A coagglutination test for the detection of *Brucella abortus* antigen and antibody in cows' milk

by

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INTRODUCTION

Bovine brucellosis is a disease characterized by abortion and a subsequent high rate of infertility in cows. Brucella abortus has been identified as the etiological agent. The primary clinical sign of the disease is abortion, although most cows develop a chronic, focal, nonpurulent, interstitial mastitis which is apparent at clinical examination. As many as nine of ten cows infected with B. abortus excrete the organism continuously or intermittently in milk (20).

Currently, bacteriological recovery of viable organisms or detection of Brucella antibody by the Brucella ring test (BRT) are the most widely accepted methods for diagnosis of udder infection by B. abortus. However, both methods have limitations. The sensitivity of the BRT is influenced by the size of the herd contributing to the pooled milk sample. The test sensitivity is adjusted according to the size of the herd (1). Even by increasing the sensitivity, the BRT is not as effective in detecting infection in large herds as compared to small herds (104). Cultural techniques are essential in definitive diagnosis, but also have limitations (98):

1) excretion of B. abortus from the udder may be intermittent, requiring several isolation attempts to validate the results

2) identification of the organism is delayed since an incubation period of four to five days is required

3) the high cost in time, labor and materials

In recent years a rapid slide coagglutination technique has been developed and used to identify antigens from both gram-positive and gram-negative bacteria. This test utilizes a strain of Staphylococcus aureus (Cowan I) which contains protein A in the cell wall. The basis for the staphylococcal coagglutination test is the ability of protein A to bind the Fc fragment of immunoglobulin G, leaving the Fab fragments free to participate in antigen-antibody reactions. The addition of homologous antigen to antibody-coated staphylococci results in a coagglutination reaction.

The coagglutination technique may be applicable in the detection of B. abortus antigen and B. abortus antibody in milk from Brucella-infected cows. Conceivably, soluble Brucella antigens which could not be detected by cultural techniques could be present in milk from infected animals. The coagglutination test would be a feasible method for detecting Brucella antigens since coagglutination occurs not only with whole cells, but also soluble antigens (134). Conversely, Brucella antibody in milk may be bound to protein A via the Fc fragment and reacted with known concentrations of B. abortus antigen.

The purpose of this study was to determine if the coagglutination test is a simpler, more specific and more sensitive test than the standard procedures for the diagnosis of udder infection by B. abortus. Research in developing and evaluating the coagglutination test for detection of B. abortus antigen and B. abortus antibody are described.

REVIEW OF LITERATURE

Bovine Brucellosis - A Historical Review

Brucella abortus was first identified as the etiological agent of bovine brucellosis by Bang in Denmark in 1897 (3). He isolated the organism from aborted bovine fetuses and fetal membranes and was able to reproduce the disease in healthy pregnant heifers. Bang's discovery was later confirmed by Preisz in Hungary (105), McFadyean and Stockman in Great Britain (93) and McNeal and Kerr in the United States (94).

In 1894 Schroeder reported the presence of tuberculous-like lesions in the liver and spleen of guinea pigs that had been inoculated intraperitoneally with milk suspected of containing tubercle bacilli (119). The gross pathological appearance resembled tuberculosis, although no acid-fast bacilli were isolated. The nature of the disease was unknown. Zeller conducted similar experiments and observed lesions of undetermined etiology which he classified as pseudotuberculosis (139).

It was not until 1911 that Schroeder and Cotton (120) and Mohler and Traum (96) independently isolated Brucella abortus from milk by guinea pig inoculation. Other

investigators substantiated their results shortly thereafter (128, 141). In retrospect, Schroeder concluded that the abortion bacillus must have been present in the milk samples of their earlier studies. His data indicated that the organism persisted in the udders of infected cows and was eliminated intermittently or continuously in the milk of animals that no longer aborted. Cotton reported that eighteen of nineteen cows studied were naturally infected with B. abortus and all but one shed the organism in milk for periods varying from six months to four years and three months (19).

Pathogenesis of Udder Infection

Infection with B. abortus in susceptible animals occurs naturally by exposure to contaminated materials either through ingestion or penetration of the mucous membranes or intact skin (12, 130, 131). After the initial invasion of the body, the bacteria enter the lymphatics and are transported to the regional lymph nodes, usually within polymorphonuclear leukocytes. Most Brucella are ingested initially by these cells. The polymorphonuclear leukocytes are ineffective against Brucella, which resist digestion and multiply within the cell (22). Brucella are either

contained within the regional lymph nodes or proliferate and are spread throughout the body within phagocytic cells, especially polymorphonuclear leukocytes, via the lymphatics and blood (90). Brucella abortus has a predilection for the male and female reproductive organs, but commonly localize in other organs and tissues such as the spleen, mammary gland, lymph nodes and bone marrow, and occasionally in joints and tendon sheaths (88). Brucella abortus persists in supramammary lymph nodes or udders of 90% or more of infected dairy cows.

The most common clinical sign of the disease is abortion although infected cows may develop a chronic, focal, nonpurulent, interstitial mastitis which is not apparent at clinical examination (117). The infection occurs commonly in one or two quarters, but rarely in all four and more frequently in the rear quarters than the front quarters (73).

Invasion of the udder by B. abortus may produce an acute, subacute and chronic inflammatory response, the latter predominating (108, 114). Lesions occur in the alveoli, the interalveolar connective tissue and less frequently in the interlobular connective tissue and along the lactiferous ducts. The histological changes are those of an acute or subacute nature when the alveoli are involved and of a chronic nature when the interstitial tissue is involved.

The changes occur in a progressive manner involving the parenchyma initially and the interstitial tissue later. Numerous papers describe the histopathological changes in Brucella-infected udders (27, 45, 51, 121, 122).

Excretion of B. abortus in milk is usually intermittent and may persist for several years (129) or in some cases for the rest of the animal's life (54). Excretion of Brucella is not constant and may vary from 200,000 per milliliter to a single organism (131, 133). The organisms accumulate in the udder during the dry period. Consequently, excretion is highest during the first days of lactation and decreases as lactation advances (133).

Excellent reviews have been written which describe the nature of Brucella infection in the mammary gland and its effect (47, 54).

