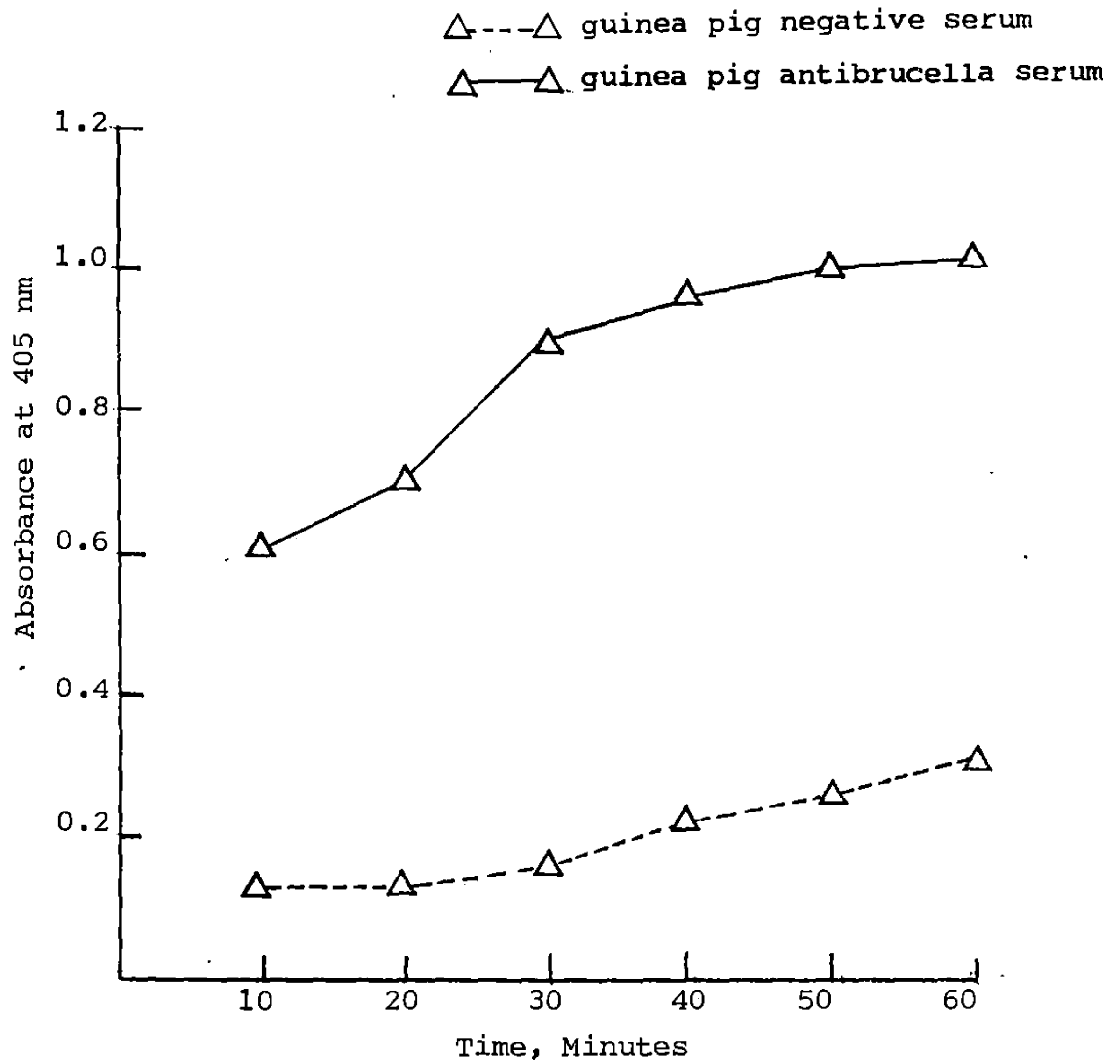

guinea pig negative serum
guinea pig antibrucella serum
Absorbance at 405 nm
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
Time, Minutes

Figure 12. Results of ELISA reactions for different enzyme-substrate incubation time intervals

(Guinea pig antibrucella serum (1:640 dilution) was incubated with a 1:4096 dilution of *B. abortus* Strain 19 cell suspension comparable to a McFarland Standard Tube No. 1 for 60 minutes. Goat antiguinea pig conjugate used at a 1:600 dilution was incubated for 30 minutes. The results (absorbance values) following enzyme-substrate incubation at 10, 20, 30, 40, 50, and 60 minutes were measured)

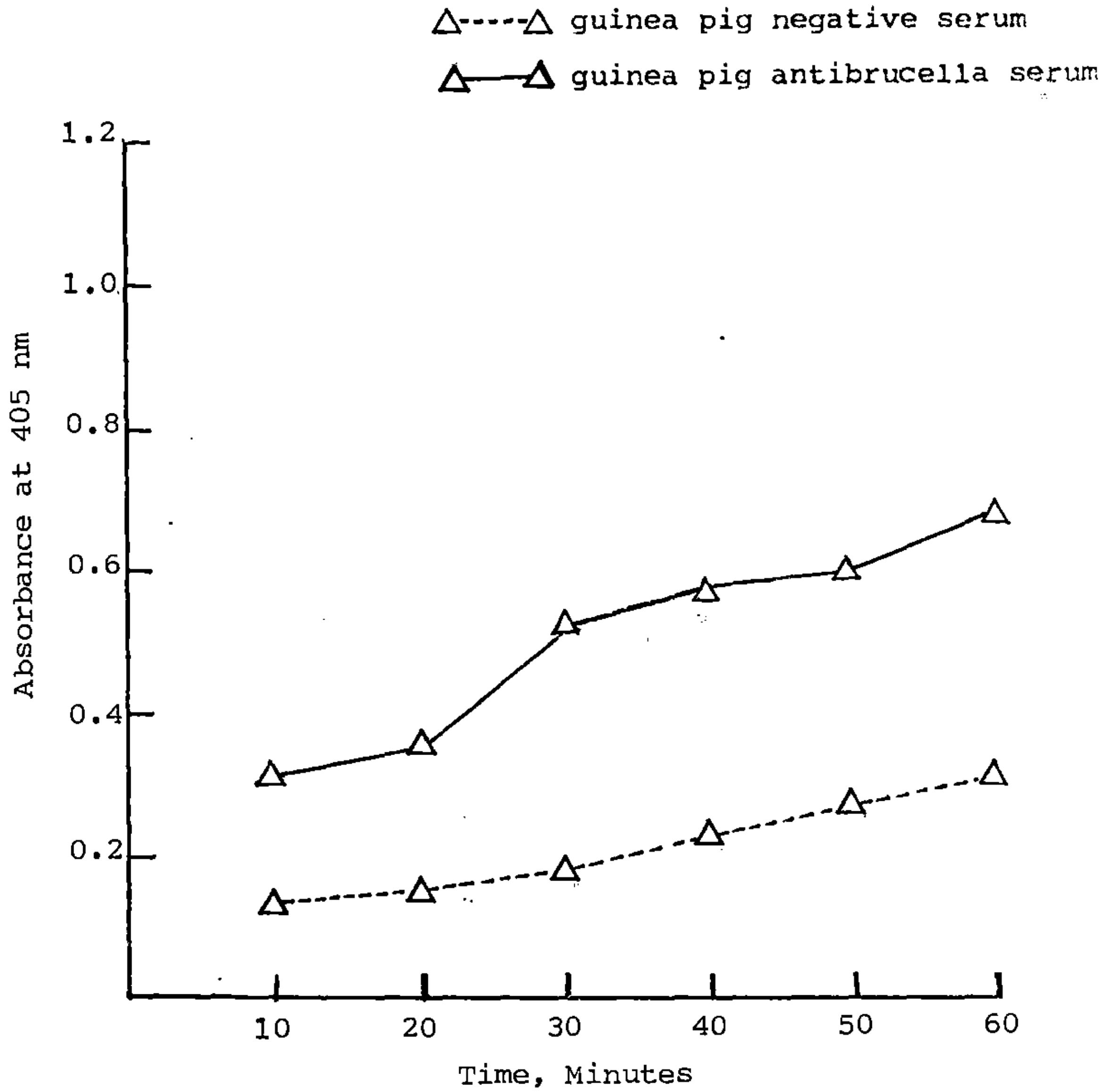

guinea pig negative serum
guinea pig antibrucella serum
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance at 405 nm
10
20
30
40
50
60
Time, Minutes