Immunoglobulins of the Brucella-infected Mammary Gland

In addition to the cellular defense mechanism, the host responds to the invasion of B. abortus by the production of specific immunoglobulins. Brucella antibodies are present in milk and may be demonstrated in milk or whey. Most evidence indicates that the antibodies are locally produced in the mammary gland. One of the first attempts to determine the source of Brucella agglutinins in cows milk was conducted

by Smith, Orcutt and Little (129). In this study heat-killed B. abortus was injected into one or more quarters of the udders of cows and the agglutinins in blood and milk measured. They noted an increased agglutinin titer in the injected quarters as well as an increased seroagglutinin titer. They concluded that each quarter produced agglutinins as a result of direct stimulation by B. abortus. It appeared that specific antibodies were unable to pass from the blood to the milk, but passed readily from the udder to the blood. Kerr, Pearson and Rankin also concluded that specific antibodies to B. abortus were synthesized locally in the udder when infused with killed B. abortus (62). Since the noninfused control quarters failed to show a whey agglutinin titer, it was assumed that no infiltration of serum globulins into the milk occurred. Similar studies were performed in sheep to determine whether the infusion of antigens into the dry mammary glands of pregnant ewes produced local immunity (76). The results indicated that during lactation most of the specific antibody was of local origin.

Beh analyzed the distribution of Brucella antibody among immunoglobulin classes in serum and whey of cattle (6). The quantity of specific Brucella antibody present in milk whey and colostral whey of naturally infected cattle was determined using the single radial immunodiffusion

technique. In milk whey 54% of the total IgA was specific Brucella antibody, whereas in colostral whey 24% of the total IgA was specific for B. abortus. A high proportion of IgG1 in both colostral whey (36%) and milk whey (46%) was specific antibody. Specific Brucella IgG2 was not detected in colostral whey and only 7% of the total IgG2 detected in milk whey was specific for B. abortus. Less than 4% of the total IgM in the colostral whey sample was specific Brucella antibody. Specific IgM was not present in two of three milk whey samples, however, 30% of the total IgM present in the third sample was specific Brucella antibody.

Purified preparations of IgG1, IgG2, IgA, IgM and low molecular weight antibody from serum and whey of Brucella positive cattle were tested for Brucella antibody activity in the Brucella ring test (BRT) and other commonly used diagnostic tests (5). The results indicated that IgM and IgA antibody reacted more quickly in the BRT than IgG1 and IgG2. Low molecular weight antibody and IgG2 appeared to have less affinity for the fat globule membrane than the antibody in the other classes of immunoglobulins. Collin also concluded that purified preparations of milk immunoglobulins, IgM and IgA, from a naturally Brucella-infected udder reacted in the BRT, whereas, IgG1 and IgG2 did not (18).

Diagnosis of Udder Infection

The definitive diagnosis of brucellosis is based on the isolation and identification of B. abortus. In the absence of a positive culture, a presumptive diagnosis is made based on a positive specific serological response. Udder infection is detected in animals that excrete specific Brucella antibodies in milk by the BRT. The BRT is a satisfactory test for the surveillance of dairy herds (1). The major limitation of the test is the dilution factor which occurs in large dairy herds where large quantities of milk are stored in bulk tanks. Roepke and Stiles concluded that a single BRT would have a 65% probability of detecting one reactor cow in more than 95% of the herds in most dairy states if the tests were conducted on bulk tank samples (111). The sensitivity of the test is varied according to the size of the herd (1). Holm, et al., (52) and Ferguson and Robertson (29) concluded that the individual BRT could be applied as a routine method for diagnosis of brucellosis. Pietz (104), however, states that the BRT should be used only as a supplemental test on individual animals since udder infection follows systemic infection and there usually is not a significant level of antibodies in milk until after the udder is infected.

Other agglutination tests have been devised for the detection of Brucella antibodies in milk, including the whey plate test (97, 137). Cameron considered the whey plate test an adequate substitute for blood tests for diagnosis of reactors in problem herds (16). The same group later recommended that the whey plate test be used in conjunction with blood tests (9). Roepke, et al., stated that the whey plate test was not suitable as a diagnostic test since Brucella was isolated from 16% of the milk samples which were whey plate test negative (112). Individual milk or whey tests are considered supplemental tests in the diagnosis of brucellosis.

Staphylococcal Protein A

Background

Jensen first noted that the cell wall of most strains of Staphylococcus aureus contained an extractable product which precipitated in agar gel with normal human sera (57). This was considered a true antigen-antibody reaction with natural antibodies. He designated the cell wall extract "antigen A" and suggested it was a polysaccharide. Simultaneous work by Beiser, et al., also showed that bacterial extracts of S. aureus precipitated human immunoglobulin G (7). Analysis of Jensen's antigen A preparation after

purification by electrophoresis indicated it was composed primarily of protein (84, 85, 86). Grov, Myklestad, and Oeding labeled the cell wall extract protein A to distinguish it from polysaccharide A (40). Forsgren and Sjöquist further investigated the nature of the reaction between protein A and human gamma globulin (33). Their results showed that the reaction is not a true antigen-antibody reaction, but rather, the reactive site of the gamma globulin is restricted to the Fc fragment.

Physicochemical and biological properties of protein A

Protein A of S. aureus could be detected on cell walls of intact bacteria by use of radioactively labeled myeloma globulins (68). Ninety percent (141 strains of 156 total) of the strains of S. aureus tested contained protein A. Lachica noted a high frequency of occurrence of protein A among strains of S. aureus of nonhuman origin (74). Most strains of S. aureus synthesize protein A, however, there is a variation in the amount produced in individual strains (68, 74). Analysis of staphyloccal protein A reactivity by electron microscopy shows that protein A resides in the outermost layer of the cell wall and is evenly distributed over the whole cell (82). Further analysis of protein A using enzymatic digestion demonstrated that it is a cell wall

component and that it is covalently linked to the peptidoglycan structure (127). Kronvall, et al., estimated that 80,000 protein A residues may be present on S. aureus strain Cowan I (70). In addition to formation of cell-bound protein A, most strains release protein A extracellularly. Movitz reported the secretion of extracellular protein A in various strains (ranging from 8% to 30%) during the exponential growth phase of S. aureus (100).

The physicochemical properties of protein A have been described (11, 35). Protein A is a single polypeptide chain of molecular weight 42,000 as determined by sedimentation analysis and gel chromatography. The affinity constant for human or rabbit IgG is approximately 10^8 l/mole (59, 70). Sjöquist determined that one mole of protein A binds two moles of IgG (126).

Hjelm, et al., employed partial tryptic digestion of purified protein A and of intact S. aureus in order to generate the smallest possible fragment of protein A still retaining the ability to bind the Fc fragment of IgG (50). Six active fragments were isolated from purified protein A and two active fragments from intact S. aureus by affinity chromatography. All fragments contained only one active site and appeared to have similar amino acid sequences. Sjödahl demonstrated that protein A contains three highly homologous

Fc-binding regions each consisting of more than 50 amino acids (123).

The biological function of protein A reactivity has been investigated. Dossett suggests that protein A may be anti-phagocytic by masking important antigenic sites or by competing with polymorphonuclear leukocytes for γG sites on the Fc fraction (23). The in vivo administration of protein A gives rise to the same types of hypersensitivity phenomena as those resulting from antigen-antibody reactions. Protein A is capable of evoking an anaphylactic-like reaction in guinea pigs (43) and an Arthus-like reaction in rabbits (42). Furthermore, the binding of IgG to protein A activates complement (66, 124, 132).

Interaction between immunoglobulins and protein A

Forsgren and Sjöquist found that protein A of S. aureus precipitated normal rabbit γG globulin (34), normal guinea pig serum (31, 32) and normal human serum (33) nonspecifically. He demonstrated that the reactivity between protein A and human γG globulin is not a true antigen-antibody reaction. Diffusion in agar gel of protein A and papain fragments of normal γG globulin indicated that the reaction was mediated through the Fc fragment rather than the Fab fragment (33). Lind investigated the difference between

protein A reactivity of S. aureus and the specific adsorption of homologous antibodies by means of ferritin-labeled rabbit serum globulins and electron microscopy (82). In an experiment where rabbit antiferritin globulin was bound to S. aureus prior to exposure to the antigen (ferritin), he demonstrated that the antibody-combining sites were free after the globulin combined with protein A and confirmed the Fc fragment involvement. Attempts to find a fragment of IgG smaller than the Fc fragment that reacts with protein A have not been successful (28, 65, 135).

The ability of immunoglobulins to react with protein A is primarily confined to γG globulins among serum proteins. The protein A-binding structures in the Fc fragment in human IgG can be detected only in subclasses IgG1, IgG2, and IgG4 (64), whereas in the mouse, IgG2a, IgG2b, and IgG3 are reactive, but not IgG4 (69). Kronvall, et al., examined γG globulin from species representing seven classes and 30 orders of living vertebrate for reactivity with protein A by agar gel diffusion (71, 72). No positive reactions were observed among Amphibia and Reptilia, but IgG from all 65 mammalian species tested reacted with protein A. The reactivity between protein A of S. aureus Cowan I and serum globulins from different species was found to vary (41). Adsorption of normal sera from man, dog and swine with Cowan I removed all

but traces of γG globulin when analyzed by immunoelectrophoresis (81). However, adsorption of normal sera from two other species, the sheep and the cow, removed the slow γG globulin (IgG2), but not the fast γG globulin (IgG1). Goudswaard, et al., reported that the binding of ruminant IgG1 was very poor (37).

Contrary to early reports (31, 80, 125) that protein A does not have the capacity to bind immunoglobulins other than IgG, recent evidence indicates other immunoglobulin classes are reactive. Bennell and Watson examined the interaction of porcine and ovine serum and colostral immunoglobulins with protein A (8). All classes and subclasses of immunoglobulins in the serum and whey bound protein A to some extent. The protein A binding capacity of ovine serum and colostral immunoglobulins of the same class and subclass were distinctly different.

The binding of some IgM produced by myeloma cells to protein A has been reported in humans (46, 83), mice (87) and rats (95). Grov has shown that human IgM was fixed to a protein A-Sepharose column and concluded that the reactive sites were localized in the Fc region (38). Human colostral IgA (39, 92) and human monoclonal IgA proteins (46, 115) have demonstrated protein A reactivity. It has been suggested that the binding of human IgM and IgA to

staphylococcal protein A may be a property of distinct subclasses (46, 87, 115). It has recently been shown that polyclonal IgM and IgA of the pig, dog and cat may be separated into protein A reactive and nonreactive fractions (37). A previous study on a single IgD and a single IgE myeloma protein suggested that these classes are nonreactive with protein A (69). However, Johansson and Inganäs found that polyclonal human IgE interacts with protein A (58). The same investigators found that the IgE peptic fragment Fab'_2 and not Fc" retained the ability to react with protein A (55).

Applications

The capacity of staphylococcal protein A to bind IgG via the Fc fragment has numerous applications in immunological methodology (36). Purification of immunoglobulins and fractionation of subclasses have been conducted by affinity chromatography on protein A-Sepharose. Intact staphylococci are useful as a solid phase adsorbent for isolating antigen-antibody complexes and membrane antigens and receptors. Protein A has also been used in the study of surface antigens on intact cells.

The property of staphylococcal protein A to nonspecifically bind IgG has also been applied in diagnostic microbiology. S. aureus cells are coated with antibody via the

Fc fragment leaving the Fab fragment free to participate in antigen-antibody reactions. The addition of homologous antigen to the antibody-coated staphylococci results in an agglutination, or rather, a coagglutination reaction. The coagglutination technique was first described by Kronvall in 1973 as a rapid slide agglutination test for typing pneumococci (64). The rapid slide coagglutination test has been used to identify antigens from both gram positive and gram negative bacteria. Staphylococci coated with antibodies directed against streptococcal group-specific antigens have been used in the identification and serological grouping of streptococci (17, 30, 44, 77, 89). Edwards and Larson developed a more rapid technique for grouping beta-hemolytic streptococci in which the coagglutination reaction is performed directly on a colony on the primary isolation plate (26). The coagglutination test has been utilized as a method for identifying Neisseria gonorrhoeae (21, 79) and Neisseria meningitidis (61, 103, 140). Tests were done on slides with suspensions of cultured organisms or cerebrospinal fluid, or directly on agar plates. Suksanong and Dajani employed a coagglutination test and detected Haemophilus influenzae type b antigen in 58% of the sera, 67% of the urine and 46% of the cerebrospinal fluid samples from patients with H. influenzae type b septicemia (134). Salmonella typhi antigens were detected in 97% of the urine

samples from patients with typhoid fever using the coagglutination test (110). Various other bacteria have been identified and grouped using the coagglutination technique: mycobacteria (60), Escherichia (53, 63), Pasteurella multocida (109), and Salmonella and Shigella (25, 116).

The coagglutination test has been described as a specific and sensitive rapid slide test. It was recently reported that the staphylococcal coagglutination test is more sensitive than counterimmunoelectrophoresis (24, 136, 138) and latex agglutination (136) in detecting bacterial antigens in body fluids.

MATERIALS AND METHODS

General Procedures

Experimental animals

Ninety-three of the cattle used in this study were Hereford-Angus cross heifers that were utilized in a vaccination experiment in the Brucellosis Research Unit at the National Animal Disease Center (NADC), Ames, Iowa. The heifers were challenged during midgestation (15 months post-vaccination) with virulent Brucella abortus strain 2308. The challenge dose was 1.3×10^7 colony forming units (CFU) and was administered by the conjunctival route.