Figure 13. Results of ELISA reactions for different enzyme-substrate incubation time intervals

(Guinea pig antibrucella serum (1:640 dilution) was incubated with a 1:16384 dilution of *B. abortus* Strain 19 cell suspension comparable to a McFarland Standard Tube No. 1 for 60 minutes. Goat antiguinea pig conjugate used at a 1:600 dilution was incubated for 30 minutes. The results (absorbance values) following enzyme-substrate incubation at 10, 20, 30, 40, 50, and 60 minutes were measured)

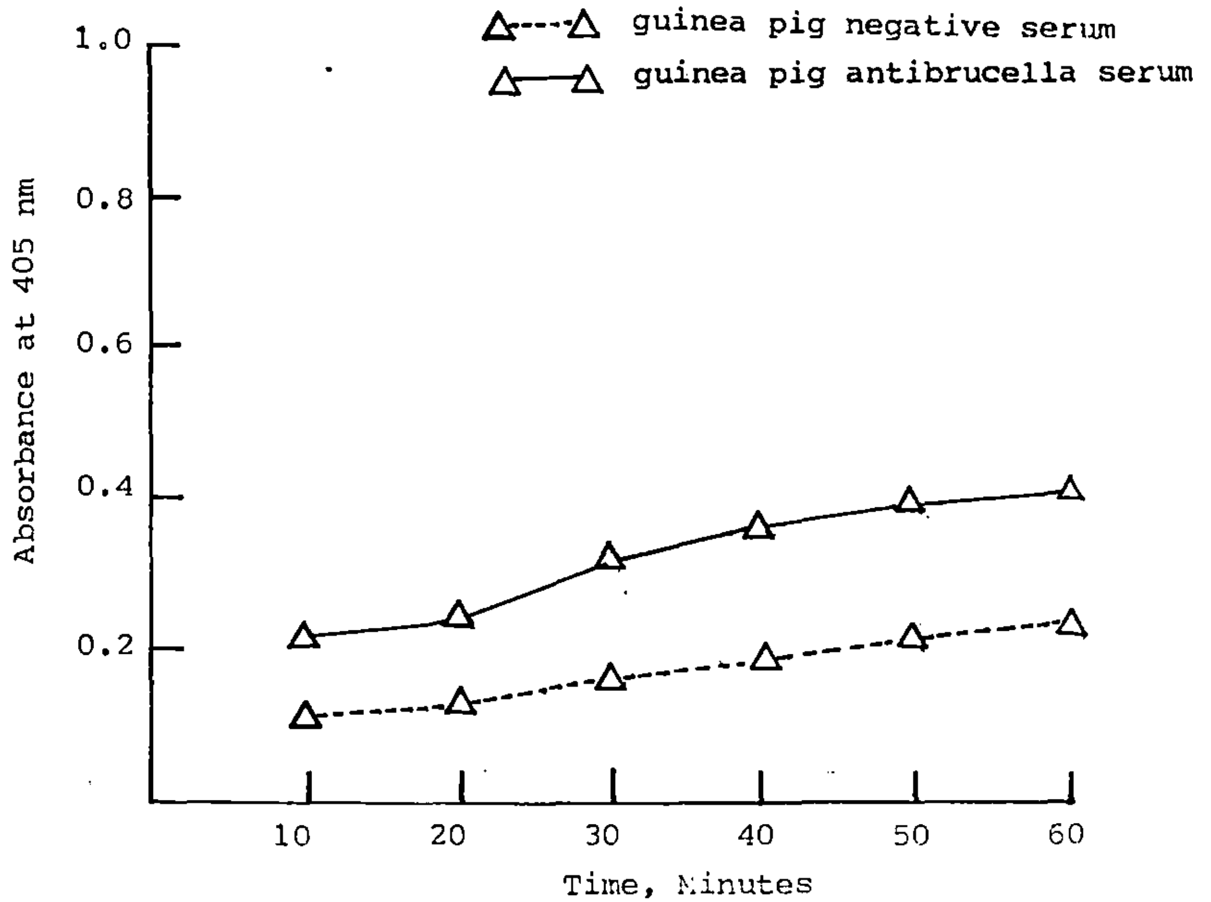
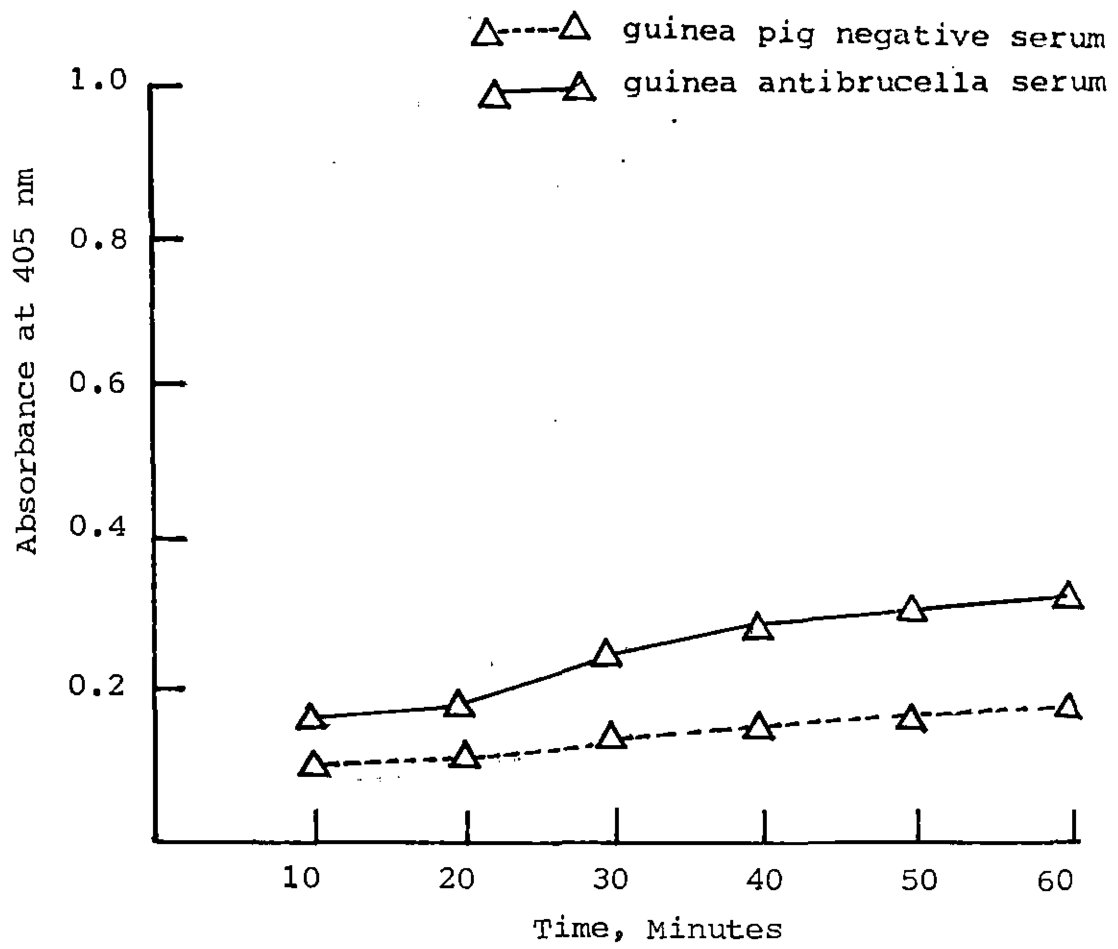

guinea pig negative serum
guinea pig antibrucella serum
1.0
0.8
0.6
0.4
0.2
Absorbance at 405 nm
10
20
30
40
50
60
Time, Minutes

Figure 14. Results of ELISA reactions for different enzyme-substrate incubation time intervals

(Guinea pig antibrucella serum (1:640 dilution) was incubated with a 1.65536 dilution of <u>B. abortus</u> Strain 19 cell suspension comparable to a McFarland Standard Tube No. 1 for 60 minutes. Goat anti-guinea pig conjugate used at a 1:600 dilution was incubated for 30 minutes. The results (absorbance values) following enzyme-substrate incubation at 10, 20, 30, 40, 50, and 60 minutes were measured)



guinea pig negative serum
guinea antibrucella serum
1.0
0.8
0.6
0.4
0.2
Absorbance at 405 nm
10
20
30
40
50
60
Time, Minutes