Seventeen Holstein-Friesian cows from the Mastitis Research Herd at NADC were used as controls. The cows ranged in age from three to nine years and were chosen for controls because they had not been vaccinated with or exposed to B. abortus and their previous exposure to Staphylococcus aureus was known. The bacteriological and clinical mastitis history prior to the experiment is summarized in Table 12 of the Appendix. Several cows had a history of intramammary staphylococcal infection. All but three were free of S. aureus infection at the time of sampling.

Milk collections

Milk was collected at one and two weeks postpartum. Additional samples were collected from some cows that continued to excrete Brucella abortus in the milk. Each teat was disinfected with an alcohol swab and allowed to dry. The first two streams of milk were discarded and then 20 ml was collected from each quarter directly into a sterile glass tube. Each sampling provided five samples per cow (four quarter samples and one composite). Equal portions of milk from each quarter sample were combined in a fifth tube for the composite sample.

Bacteriological and serological procedures

All milk samples were cultured directly on tryptose agar containing 5% bovine serum, antibiotics, and ethyl violet as described by Alton, et al. (1). Two culture plates per sample were inoculated with 0.2 ml of the gravity cream. The inoculated plates were incubated at 37°C and examined on the fourth day postinoculation for Brucella colonies.

The presence of Brucella agglutinins was determined by the Brucella ring test (BRT) using the serial dilution method (137). Two-fold serial dilutions were made beginning at a 1:10 dilution. The highest dilution in which the intensity of color in the cream layer was deeper than that in the

skim portion was recorded as the end-titer of the sample. A BRT titer greater than 1:20 was interpreted as a positive reaction.

Preparation of whey

Whey was prepared by adding two or three drops of a Rennin[1] solution (1:50 dilution) per 10 ml of milk and incubating for two hours at 37° C. After centrifugation at 1000 x g for ten minutes, the whey was decanted and filtered through glass wool[2] to remove the milk fat. The whey samples were frozen at -70° C until tested.

The presence of Brucella agglutinins in whey was determined by the whey plate test (WPT) (137). Whey plate test reactions greater than 1:25 were considered positive.

Preparation of Staphylococcus aureus stock cultures

To assure the uniformity of the bacterial cultures throughout the study, frozen stock cultures of Staphylococcus aureus strains Cowan I[3] (protein A positive) and Wood 46[4]

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[1]Difco Laboratories, Detroit, MI.

[2]Corning Glass Works, Corning, NY.

[3]Provided by R. B. Rimler, NADC, Ames, IA.

[4]Provided by J. S. McDonald, NADC, Ames, IA.

(protein A negative) were maintained as a source of inoculum. The two staphylococcal strains were grown on tryptose agar in Roux flasks and incubated 18 hours at 37° C. The cells were harvested by washing the agar surface with 15 ml of double-strength sterile skim milk and freezing in one ml aliquots at -70° C.

Preparation of stabilized Staphylococcus aureus

Cowan I Stabilized suspensions of S. aureus strain Cowan I were prepared by the method of Kronvall (64). Flasks containing 500 ml of trypticase soy broth were inoculated with 0.2 ml of the stock culture. The cultures were incubated at 37° C for 18 hours on a shaker[1] and the cells harvested by centrifuging at 10,000 x g for five minutes in 250 ml bottles. The harvested cells from two containers were combined in a 50 ml centrifuge tube and washed three times in phosphate-buffered saline (PBS; 0.12 M NaCl-0.03 M Na_2HPO_4 at pH 7.3). The cells were resuspended in 45 ml of PBS containing 0.5% formalin and kept at room temperature for three hours. The cells were again washed three times in PBS and adjusted to a concentration of 10% (vol/vol). The suspension was heated at 80° C for one hour in a water bath

[1]Gyrotary Shaker, New Brunswick Scientific Co., New Brunswick, NJ.

to stabilize the protein A on the cell surface. The formaldehyde and heat treatment inactivates the enzymes that cause autolysis.

Wood 46 S. aureus strain Wood 46 was prepared either by the method of Kronvall previously described or by heat-killing. To prepare the heat-killed suspension an 18 hour broth culture was autoclaved at 121° C for 15 minutes and allowed to cool to room temperature. The cells were then washed three times and adjusted to a concentration of 10% (vol/vol).

A Coagglutination Test for the Detection of Brucella abortus Antigen

Basic procedures

Antisera All antisera used in this study were provided by the Brucellosis Research Unit, NADC, Ames, IA.

Preparation of antibody-coated staphylococci The stabilized suspension of S. aureus strain Cowan I was coated with antibody by mixing 0.1 ml of Brucella antiserum with 1.0 ml of the cell suspension and holding at 25° C for one hour. The coated staphylococci were washed once with PBS containing 0.1% sodium azide. This final suspension was used as the coagglutination reagent and was stable at 4° C for four weeks.

Coagglutination test Coagglutination tests were conducted on a glass plate by adding 0.05 ml of antibody-coated staphylococci to 0.05 ml of the whey sample to be tested. The plate was rotated at two minutes and results read after four minutes. Visible agglutination within four minutes was considered positive. Stabilized uncoated staphylococci and normal rabbit serum-coated staphylococci were used as controls. Whey samples which agglutinated controls were adsorbed with killed S. aureus Cowan I and retested.

Adsorption of whey samples Whey samples were adsorbed with S. aureus strain Cowan I to eliminate false positive reactions by removing antibodies specific to S. aureus and nonspecific IgG molecules. Only those whey samples giving positive reactions with test controls were adsorbed. One ml of the 10% suspension of stabilized Cowan I cells was added to 10 ml of whey and held at 37° C for two hours. The cells were sedimented by centrifugation and the whey recovered for testing.

Test development

Experiment 1 - Titration of Brucella abortus antigen in saline, milk and whey using the coagglutination reagent Various concentrations of B. abortus antigen were added to normal milk and normal whey and titrated using the coagglutination reagent. Preliminary titrations were performed in

saline to determine if the coagglutination test is a feasible method for detecting B. abortus antigen. Test samples were simulated using milk and whey as diluent and titrations repeated to determine the minimal concentration of B. abortus antigen that can be detected by the coagglutination test. Milk collected from the Iowa State University dairy herd was used as diluent. The milk was a composite sample from more than 25 cows and was negative to the BRT. Whey was prepared from the composite sample.

One milliliter of standard USDA tube agglutination test antigen[1] was added to 44 ml of 0.85% saline to give a concentration of 1 mg of antigen per 1 ml of saline. The antigen was serially diluted ten-fold in saline, milk and whey. Each dilution was tested with S. aureus coated with rabbit anti-B. abortus 2308 (whole cell) serum and read after four minutes. In addition to the coagglutination test controls previously described, an antigen control (antigen plus diluent) was included.