Figure 15. Comparison of ELISA results (ratio) obtained using guinea pig antibrucella serum and guinea pig negative control serum at different enzyme-substrate incubation times

(Guinea pig antibrucella serum and guinea pig control serum were each used at a 1:640 dilution; the sera were incubated with different concentrations of *B. abortus* Strain 19 antigen for 60 minutes. Goat antiguinea pig conjugate was used at a 1:600 dilution and incubated for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)

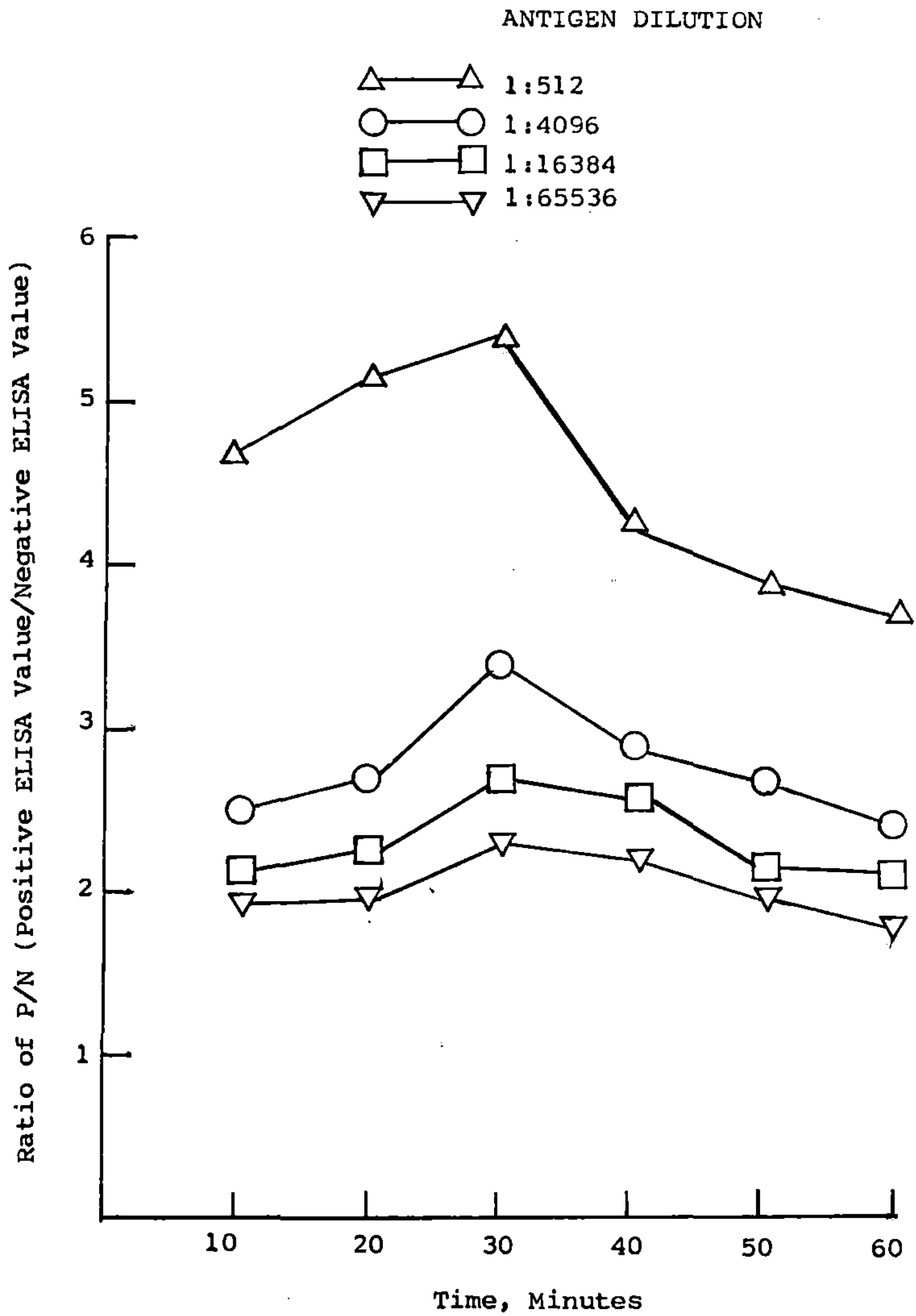

ANTIGEN DILUTION
1:512
1:4096
1:16384
1:65536
Ratio of P/N (Positive ELISA Value/Negative ELISA Value)
6
5
4
3
2
1
10
20
30
40
50
60
Time, Minutes