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[1]The USDA National Veterinary Services Laboratories, Ames, IA.

Experiment 2 - The coagglutinating reactivity of S. aureus Cowan I coated with various antisera against B. abortus The reactivity between protein A of S. aureus Cowan I and serum globulins from various species has been reported to vary. Adsorption of normal sera from man, dog and swine with Cowan I removed all but traces of IgG. Adsorption of normal sera from two other species, the sheep and cow, removed the slow γG-globulin (IgG2) but not the fast γG-globulin (IgG1) (81). The objective of this study was to determine the most suitable antiserum for use as a coagglutination reagent for detecting B. abortus antigen in whey.

A comparison of the coagglutinating reactivity of S. aureus Cowan I coated with various antisera against B. abortus was conducted. Experimental coagglutination reagents were prepared by coating staphylococcal cells with B. abortus antisera obtained from cattle and rabbits. Antisera tested included:

1. Bovine anti-B. abortus 2308 (whole cell)
2. purified bovine anti-B. abortus strain 19 IgG1
3. purified bovine anti-B. abortus strain 19 IgG2
4. rabbit anti-B. abortus 2308 (whole cell)
5. rabbit anti-B. abortus strain 19 lipopolysaccharide (LPS)

Coagglutination tests using these five different

reagents were conducted with normal whey (BRT and WPT negative pooled milk sample from Iowa State University dairy herd) to which B. abortus tube agglutination test antigen was added. The whey was serially diluted ten-fold, beginning at a concentration of 1 mg of antigen per 1 ml of whey and titrated with each experimental coagglutination reagent. Tests were read at one minute intervals for eight minutes. Uncoated S. aureus cells and normal rabbit serum or normal bovine serum coated S. aureus cells were used as controls.

Experiment 3 - Evaluation of a microcoagglutination test

Part I. Titration of B. abortus antigen in whey

A microcoagglutination test was evaluated to determine its sensitivity in detecting B. abortus antigen in whey. The purpose of this experiment was to determine if the microtiter method was more sensitive than the rapid plate method, i.e., capable of detecting smaller quantities of Brucella antigen.

Preliminary trials were conducted with normal whey to which B. abortus tube agglutination test antigen was added at an initial concentration of 1000 µg/ml of whey. Two-fold serial dilutions were made in disposable U-bottom microtiter plates[1] by transferring 50 µl of whey-antigen mixture to 50

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[1]Linbro Scientific Co., Hamden, Conn.

µl of PBS with microdiluters from well 1 through well 11. Well 12 served as reagent control (coagglutination reagent plus diluent). Rabbit anti-B. abortus 2308 (whole cell) serum-coated cells were standardized at an optical density of 0.4 at wavelength 520 A[1] and 50 µl of this suspension was added to each well. The procedure was repeated with cell suspensions of 0.6 and 0.8 optical density. Tests were incubated at 37° C and read macroscopically at 24 and 48 hours. Formation of a solid pellet or "button" with a well-defined edge was considered negative. Formation of a layer of cells with irregular edges or "shield" was interpreted as a positive reaction.

Part II. The effect of pH on the microcoagglutination test Since the reagent controls in part I were positive, this experiment was designed to study the effect of pH on the microcoagglutination test and to determine the optimal pH. A one percent suspension of rabbit anti-B. abortus 2308 (whole cell) serum-coated S. aureus cells was prepared at the following pHs: 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, and 9.00 using Sorensen's phosphate buffer as diluent. Specific quantities of 0.06 M Na_2HPO_4 and 0.06 M KH_2PO_4 were mixed to obtain each pH (78). B. abortus tube

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[1]Spectronic 20, Bausch and Lomb, Rochester, NY.

agglutination test antigen was added to normal whey at a concentration of 100 µg/ml. The whey-antigen mixture was serially diluted two-fold by transferring 50 µl of whey to 50 µl of diluent (Sorensen's buffer) at the respective pH from well 1 through well 11. Well 12 served as the reagent control. Fifty microliters of the 1% (vol/vol) suspension of the antibody-coated cells at the eight different pHs were added to all wells in each corresponding row. Microtiter plates were incubated at 37° C and read at 24 and 48 hours. The procedure was repeated with suspensions of the coagglutination reagent prepared at optical densities 0.4, 0.6, and 0.8.

Experiment 4 - Precipitation reactions between various Brucella antisera and whey from Brucella-infected cows Because S. aureus Cowan I cells coated with rabbit anti-B. abortus 2308 (whole cell) serum aggregated in normal whey this experiment was done to evaluate the specificity of the reactions by using a gel diffusion technique.

Double immunodiffusion was performed in a medium containing 1% agarose of electrophoretic grade[1], 0.067 g Tris

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[1]ICN Pharmaceuticals, Cleveland, Ohio.

[tris(hydroxymethyl)aminomethane][1], 0.7 g Tris hydrochloride[2], 8.5 g NaCl and 10 mg protamine sulfate per 200 ml of distilled water. Ten milliliters of the melted agarose medium was poured into a 100 mm x 15 mm plastic petri plate. A pattern of one central well surrounded by six peripheral wells (3 mm in diameter) was cut in the agar 3 mm from each other. Four to six patterns were cut per plate.

Four different antisera obtained from rabbits hyperimmunized with B. abortus were analyzed for their ability to produce precipitin lines with whey from Brucella-infected cows by the agar-diffusion method. Antisera were prepared against B. abortus 2308 (whole cells), FAO/WHO reference strain B. abortus 544, B. abortus 2308 (cell surface protein) (CSP), and B. abortus strain 19 (CSP). Normal whey samples were included as controls. Concentrated soluble B. abortus strain 1119-3 antigen[3] was used as a positive antigen control. The wells were filled to agar surface with reactants. Reactions were read 18-24 hours after incubation at 37° C in a humidified chamber.

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[1]Trizma base, Sigma Chemical Co., St. Louis, Mo.

[2]Trizma-HCL, Sigma Chemical Co., St. Louis, Mo.

[3]Provided by J. M. Patterson, NADC, Ames, Ia.

Evaluation of the coagglutination test for Brucella antigen

Experiment 1 - Comparison of the coagglutination test, the Brucella ring test and direct culture for the diagnosis of udder infection by B. abortus A comparison of the coagglutination test, the Brucella ring test and direct culture for the diagnosis of udder infection was made. Milk samples were collected from one cow (Y-65) which was experimentally infected with B. abortus strain 2308 and consistently excreted Brucella organisms from two quarters. Milk was collected from each quarter every other day for two and a half months postpartum. Samples were examined by direct culture for the presence of Brucella organisms and by the BRT for Brucella antibodies as indicators of infection. Samples were also tested by the coagglutination test using cells coated with rabbit anti-B. abortus 2308 (whole cell) serum. Samples from the control herd were tested by the same procedures.