Figure 16. Colony counts of different dilutions of *Brucella abortus* Strain 19

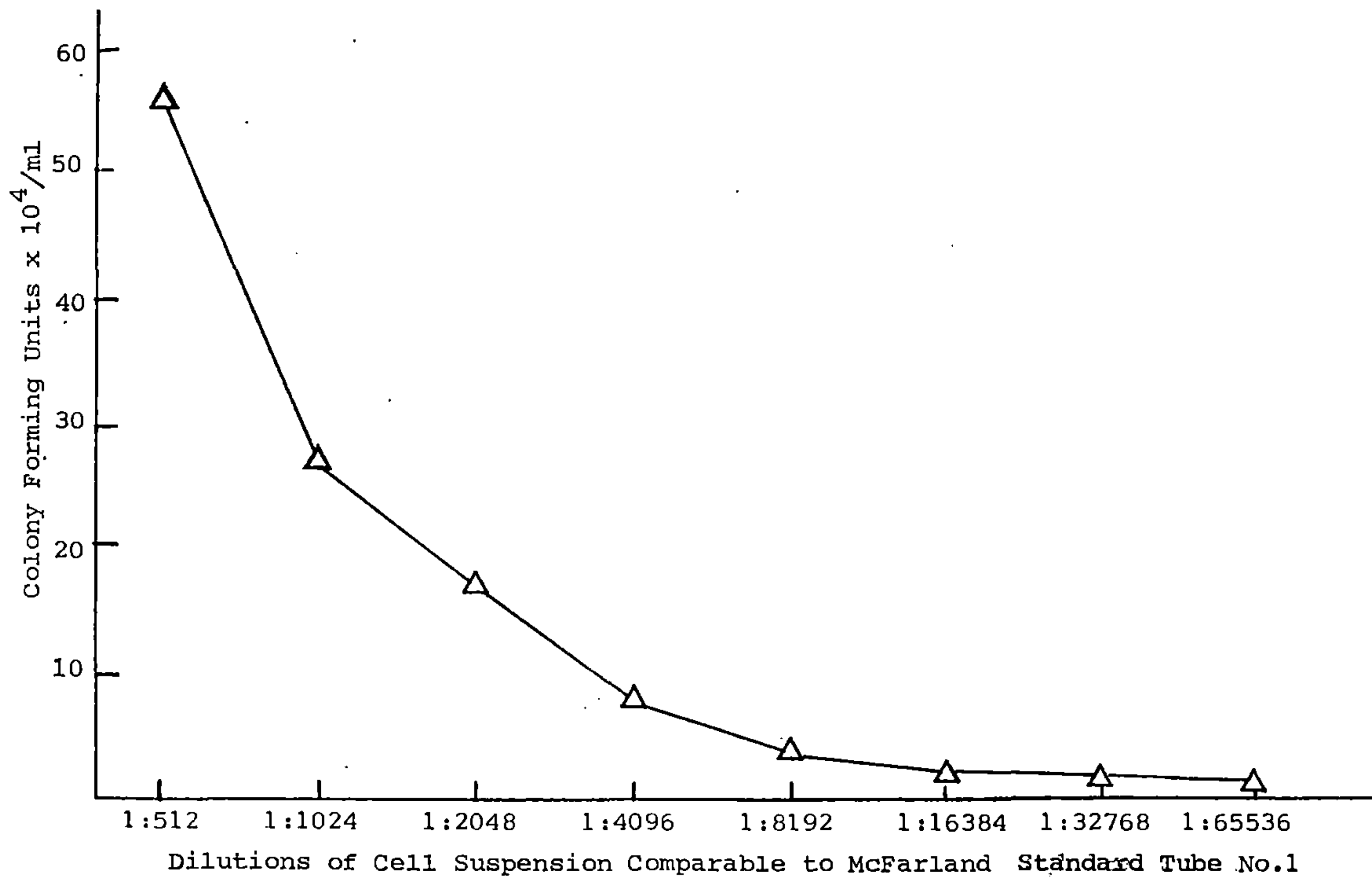
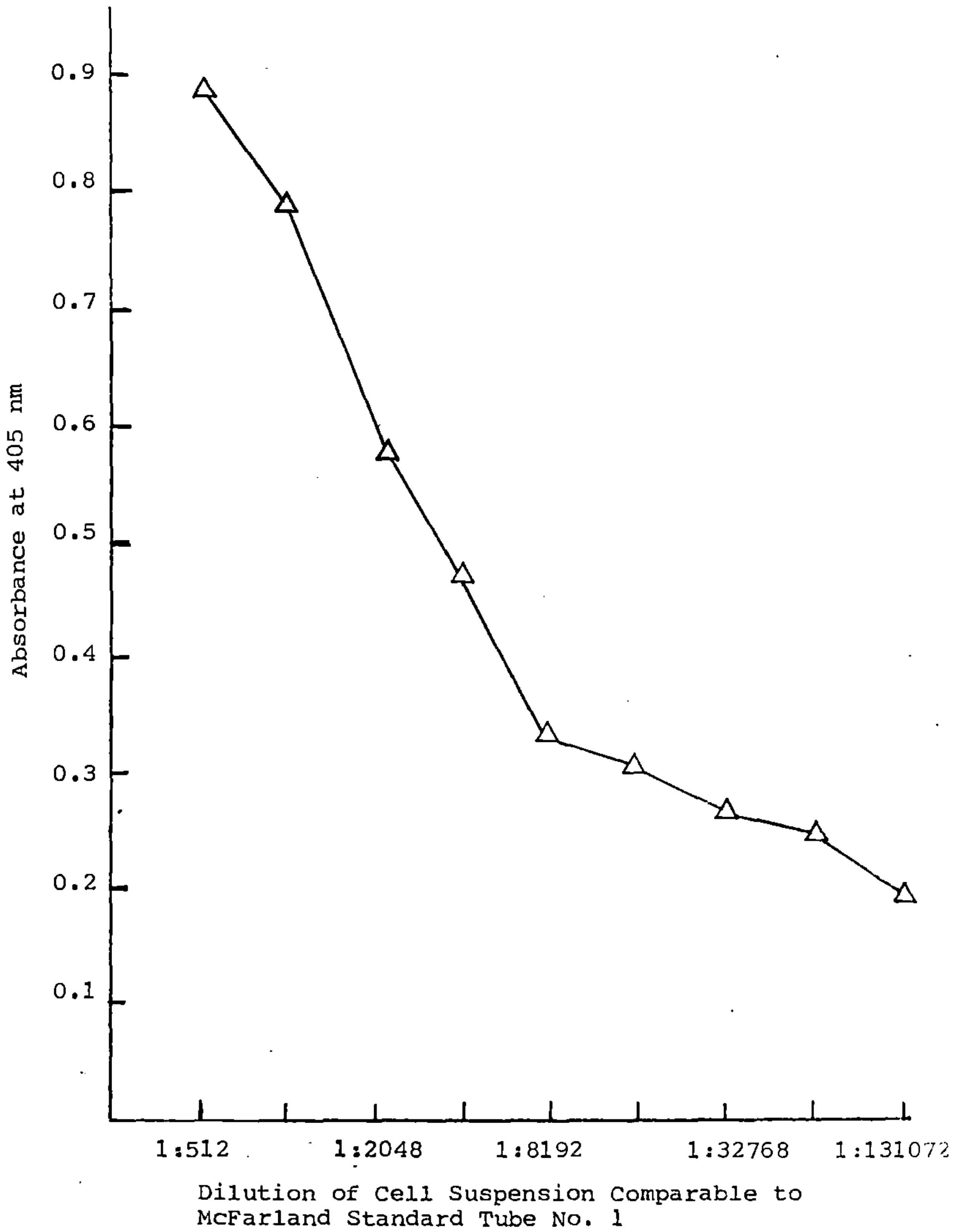

60
50
40
30
20
10
Colony Forming Units x 10^4/ml
1:512
1:1024
1:2048
1:4096
1:8192
1:16384
1:32768
1:65536
Dilutions of Cell Suspension Comparable to McFarland Standard Tube No.1

Figure 17. Results of ELISA reaction for different dilutions of *B. abortus* Strain 19 antigen

(Guinea pig antibrucella serum (1:640 dilution) was incubated with serial dilutions of a *B. abortus* Strain 19 cell suspension comparable to a McFarland Standard Tube No. 1 for 60 minutes. Goat antiguinea pig conjugate was used at a 1:600 dilution and incubated for 30 minutes. The results were measured at 30 minutes enzyme-substrate reaction time)



0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance at 405 nm
1:512
1:2048
1:8192
1:32768
1:131072
Dilution of Cell Suspension Comparable to McFarland Standard Tube No. 1

DISCUSSION

Comparison of the ELISA results of different methods for coating antigen onto microcuvettes indicated that a significant increase in sensitivity was obtained using B. abortus Strain 19 antigen diluted in Na_2CO_3 buffer containing carbodiimide and subsequently incubated with NH_4Cl. The binding of antigen to a solid phase (polystyrene microcuvette) has been proposed to involve hydrophobic adsorption and/or covalent bonding (19, 33,110). Carbodiimide compounds have been used to covalently couple proteins to other proteins and to small molecular weight compounds (5,10). Since each protein may have a different binding capacity to polystyrene and different adsorption characteristics, the influence of carbodiimide may vary for different antigens (19,33,42,43). The covalent binding activity of carbodiimide involves amino group(s) and/or carboxyl group(s); therefore, the increased binding of B. abortus Strain 19 whole cell antigen to the microcuvette by incubation with carbodiimide may have been due to the increased covalent binding of the carboxyl group and/or the amino group on the surface of the whole cell or of the cell fraction (broken during the antigen preparation) to the polystyrene surface (87,110). However, the specific mechanism(s) of the

increased binding of Brucella whole cell antigen to microcuvette surface by carbodiimide are not well-understood.

The suggested activity of NH_4Cl was to neutralize residual active charge present on the cuvette surface and thus decrease nonspecific binding. When negative control serum was used in the studies described herein marked decreases in nonspecific binding were observed in NH_4Cl treated cuvettes. Increases in ELISA reactions were observed using guinea pig antibrucella serum when cuvettes were treated with NH_4Cl; however, the difference was not significant when compared with the reactions in cuvettes incubated with carbodiimide alone. The results of preliminary studies revealed that nonspecific background ELISA reactions were observed when rabbit antiguinea pig conjugate was used; a possible explanation for this is that the conjugate was produced in a rabbit that had been previously exposed to agent(s) that induced antibodies which cross react with the Brucella on the cuvette surface. It should be emphasized that an increase in ELISA reactivity was obtained through the use of an affinity purified goat antiguinea pig IgG labeled with horseradish peroxidase.

The results observed in the studies indicated that ELISA reactions increased when antigen-antiserum incubation

time, conjugate incubation time, or enzyme-substrate reaction time intervals were increased. However, the reactions were not markedly increased when antigen-antiserum incubation times were greater than 60 minutes, or conjugate incubation times were greater than 30 minutes. Detectable ELISA reactions increased as the enzyme-substrate reaction time increased; however, at 30 minutes reaction time, the ratios of positive serum ELISA absorbance values were higher as compared to the negative serum ELISA absorbance values for all conditions studied. After 30 minutes enzyme-substrate reaction time, the increase in ELISA reactions did not provide for greater differentiation.

Viable bacterial count is a routine procedure used in the standardization of vaccine containing live *Brucella* cells; it is also used frequently in research (1). The results from the viable count in this study provided information on the number of *Brucella* cells in each antigen dilution. However, for ELISA, the *Brucella* cells used herein were heat-killed whereas, for viable count, the *Brucella* used were live cells. The cells used in these two studies were different; therefore, only a relative comparison can be made between viable count results and ELISA results.