Experiment 2 - Further comparison of the Brucella ring test, direct culture and the coagglutination test for diagnosis of udder infection by B. abortus using a different coagglutination reagent Since S. aureus cells coated with rabbit anti-B.abortus 2308 (whole cell) serum aggregated when tested with whey from the control herd in experiment #1, the

experiment was repeated using a different coagglutination reagent. Stabilized staphylococcal cells were coated with rabbit anti-B. abortus 2308 (CSP) serum. In contrast to the whole cell antiserum, this serum did not show a precipitation reaction when tested by gel diffusion with normal whey.

A Coagglutination Test for the Detection of Brucella abortus Antibody

Basic procedures

Adsorption of whey All whey samples were adsorbed with S. aureus Wood 46, a protein A negative strain, to eliminate any antibodies specific to S. aureus. The whey was adsorbed once with a 10% suspension of S. aureus for one hour at 37° C at a proportion of 0.1 ml of the suspension per 1.0 ml of whey. The mixture was centrifuged at 1000 x g for 30 minutes and the supernatant removed for testing.

Preparation of standardized suspension of S. aureus A stabilized suspension of S. aureus Cowan I was prepared at an approximate concentration of 10% as previously described. Then the bacterial concentration of the suspension was more accurately standardized by a packed-cell volume method using Hopkins vaccine tubes.[1] The following quantities were

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[1]Kimble Co., Toledo, Ohio.

placed in a Hopkins vaccine tube with PBS and centrifuged at 1000 x g for 30 minutes: 0.2 ml, 0.3 ml, and 0.5 ml. The packed-cell volume of each lot of cells was determined.

Preparation of coated staphylococci A stabilized suspension of S. aureus strain Cowan I was coated with Brucella antibody present in the whey by adsorption. One milliliter of whey was mixed with 0.2 ml of the 10% suspension of staphylococcal cells and held at room temperature for one hour. The coated staphylococcal cells were restored to 1% by resuspending in PBS.

Coagglutination test The coagglutination test was performed by combining 0.05 ml of the coated S. aureus cells and 0.05 ml of B. abortus tube agglutination test antigen (1:200 dilution) on a glass plate. The glass plate was rotated at two minutes and the results read after four minutes.

Test development

Experiment 1 - Evidence for the reaction between anti-Brucella whey immunoglobulins and staphylococcal protein A The basis for the coagglutination reaction is the ability of protein A to bind the Fc fragment of immunoglobulin G leaving the Fab fragments free to participate in antigen-antibody reactions. This study was conducted to demonstrate that

whey immunoglobulins from Brucella-infected cattle react with protein A of S. aureus.

Agglutination tests The ability of Brucella antibodies to react with protein A was tested by determining the agglutination titer of whey before and after adsorption to S. aureus. Agglutination titers of whey from Brucella-infected cows were determined by the mercaptoethanol tube test (1) and the whey tube test (137). For immunoglobulin adsorption, one ml of a 10% suspension of S. aureus Cowan I was centrifuged at 1000 x g for 30 minutes and the supernatant removed. Whey (0.5 ml) was added to the pellet and the mixture was vortexed. After incubation at 25° C for one hour, the bacteria were sedimented by centrifugation and the whey removed for testing.

Immunoelectrophoresis Immunoelectrophoretic analysis was performed using an LKB apparatus[1] on glass plates covered with 12 ml of a 1% agarose gel in Tris-barbiturate buffer of pH 8.6. The buffer was prepared by combining 22.4 g diethylbarbituric acid, 44.3 g Tris, 0.533 g calcium lactate and 0.65 g NaN_3 in one liter of distilled water. The concentrated buffer was diluted 1:4 before use.

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[1]LKB 2117 Multiphor, LKB Produkter, Bromma, Sweden.

A pattern of five wells and four troughs were cut in the agar as shown in Figure 2. Whey samples from Brucella-infected cows were analyzed before and after adsorption with S. aureus strains Wood 46 and Cowan I. Whey was placed in the wells and electrophoresed for one hour at 10 V/cm. The troughs were filled with anti-bovine immunoglobulin preparations and incubated at room temperature for 24 hours. Immunoelectrophoretic patterns of adsorbed samples were compared to the patterns of the unadsorbed samples.

Experiment 2 - Determination of the optimal Brucella antigen concentration This experiment was done to determine the optimal antigen concentration to be used in the coagglutination test for the detection of B. abortus antibody in whey. Based on BRT and WPT results, eleven positive and six negative samples were selected for testing. S. aureus Cowan I cells were coated with B. abortus antibody by adsorbing whey from Brucella-infected cows as previously described. Antigen titrations were performed by combining 0.05 ml of coated staphylococcal cells and 0.05 ml of B. abortus tube agglutination test antigen at dilutions of 1:100, 1:200, and 1:400. The tests were read at 30 second intervals over a period of two minutes.

Evaluation of the coagglutination test for Brucella antibody

Experiment 1 - Evaluation of the coagglutination test for the detection of Brucella antibody in the whey of experimentally infected cows Seven hundred and eight whey samples from 93 cows experimentally exposed to B. abortus 2308 were tested for antibody to B. abortus by the coagglutination test, the Brucella ring test and the whey plate test. Whey samples from nonexposed animals were similarly tested.

Experiment 2 - The differentiation of specific and nonspecific agglutination reactions in the coagglutination test The occurrence of nonspecific serum agglutinins to B. abortus has been demonstrated (49). The level of nonspecific agglutinins is usually diagnostically insignificant, however, many false positive reactions in agglutination tests have been attributed to this factor. Nonspecific agglutinins in whey were considered as one possible explanation of the 'false positive' coagglutination reaction occurring in BRT and WPT negative samples. Several procedures for differentiating specific and nonspecific agglutination reactions were applied in this study in an attempt to eliminate false positive coagglutination reactions. Whey samples which exhibited negative results to the BRT and WPT, but positive results to the coagglutination test were selected

for testing. The results were compared to those of a duplicate test in which the whey samples were untreated.

Part I. A heat inactivation test Heat inactivation tests for differentiating specific and nonspecific agglutination reactions in sera from Brucella-infected cattle have been described. Nonspecific agglutinins, which were associated with suspect Brucella titers, were inactivated when treated with heat (2, 48, 99). In this experiment, a heat inactivation test was employed to differentiate specific and nonspecific agglutination reactions in the coagglutination test.

Whey samples were heat-treated in a 56° C water bath for 30 minutes. After heat inactivation, the samples were cooled and tested for Brucella agglutinins by the coagglutination test.

Part II. Low pH coagglutination test A plate agglutination test for bovine brucellosis has been described which distinguishes between specific and nonspecific agglutinins using a stained Brucella antigen buffered at pH 4.0. Sera containing nonspecific agglutinins gave negative reactions at pH below 4.25. In contrast, low-titer sera containing specific agglutinins were either unchanged or decreased one dilution at pH values between 3.8 and 4.25 (113). This experiment was designed to determine the

influence of low pH on the specificity of the agglutinins reacting in the coagglutination test.

The coagglutination test was conducted by prescribed procedures except coated staphylococcal cells were resuspended in a buffered diluent of pH 4.0. The diluent was prepared in the same manner as the USDA buffered Brucella antigen diluent, but without phenol (1). The pH was adjusted to 4.0 with sodium hydroxide.

Part III. Chelation of divalent cations The use of ethylenediaminetetraacetate (EDTA) to reduce nonspecific agglutination of B. abortus in the standard tube agglutination test has been described. Addition of EDTA to serum samples prevented 80% of nonspecific Brucella agglutination reactions with no interference in specific reactions (118). Nielsen, et al., described the use of EDTA and EGTA (ethylene-glycol-bis-(B-aminoethyl ether)N,N-tetraacetic acid) to reduce nonspecific agglutination of B. abortus in the standard tube agglutination and plate agglutination tests (102).

The purpose of this experiment was to determine the effect of chelating agents on the specificity of the coagglutination test. The coagglutination test was modified by

incorporating 0.1 mg of disodium EDTA[1] per 1.0 ml of whey after adsorption with S. aureus Wood 46.

Experiment 3 - Coombs test for the detection of incomplete antibodies in whey The positive coagglutination reaction in BRT and WPT negative samples may be attributed to the presence of incomplete antibodies in the whey. In this experiment the anti-globulin or Coombs test (1) was employed for the detection of incomplete antibodies. A series of BRT-WPT nonreactor whey samples which were positive by the coagglutination test was tested.

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[1]Fisher Scientific Co., Fair Lawn, New Jersey.

RESULTS

A Coagglutination Test for the Detection of *Brucella abortus* Antigen

Test development

Experiment 1 - Titration of B. abortus antigen in saline, milk, and whey using the coagglutination reagent

Brucella abortus antigen was added to saline, normal milk, and normal whey and titrated using the coagglutination reagent. The results are shown in Table 1. Preliminary titrations in saline demonstrated that the coagglutination test was a feasible method for detecting *B. abortus* antigen. Positive reactions were observed when test samples were simulated using whey as diluent. The minimal quantity of detectable antigen in saline and whey was between 10 and 100 μg/ml. An unexpected negative reaction was observed at an antigen concentration of 10,000 μg/ml of saline. Whole milk was not a suitable test sample since false positive reactions occurred at all antigen concentrations; also in all test and antigen controls. The reaction appeared to be a sedimentation of milk solids rather than an agglutination reaction.

Table 1. Titration of *Brucella abortus* antigen in saline, normal milk and normal whey

Diluent	Antigen concentration (μg/ml)	Coagglutination reagent[a]	Coagglutination reaction: Controls: Uncoated *S. aureus*	Coagglutination reaction: Controls: Normal rabbit serum coated *S. aureus*	Coagglutination reaction: Controls: Ag control (antigen + diluent)
Saline	10,000	-	-	-	-
	1,000	+	-	-	-
	100	+	-	-	-
	10	-	-	-	-
	1	-	-	-	-
Whey	10,000	+	-	-	-
	1,000	+	-	-	-
	100	+	-	-	-
	10	-	-	-	-
	1	-	-	-	-
Milk	10,000	A[b]	A	A	A
	1,000	A	A	A	A
	100	A	A	A	A
	10	A	A	A	A
	1	A	A	A	A

[a] *S. aureus* cells coated with rabbit anti-*B. abortus* 2308 (whole cell) serum.

[b] Atypical reaction; not specific antigen-antibody reaction.

Experiment 2 - The coagglutinating reactivity of S. aureus Cowan I coated with various antisera against B. abortus A comparison of the coagglutinating reactivity of S. aureus Cowan I coated with various antisera against B. abortus was made. Brucella antigen added to normal whey was titrated with the five different experimental coagglutination reagents (Table 2).

Positive reactions occurred with all experimental coagglutination reagents at an antigen concentration of 100 µg/ml of whey within two minutes. Within four to six minutes, positive reactions occurred at all antigen concentrations in titrations utilizing the three different bovine sera. These reactions did not appear to be a specific Brucella antigen-antibody reaction since positive reactions also occurred in titrations utilizing normal bovine serum. For this reason, bovine immune sera was not suitable for use in the coagglutination test. The strongest and most rapid agglutination occurred with cells coated with rabbit anti-B. abortus 2308 (whole cell) serum. The smallest quantity of detectable antigen was between 10 and 100 µg/ml of whey. The same quantity of antigen was detected when using cells coated with rabbit anti-B. abortus strain 19 LPS serum, although the reaction was slower and less intense. Based on

Table 2. Titration of B. abortus antigen using different coagglutination reagents

Antiserum	Antigen concentration µg antigen per ml normal whey	Coagglutination reaction read at one minute intervals 1	2	3	4	5	6	7	8
Rabbit anti-*B. abortus* 2308 (whole cell)	1000	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	0	-	-	-	-	-	-	-	-
Bovine anti-*B. abortus* 2308 (whole cell)	1000	+	+	+	+	+	+	+	+
	100	-	+	+	+	+	+	+	+
	10	-	-	-	+	+	+	+	+
	1	-	-	-	+	+	+	+	+
	0.1	-	-	-	+	+	+	+	+
	0	-	-	-	+	+	+	+	+
Rabbit anti-*B. abortus* strain 19 LPS	1000	-	+	+	+	+	+	+	+
	100	-	-	+	+	+	+	+	+
	10	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	0	-	-	-	-	-	-	-	-
Purified bovine anti-*B. abortus* strain 19 IgG1	1000	+	+	+	+	+	+	+	+
	100	-	+	+	+	+	+	+	+
	10	-	-	-	-	-	+	+	+
	1	-	-	-	-	-	+	+	+
	0.1	-	-	-	-	-	+	+	+
	0	-	-	-	-	-	+	+	+
Purified bovine anti-*B. abortus* strain 19 IgG2	1000	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+
	10	-	-	-	-	+	+	+	+
	1	-	-	-	-	+	+	+	+
	0.1	-	-	-	-	+	+	+	+
	0	-	-	-	-	+	+	+	+

these results, rabbit anti-B. abortus 2308 (whole cell) serum was selected for the preparation of the coagglutination reagent.