The finding in this study revealed that to establish optimal conditions for the ELISA, it is necessary to

carefully select concentrations of antiserum and conjugate as well as the incubation time intervals for each reagent used. The addition of carbodiimide to a suspension of *Brucella* increased the sensitivity of the ELISA for detecting the presence of antigens. Moreover, the incubation with NH_4Cl decreased nonspecific ELISA reactions in negative controls. No information is available on the detection of *Brucella abortus* shed in vaginal discharge by ELISA; previous reports concerned the detection of the *Brucella* antibodies from serum or milk (2,4,14,20,21,26, 40,55,61,62,64,94,95). The detection of *Brucella* could be useful in identifying infected animals that shed organisms. Therefore, further studies on the application of ELISA developed herein to field cases may be useful in the early detection of animals that shed *Brucella*; this could provide information of value in the eradication of brucellosis in cattle.

SECTION II: APPLICATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF *BRUCELLA* ANTIGENS IN VAGINAL DISCHARGE OF COWS

Application of an enzyme-linked immunosorbent assay for detection of *Brucella* antigens in vaginal discharge of cows

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SUMMARY

An enzyme-linked immunosorbent assay (ELISA) was developed for detecting *Brucella abortus*. Carbodiimide cyanamide was used as an antigen coupling agent and NH_4Cl was used to neutralize residual active charges present on the cuvette surface. Suitable ELISA reactions were observed in 41 vaginal swabs diluted in phosphate-buffered saline. The ELISA described may be useful under controlled laboratory conditions for detecting *B. abortus*-infected cows shedding bacteria in vaginal secretions.

INTRODUCTION

The diagnosis of brucellosis in cattle is usually made on the basis of serological tests (73). However, bacteriologic confirmation by the examination of appropriate specimens is often required (1). When culturing is performed to confirm a diagnosis of brucellosis, milk and/or tissues are commonly used (1). The culturing of the vaginal discharge has probably not been a routine practice because of the short time that Brucella are shed. Generally, infected cattle shed massive quantities of Brucella through the vaginal discharge for the first two weeks after normal or abnormal parturition. Shedding then usually diminishes for 4 to 5 weeks, after which 5 to 10% of the cows will shed intermittently for up to 2 years (16,34,35,36). The detection and removal of cattle shedding Brucella at the time of calving would be an effective method of reducing the exposure potential in infected herds. Consequently, a relatively simple rapid test for detecting Brucella antigens in the vaginal discharge of cattle would be of practical value.

Previous reports have been made on the application of enzyme-linked immunosorbent assays (ELISA) for detecting Brucella antibodies in the milk and sera of B. abortus-infected cattle (57,59,67). However, no information is

available on the use of ELISA for detecting *Brucella* antigens in secretions of the vaginal tract.

The purpose of this investigation was to apply the ELISA that was developed in previous research for determining the presence of *Brucella* antigens in swabs of vaginal secretions collected from cows experimentally exposed to *B. abortus* Strain 2308.

MATERIALS AND METHODS

Antigen

Brucella abortus Strain 19 vaccine was reconstituted with diluent (National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA). Two hundred µl of the diluent was used to inoculate 5 ml quantities of tryptose broth which was incubated at 37°C for 72 hours. A 2 ml amount of the 72-hour broth culture was used to inoculate Roux Flasks containing tryptose agar; the cultures were incubated at 37°C for 4 days. The cells were harvested by washing the surface with sterile phosphate-buffered saline. The cell suspensions were autoclaved at 121°C for 15 minutes, and then washed two times with phosphate-buffered saline. The killed cell suspension density was adjusted to a McFarland Standard Tube No. 1; optical density was measured at 480 nm on a Beckman Spectrophotometer Model No. 25 (OD=0.377). Serial two-fold dilutions from 1:512 to 1:65536 were made using 0.1 M Na_2CO_3 (pH 9.6).

Antiserum

Guinea pig antibrucella serum was obtained from National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA. The hyperimmune serum was collected from guinea pigs inoculated subcutaneously with *B. abortus* Strain 2308. Block titrations of the sera and *Brucella* antigen were conducted to determine the optimal serum concentration (1:640) for ELISA (see section I).

Conjugate

Affinity purified goat antiguinea pig IgG antibody was labeled with horseradish peroxidase[1] by modification of the method of Nakane and Kawaoi (63). A 1:600 dilution of conjugate (Lot No. FA 04), was used to obtain suitable ELISA reactions.

Substrate

A working solution of substrate was prepared, using 3% hydrogen peroxide and 2,2'-azino-di-(3-ethyl benzthiozaline 6-sulfonate) (ABTS) in 0.05 *M* citric acid.[2]

[1]Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD.

[2]The procedure for preparing ABTS was obtained from Dr. M. L. Bartlett, Los Alamos Scientific Laboratory, Los Alamos, NM.

Swab Samples

Swab samples of vaginal secretions were collected immediately after parturition or abortion from each of 65 cows experimentally exposed to B. abortus Strain 2308. The vulvar region of each cow was wiped clean by a cotton swab saturated with isopropyl alcohol. A sterile, disposable culture swab enclosed in a plastic sheath[1] was inserted into the vagina, and the swab was then extended about ten inches into the vagina. The vaginal secretions were absorbed for 10-15 seconds. The swab was withdrawn about half-way into the external sheath, and the entire swabbing instrument was removed. The external plastic sheath was then cut with a disinfected snipper approximately two to three inches distal to the inner swab. The swab was pushed through the protective sheath and inserted into a sterile culture tube containing 0.5 ml of tryptose broth. The vaginal swab was cut off and the tube was recapped. One set of vaginal swabs were processed for the isolation of Brucella, as described previously (6). Tissues were collected at necropsy for cultural examination. Duplicate swabs were collected and placed in 0.5 ml tryptose broth and stored at -20°C. The specimens were thawed immediately before conducting ELISA. One and five tenths ml of tryptose

[1]Kalayjian Industries, Long Beach, CA.

broth was added to each of 24 swabs, and 1.5 ml of phosphate-buffered saline was added to each of 41 swabs. Tubes containing the specimens were placed in a 70°C water for 3 hours to kill the *Brucella*.

Procedure for ELISA Test

The ELISA was conducted by modification of a procedure described previously (57). Fifty µl of serial dilution of McFarland Standard Tube No. 1 (1:512 to 1:65,536) of the heat-killed *Brucella abortus* Strain 19 (positive controls) or of a swab suspension was added to separate wells of a Gilford cuvette. Fifty µl of carbodiimide[1] (1 mg/ml) was added to each well. The cuvettes were allowed to stand at 4°C for 16 hours and washed three times with PBS (pH 7.4). Then 100 µl of 0.1 *M* NH_4Cl was added to wells. After 30 minutes incubation, the cuvettes were washed 3 times with 0.5 *M* NaCl containing 0.5% Tween 80 adjusted to pH 7.5 with 1 *N* NaOH. Fifty µl of antiserum diluted to 1:640 using 0.5 *M* NaCl buffered to pH 7.4 containing 1% Tween 80 and 1% bovine albumin was added and incubated for 1 hour at 22°C on a horizontal shaker[2] at 90 rpm. Following the incubation period, the cuvettes were washed eight times with 0.5 *M* NaCl containing

[1] Sigma Chemical Co., St. Louis, MO. Lot No. C2388.

[2] Arthur H. Thomas Co., Philadelphia, PA.

0.5% Tween 80 adjusted to pH 7.5 with 1 N NaOH. Fifty µl of 1:600 conjugate was added to separate wells of the cuvettes and incubated for 30 minutes; then the cuvettes were washed eight times with the same wash solution to remove excess conjugate. One hundred µl of substrate solution was added to each well and incubated for 30 minutes at 22°C. A Gilford PR-50 Processor Reader[1] was used at a wavelength of 405 nm to determine the color intensity of the ELISA reactions.

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[1]Gilford Instruments, Oberlin, OH.

RESULTS

Optimal ELISA reactions were observed at 30 minutes using a 1:640 dilution of hyperimmune guinea pig antibrucella sera and a 1:600 dilution of goat antiguinea pig IgG labeled with horseradish peroxidase.

The results of ELISA on vaginal swabs placed in tryptose broth are shown in Tables 11 and A14. Eleven cows positive on bacteriologic examination were positive on ELISA. Of the 13 vaginal swabs collected from culture negative cows, 4 had negative ELISA reactions, 5 had suspicious reactions, and 4 had positive reactions. *Brucella abortus* was isolated from tissues of two cows with negative culture results on vaginal discharge and suspicious reactions on ELISA.

ELISA results on 41 vaginal swabs placed in 1.5 ml of phosphate-buffered saline are summarized in Table 2 and Table A15. Positive ELISA reactions were observed in 23 of 25 vaginal specimens positive on cultural examination; the other two had suspicious ELISA reactions. Of the 16 swab specimens collected from cows negative on cultural examination, 14 were negative on ELISA and 1 had a suspicious reaction one was positive on ELISA. At necropsy, *B. abortus* Strain 2308 was isolated from the retropharyngeal lymph nodes of the cow that had been negative on culture and positive on

ELISA. Also, *B. abortus* was isolated from supramammary lymph node of one animal suspicious on ELISA and negative on bacteriologic examination of vaginal secretion.

DISCUSSION

Bacteriologic examinations can be used for detecting Brucella shed in the vaginal discharges of cows. However, these procedures have not been widely used in herds where brucellosis has been diagnosed because they are laborious and time-consuming. The results presented herein on the use of an ELISA for detecting Brucella in experimentally exposed cows, indicate this procedure may be a practical method for detecting cows shedding Brucella. Since the results would be available in a few hours, shedding animals could be removed immediately, thereby, minimizing spread to other susceptible cattle in the herd.

A previous investigation indicated that an improved culture method, using a selective enrichment broth, was useful in detecting cows shedding low numbers of Brucella in the vaginal discharge (6). The results of the study revealed an important correlation between the ELISA and the isolation of Brucella by the direct streaking of swabs of vaginal discharge onto plates of agar medium (1). Further studies are needed to compare, standardize, and evaluate the ELISA using the improved culture method on naturally infected cows over an extended period of time following parturition or abortion.

Changes in management practices, such as the use of dry pens and maternity stalls, was shown to accelerate the eradication of brucellosis in chronically infected dairy herds (99). These management practices were effective in eliminating brucellosis because the exposure potential was reduced by the segregation of parturating cows from most of the animals in the herd.

The vaccination of adult cattle with reduced doses of _B. abortus_ Strain 19 vaccine has been reported to be a practical method for reducing brucellosis in large cattle populations (65). This procedure was effective in controlling brucellosis because vaccination increases the resistance of most animals. It would appear that brucellosis could be eliminated even more rapidly by the detection and removal of animals shedding _Brucella_ through the vaginal discharge at parturition in herds using improved management and/or adult vaccination procedures. In addition, a simple, rapid ELISA for detecting _Brucella_ antigens in vaginal discharges, would be an effective supplemental method to detect recently infected herds, especially in areas where brucellosis has been reduced to low levels. An ELISA conducted on aborting cows by veterinary practitioners should detect infected herds rapidly and provide more accurate results than _Brucella_ Ring Test and market cattle testing procedures that are presently being used.

Table 1. Comparison of results of ELISA and of culture on vaginal discharge from 24 cows experimentally exposed to Brucella abortus Strain 2308 (Swab specimens were suspended in 1.5 ml tryptose broth; the results were observed at absorbance of 405 nm at 30 minutes substrate reaction time)

Culture results	ELISA results		
	Positive	Suspect	Negative
Positive	11	0	0
Negative	4	5	4

Table 2. Comparison of results of ELISA and of culture on vaginal discharge from 41 cows experimentally exposed to Brucella abortus Strain 2308 (Swab specimens were suspended in 1.5 ml phosphate-buffered saline; the results were observed at absorbance of 405 nm at 30 minutes substrate reaction time)

Culture results	ELISA results		
	Positive	Suspect	Negative
Positive	23	2	0
Negative	1	1	14

GENERAL DISCUSSION

Enzyme-linked immunosorbent assays (ELISA) have been used for detecting antibodies in bacterial, viral, and certain parasitic diseases of cattle and other animals (79,83,84,102,104). Recent reports indicate that ELISA is a reliable test for detecting antibodies in milk and serum of cows from which *B. abortus* was isolated (14,39, 40,84,90). The results obtained in this study reveal that the ELISA can also be used for the detection of *Brucella* antigens in vaginal discharge of cattle that were experimentally exposed to *Brucella abortus* Strain 2308.

The results from the section I of this study indicate that variations in ELISA protocol produced different results. These included alterations in the *Brucella* antigen coating methods, antiserum concentrations, conjugate concentrations and the reaction time intervals of *Brucella* antiserum, conjugate, and enzyme-substrate. Therefore, it is apparent that most of these factors may influence ELISA reactions. The sensitivity of ELISA was increased when carbodiimide was added to antigen suspensions. This may have been due in part to an increase in covalent binding between antigens and the surface of the microcuvettes (10, 19,87). Therefore, carbodiimide was added when studies were made for the detection of *Brucella* antigen in vaginal swab

suspensions.

The active charge sites which remained on the microcuvette surface after the incubation of the antigens and antibodies were neutralized by the addition of NH_4Cl solution (a blocking agent). Therefore, the nonspecific binding of the active charge sites and other proteins in the specimen were minimized; this provided a distinct difference in ELISA reactions observed between positive samples and negative samples.

Several different enzymes have been used to label antigens and antibodies used in ELISA (96,97). The enzyme must be stable, highly reactive, available in a purified form, inexpensive and yield conjugates that are not hazardous. In addition, a convenient substrate detector system must be available. Enzymes which have been used include acetyl cholinesterase, β-D-galactosidase, glucoamylase, glucose oxidase, β-D-glucuronidase, lactate dehydrogenase, lactoperoxidase, ribonuclease and tyrosinase (97). However, alkaline phosphatase and horseradish peroxidase are now preferred because they can react with a wide variety of substrates (96,97). In this study, affinity purified goat antiguinea pig IgG labeled with horseradish peroxidase was used because it was stable and produced low ELISA reactions on negative samples.

The technique for the collection of vaginal swab

samples is very important; vaginal samples for ELISA or for culture must be carefully collected to avoid contamination. The vulvar region should be cleaned and swabbed with a cotton ball saturated with isopropyl alcohol before sampling. A sterile, disposable culture swab enclosed in a plastic sheath should be used to obtain the swab specimen from the anterior region of the vagina near the cervix. This will minimize the possibility of contamination of the swab specimen.

Infection by B. abortus can stimulate both the cellular and humoral immune responses of the host. Complement-dependent phagocytosis has been reported to play an important role in the engulfment of Brucella by leukocytes in blood (51,78); however, the intracellular location of Brucella in leukocytes due to this immune response may decrease the possibility of their isolation or detection on ELISA. Natural immunoglobulins in vaginal secretion of cows include those synthesized locally or selectively transported to the region (29, 105). Studies have shown that serum components derived by transudation provided for a much higher proportion of total protein in vaginal mucus than in other external secretions. The mechanism of antibody immunity at the vaginal mucous membrane surface, and the role of the serum-derived immunoglobulins remain unclear.

A previous investigation indicated that an improved culture method using a selective enrichment broth containing tryptose broth base, hemin, bacitracin, cycloheximide, nalidixic acid, polymyxin B sulfate, vancomycin, and sodium amylosulfate (SAS) was useful in detecting cows shedding low numbers of *Brucella* in vaginal discharge (6). Sodium amylosulfate (SAS) has been shown to be an inhibitor of both the cellular and humoral antimicrobial systems (7,11, 31,32,38,48,49,50); therefore, when vaginal mucus samples were placed in the enrichment broth that contained SAS, the antimicrobial activity in vaginal secretion was limited. The addition of SAS to the enrichment broth appeared to have a potential advantage in increasing the recovery of *B. abortus* from the vaginal mucus samples of cows experimentally inoculated with *B. abortus* Strain 2308; therefore, additional studies should be conducted to determine the value of SAS in increasing ELISA reactions in *B. abortus* infected cattle shedding *Brucella* in the vaginal discharge.

Bacteria, other than *B. abortus*, were in most cases effectively controlled by inhibitors incorporated in the enrichment broth; but in some instances, colonies of contaminants were more numerous on plates subcultured from the enrichment broth than on the direct streak plates (6,12). In this research study, all vaginal swab samples were collected and suspended in 0.5 ml of tryptose broth as transport

media, and a comparison was made using PBS and tryptose broth as the diluents. The results of these experiments revealed that swab samples diluted in 1.5 ml of tryptose broth provide less satisfactory ELISA results than samples diluted in PBS; this was due to increased ELISA reactions (background values) in negative specimens. The enrichment broth increases the recovery of B. abortus from the vaginal mucus of experimentally exposed cattle (6); however, the peptones and other proteins in tryptose broth, contributed to the increased ELISA background reactions. A prospective research study would be to use the enrichment broth as transport media and preserve the swab samples in PBS for ELISA. This would provide a method for increasing the possibility of detecting Brucella in vaginal swab samples, avoiding the possible background reactions from enrichment broth, and minimizing contamination.

The results reported herein reveal an important correlation between the isolation of Brucella by the direct streaking of swabs of vaginal discharge onto plates of agar medium, and the detection of Brucella by ELISA. The ELISA used was sufficiently sensitive to detect cows that shed Brucella immediately after parturition. The detectable Brucella observed by ELISA were comparable to results of culture methods utilized. The ELISA developed is a rapid method;

therefore, the results can be obtained the same day. Culture methods usually require that media be incubated for at least 10 to 14 days to obtain results. The ELISA shows promise for use by veterinary practitioners to detect and remove cattle shedding *Brucella* before culture results can be obtained. The shedding of *Brucella* through vaginal discharges at the time of abortion or normal parturition, is considered the most significant way of transmitting the disease from infected to noninfected cattle (53). Therefore, the early diagnosis and removal of cattle shedding *Brucella* should minimize the transmission of infection and increase the possibility of eradicating brucellosis from cattle. Further investigations are needed to compare, standardize, and evaluate the ELISA, using different transport media and diluents in herds naturally infected with *B. abortus*.

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APPENDIX

Table A1. Results of enzyme-linked immunosorbent assays for different methods of coating *Brucella abortus* Strain 19 whole cell antigen onto polystyrene cuvettes (the results were measured at 30 minutes enzyme-substrate reaction time)

Antigen dilution	PBS		Na_2CO_3		NA_2CO_3 + Carbodiimide		NA_2CO_3 + Carbodiimide + NH_2Cl	
1:512	0.564[a]	0.122[b]	0.670[a]	0.134[b]	0.844[a]	0.227[b]	0.889[a]	0.121[b]
1:1024	0.414	0.117	0.570	0.120	0.725	0.201	0.782	0.111
1:2048	0.333	0.101	0.483	0.100	0.617	0.194	0.647	0.103
1:4096	0.238	0.084	0.325	0.087	0.481	0.163	0.521	0.117
1:8192	0.182	0.074	0.245	0.072	0.347	0.121	0.377	0.101
1:16384	0.074	0.084	0.121	0.075	0.267	0.107	0.325	0.098
1:32768	0.084	0.081	0.110	0.086	0.218	0.097	0.267	0.089
1:65536	0.074	0.071	0.089	0.094	0.188	0.087	0.236	0.093
None	0.032	0.039	0.050	0.044	0.041	0.043	0.051	0.047

[a]Results using guinea pig antibrucella serum (1:640 dilution).

[b]Results using guinea pig negative control serum (1:640 dilution).

Table A2. Results of enzyme-linked immunosorbent assays for different methods of coating *Brucella abortus* Strain 19 whole cell antigen onto polystyrene cuvettes (the results were measured at 60 minutes enzyme-substrate reaction time)

Antigen dilution	PBS		Na_2CO_3		NA_2CO_3 + Carbodiimide		NA_2CO_3 + Carbodiimide + NH_2Cl	
1:512	0.647[a]	0.160[b]	0.781[a]	0.178[b]	0.986[a]	0.289[a]	1.121[a]	0.174[b]
1:1024	0.508	0.147	0.672	0.156	0.845	0.267	0.890	0.167
1:2048	0.417	0.136	0.537	0.131	0.710	0.220	0.767	0.154
1:4096	0.326	0.110	0.446	0.127	0.591	0.194	0.657	0.164
1:8192	0.223	0.089	0.338	0.113	0.434	0.175	0.450	0.137
1:16384	0.187	0.096	0.227	0.117	0.351	0.172	0.387	0.121
1:32768	0.196	0.094	0.217	0.120	0.312	0.141	0.324	0.105
1:65536	0.183	0.083	0.201	0.119	0.279	0.122	0.278	0.109

[a]Results using guinea pig antibrucella serum (1:640 dilution).

[b]Results using guinea pig negative control serum (1:640 dilution).

Table A3. Results of enzyme-linked immunosorbent assays using different dilutions of guinea pig antibrucella serum and of negative control serum (conjugate was used at a 1:300 dilution)

	Antigen dilutions							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
Guinea pig antibrucella serum dilutions								
1:40	1.477	1.319	1.171	0.958	0.874	0.701	0.637	0.597
1:80	1.231	1.187	1.024	0.837	0.647	0.510	0.474	0.431
1:160	1.113	1.002	0.889	0.761	0.578	0.422	0.384	0.337
1:320	1.112	0.897	0.804	0.709	0.502	0.394	0.357	0.311
1:640	1.054	0.881	0.791	0.679	0.498	0.387	0.341	0.307
1:1280	0.807	0.699	0.613	0.532	0.373	0.254	0.226	0.208
1:2560	0.813	0.687	0.602	0.526	0.405	0.228	0.203	0.190
Guinea pig negative serum dilutions								
1:40	0.337	0.323	0.303	0.281	0.280	0.278	0.269	0.260
1:80	0.241	0.237	0.231	0.226	0.217	0.206	0.197	0.177
1:160	0.152	0.150	0.149	0.147	0.149	0.141	0.148	0.134
1:320	0.157	0.158	0.154	0.157	0.150	0.150	0.137	0.140
1:640	0.114	0.117	0.111	0.113	0.114	0.126	0.115	0.105
1:1280	0.096	0.106	0.106	0.107	0.096	0.101	0.099	0.097
1:2560	0.087	0.094	0.089	0.096	0.089	0.104	0.100	0.101

Table A4. Results of enzyme-linked immunosorbent assays using different dilutions of guinea pig antibrucella serum and of negative control serum (conjugate was used at a 1:600 dilution)

	Antigen dilutions							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
Guinea pig antibrucella serum dilutions								
1:40	1.337	1.161	0.974	0.891	0.806	0.747	0.619	0.581
1:80	1.205	0.952	0.882	0.784	0.606	0.498	0.470	0.447
1:160	1.007	0.848	0.757	0.667	0.541	0.456	0.383	0.379
1:320	0.903	0.812	0.667	0.581	0.470	0.357	0.326	0.326
1:640	0.889	0.791	0.689	0.540	0.434	0.331	0.310	0.287
1:1280	0.712	0.581	0.487	0.383	0.282	0.199	0.186	0.174
1:2560	0.730	0.570	0.458	0.337	0.261	0.190	0.167	0.148
Guinea pig negative serum dilutions								
1:40	0.351	0.323	0.308	0.307	0.267	0.261	0.253	0.250
1:80	0.261	0.231	0.231	0.241	0.201	0.220	0.192	0.190
1:160	0.164	0.147	0.148	0.153	0.143	0.160	0.160	0.160
1:320	0.159	0.143	0.150	0.151	0.141	0.161	0.147	0.152
1:640	0.121	0.111	0.103	0.117	0.101	0.089	0.089	0.083
1:1280	0.127	0.101	0.096	0.097	0.087	0.079	0.080	0.071
1:2560	0.121	0.112	0.100	0.101	0.091	0.081	0.080	0.070

Table A5. Results of enzyme-linked immunosorbent assays using different dilutions of guinea pig antibrucella serum and of negative control serum (conjugate was used at a 1:1200 dilution)

	Antigen dilutions							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
Guinea pig antibrucella serum dilutions								
1:40	0.493	0.407	0.362	0.327	0.316	0.307	0.292	0.271
1:80	0.357	0.317	0.297	0.281	0.270	0.255	0.236	0.205
1:160	0.297	0.255	0.238	0.213	0.198	0.186	0.180	0.162
1:320	0.238	0.236	0.219	0.097	0.186	0.175	0.162	0.141
1:640	0.221	0.223	0.206	0.186	0.175	0.162	0.151	0.133
1:1280	0.137	0.138	0.138	0.137	0.117	0.075	0.079	0.080
1:2560	0.135	0.122	0.133	0.101	0.087	0.056	0.061	0.065
Guinea pig negative serum dilutions								
1:40	0.151	0.142	0.131	0.126	0.115	0.103	0.097	0.080
1:80	0.111	0.126	0.119	0.117	0.097	0.080	0.072	0.068
1:160	0.097	0.100	0.096	0.091	0.080	0.067	0.067	0.065
1:320	0.077	0.078	0.076	0.077	0.062	0.051	0.051	0.062
1:640	0.071	0.074	0.070	0.071	0.057	0.048	0.049	0.050
1:1280	0.068	0.069	0.070	0.070	0.057	0.039	0.040	0.040
1:2560	0.067	0.063	0.064	0.064	0.049	0.036	0.036	0.033

Table A6. Comparison of enzyme-linked immunosorbent assays results of goat antiguinea pig conjugate titration using a 1:640 dilution of guinea pig antibrucella serum[a]

Conjugate dilutions	Antigen dilutions							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
1:300	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
1:600	84.3%	89.7%	87.1%	79.5%	87.1%	85.5%	90.9%	93.5%
1:1200	21.0%	25.3%	26.0%	27.3%	35.1%	41.6%	44.3%	43.3%

[a]Based on the absorbance value obtained using a 1:300 conjugate dilution (see Tables A3-A5).

Table A7. Results of enzyme-linked immunosorbent assays using various time intervals for antigen-antiserum (1:640 serum dilution) incubation (a 1:600 dilution of conjugate was incubated for 15 minutes)

Antigen-antiserum incubation time (minutes)	Antigen dilution							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
15	0.339	0.292	0.258	0.192	0.169	0.121	0.114	0.106
30	0.507	0.387	0.317	0.244	0.197	0.147	0.129	0.117
60	0.634	0.530	0.356	0.300	0.244	0.163	0.157	0.150
120	0.665	0.578	0.417	0.356	0.305	0.193	0.189	0.181

Table A8. Results of enzyme-linked immunosorbent assays using various time intervals for antigen-antiserum (1:640 serum dilution) incubation (a 1:600 dilution of conjugate was incubated for 30 minutes)

Antigen-antiserum incubation time (minutes)	Antigen dilution							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
15	0.671	0.597	0.489	0.388	0.282	0.181	0.152	0.124
30	0.684	0.649	0.574	0.396	0.301	0.206	0.172	0.166
60	0.925	0.817	0.690	0.578	0.451	0.376	0.341	0.320
120	1.026	0.897	0.786	0.678	0.564	0.471	0.422	0.354

Table A9. Results of enzyme-linked immunosorbent assays using various time intervals for antigen-antiserum (1:640 serum dilution) incubation (a 1:600 dilution of conjugate was incubated for 60 minutes)

Antigen-antiserum incubation time (minutes)	Antigen dilution							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
15	0.711	0.661	0.532	0.416	0.304	0.197	0.171	0.143
30	0.730	0.690	0.579	0.425	0.347	0.223	0.201	0.183
60	0.970	0.892	0.767	0.621	0.515	0.459	0.383	0.329
120	1.103	0.921	0.810	0.697	0.607	0.508	0.431	0.384

Table A10. Results of enzyme-linked immunosorbent assays comparing various time intervals for antigen-antiserum (1:640 serum dilution) incubation using a 1:600 dilution of conjugate for 120 minutes

Antigen-antiserum incubation time (minutes)	Antigen dilution							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
15	0.771	0.687	0.547	0.435	0.341	0.219	0.178	0.158
30	0.791	0.707	0.646	0.533	0.341	0.237	0.213	0.198
60	1.003	0.920	0.794	0.638	0.565	0.474	0.415	0.365
120	1.107	0.947	0.854	0.722	0.637	0.513	0.467	0.402

Table A11. Results of enzyme-linked immunosorbent assays reactions for different enzyme-substrate incubation times using a 1:640 dilution of guinea pig antibrucella serum

Enzyme-substrate incubation time (minutes)	Antigen dilution							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
10	0.625	0.569	0.377	0.304	0.222	0.209	0.174	0.167
20	0.713	0.626	0.422	0.350	0.249	0.236	0.198	0.187
30	0.904	0.789	0.640	0.520	0.357	0.325	0.274	0.236
40	0.956	0.826	0.661	0.545	0.381	0.346	0.294	0.251
50	1.043	0.871	0.704	0.587	0.401	0.369	0.311	0.267
60	1.121	0.912	0.767	0.657	0.451	0.387	0.324	0.278

Table A12. Results of enzyme-linked immunosorbent assay reactions for different enzyme-substrate incubation times using a 1:640 dilution of guinea pig negative control serum

Enzyme-substrate incubation time (minutes)	Antigen dilution							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
10	0.133	0.131	0.131	0.120	0.101	0.097	0.094	0.087
20	0.139	0.133	0.134	0.129	0.111	0.104	0.101	0.096
30	0.170	0.164	0.167	0.147	0.129	0.121	0.117	0.103
40	0.224	0.198	0.194	0.188	0.146	0.138	0.129	0.121
50	0.268	0.226	0.231	0.213	0.186	0.176	0.146	0.136
60	0.296	0.283	0.278	0.268	0.214	0.194	0.177	0.157

Table A13. Comparison of enzyme-linked immunosorbent assays results (ratio) obtained using guinea pig antibrucella serum and guinea pig negative control serum at different enzyme-substrate incubation times

Enzyme-substrate incubation times (minutes)	Antigen dilution							
	1:512	1:1024	1:2048	1:4096	1:8192	1:16384	1:32768	1:65536
10	4.69	4.30	2.87	2.53	2.19	2.15	1.85	1.91
20	5.13	4.70	3.14	2.71	2.24	2.27	1.96	1.94
30	5.32	4.81	3.80	3.54	2.81	2.69	2.34	2.30
40	4.27	4.17	3.40	2.89	2.60	2.50	2.27	2.07
50	3.89	3.85	3.04	2.75	2.15	2.09	2.13	1.96
60	3.78	3.22	2.76	2.45	2.10	1.99	1.83	1.77

[a]P/N ratio = $\frac{\text{ELISA result of guinea pig antibrucella serum (1:640 dilution)}}{\text{ELISA result of guinea pig negative control serum (1:640 dilution)}}$.

Table A14. Comparison of results of enzyme-linked immunosorbent assays and of culture on vaginal discharge from 24 cows experimentally exposed to *Brucella abortus* Strain 2308 (swab specimens were suspended in 1.5 ml tryptose broth; the results were observed at absorbance of 405 nm at 30 minutes substrate reaction time)

Cow number	ELISA result (absorbance value)		Culture result	Cow number	ELISA result (absorbance value)		Culture result
2	0.421	+	+	61	0.280	±	-
5	0.763	+	+	64	0.277	±	-
9	0.898	+	-	65	1.007	+	-
10	0.278	±	-	66	0.236	±	-
12	0.370	+	+	69	0.162	-	-
14	0.652	+	+	72	0.174	-	-
18	0.811	+	+				
20	0.331	+	+				
21	0.839	+	+				
22	0.194	-	-				
23	0.568	+	+				
25	0.540	+	-				
27	0.288	±	-				
29	0.389	+	+				
32	0.506	+	-				
41	0.127	-	-				
47	0.707	+	+				
48	0.716	+	+				

Table A15. Comparison of results of enzyme-linked immunosorbent assays and of culture on vaginal discharge from 41 cows experimentally exposed to Brucella abortus Strain 2308 (swab specimens were suspended in 1.5 ml phosphate-buffered saline; the results were observed at absorbance of 405 nm at 30 minutes substrate reaction time)

Cow number	ELISA result (absorbance value)		Culture result	Cow number	ELISA result (absorbance value)		Culture result
3	0.976	+	+	49	0.191	-	-
6	0.344	+	+	50	0.079	-	-
8	0.850	+	+	51	0.811	+	+
11	0.195	-	-	52	0.761	+	+
13	0.591	+	+	53	0.091	-	-
15	0.495	+	+	54	0.301	+	+
16	0.190	-	-	55	0.243	±	+
17	0.086	-	-	56	0.201	±	-
19	0.491	+	+	57	0.430	+	+
24	0.540	+	+	58	0.229	±	+
26	0.366	+	+	59	0.545	+	+
30	0.161	-	-	62	0.154	-	-
31	0.147	-	-	63	0.388	+	+
33	0.174	-	-	68	0.491	+	-
34	0.426	+	+	70	0.082	-	-
35	0.535	+	+	71	0.127	-	-
36	0.318	+	+				
37	0.081	-	-				
38	0.537	+	+				
39	0.387	+	+				
40	0.107	-	-				
42	0.483	+	+				
44	0.525	+	+				
45	0.528	+	+				
46	0.321	+	+				