Experiment 3 - Evaluation of a microcoagglutination test

Part I. Titration of B. abortus antigen whey

A microcoagglutination test was evaluated to determine its sensitivity in detecting Brucella antigen in whey. B. abortus antigen was added to normal whey and titrated using antibody-coated staphylococci at three different optical densities. The minimal quantity of detectable antigen and the optimal cellular density could not be determined since positive reactions occurred in all wells, including the negative control wells. Formation of a shield in the negative control indicated that the results were not entirely due to a specific antigen-antibody reaction.

Part II. The effect of pH on the microcoagglutination test The effect of pH on the microcoagglutination test was evaluated. Preliminary trials were conducted only on the reagent controls since positive reactions were obtained in the diluent (negative) controls in Part I. Aggregation was observed in all wells containing antibody-coated cells at optical densities of 0.4, 0.6, and 0.8 (Table 3). A 1% (vol/vol) suspension of coated cells formed

Table 3. The effect of pH and cellular density on the aggregation of reagent controls

Reagent	pH	Cellular Density (OD) 0.4	0.6	0.8	1.0%[a]
Coated S. aureus[b]	5.65	+[c]	+	+	+
	6.14	+	+	+	+
	6.60	+	+	+	+
	7.04	+	+	+	+
	7.41	+	+	+	+
	8.12	+	+	+	+
	8.59	+	+	+	+[d]
	8.85	+	+	+	-
Uncoated S. aureus	5.65	+	+	+	-
	6.14	+	+	+	-
	6.60	+	+	+	-
	7.04	+	+	+	-
	7.41	+	+	+	-
	8.12	+	+	+	-
	8.59	+	+	+	-
	8.85	+	+	+	-

[a]Packed-cell volume determination (vol/vol).

[b]S. aureus cells coated with anti-B. abortus 2308 (whole cell) serum.

[c]Shield formation.

[d]Button formation.

partial buttons at pH 7.41, 8.12, and 8.59 and a complete button was formed at pH 8.85. A comparison of reagent controls using uncoated staphylococci showed shield formation at optical densities of 0.4, 0.6, and 0.8. However, when a

1% (vol/vol) suspension of uncoated cells was utilized no aggregation was observed at any of the pH values.

Titrations of B. abortus antigen were not done since shield formation occurred in diluent controls of antibody-coated staphylococci. The pH of the diluent was not a factor in the aggregation of antibody-coated staphylococci. Because of the spontaneous aggregation of coated cells, the microco-agglutination test could not be adapted for use in detecting B. abortus antigen in whey.

Experiment 4 - Precipitation reaction between various Brucella antisera and whey from Brucella-infected cows. Double immunodiffusion was used to evaluate the specificity of the reaction between various rabbit hyperimmune sera and whey from Brucella-infected cows. The resulting precipitation reactions are shown in Figure 1. A simple precipitin band was formed when B. abortus strain 1119-3 soluble antigen control was reacted with the four different Brucella antisera. The presence of precipitating antibodies in whey from infected animals was indicated by the formation of a precipitin line between whey and the soluble antigen control. A precipitation reaction was observed between whey from Brucella-infected cows and whole-cell (B. abortus 2308 and B. abortus 544) antisera. This reaction did not appear to

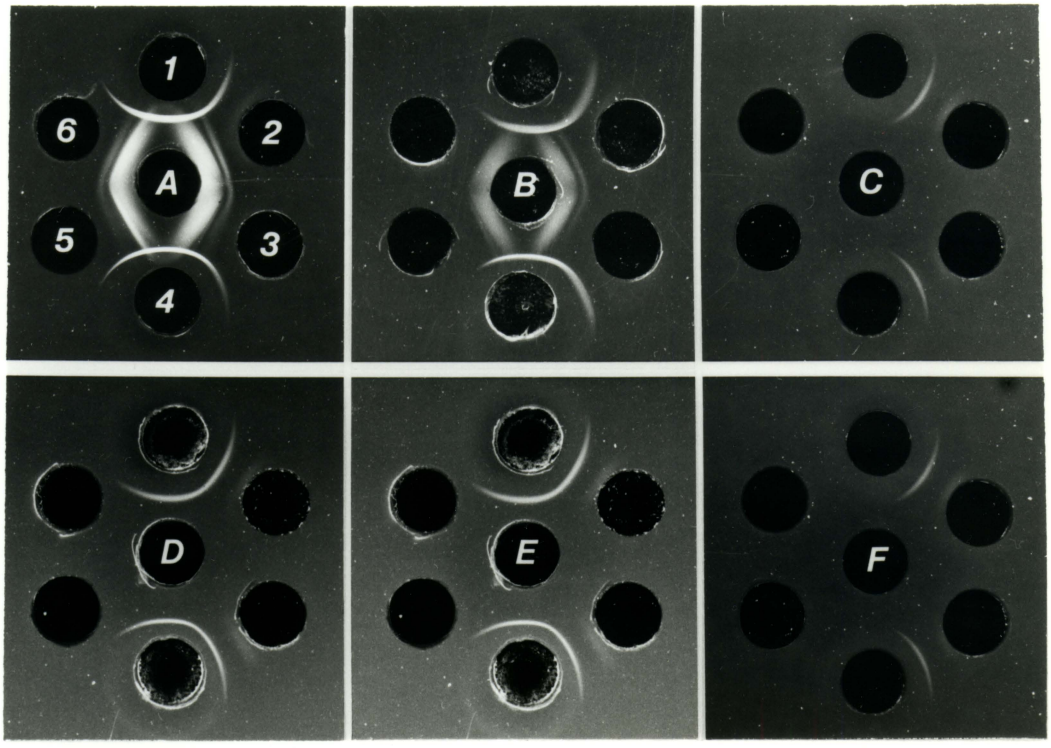
Figure 1. Precipitation reaction between various *Brucella* antisera and whey from *Brucella*-infected cows

Peripheral wells:

1 = *B. abortus* strain 1119-3 soluble antigen
2 = whey, Y-65 RR
3 = whey, Y-65 LF
4 = *B. abortus* strain 1119-3 soluble antigen
5 = normal whey, 247
6 = normal whey, ISU dairy herd

Center wells:

A = rabbit anti-*B. abortus* 2308 (whole cell) serum
B = rabbit anti-*B. abortus* 544 serum
C = normal rabbit serum 167
D = rabbit anti-*B. abortus* 2308 (CSP) serum
E = rabbit anti-*B. abortus* strain 19 (CSP) serum
F = normal rabbit serum 166


1
6
2
A
5
3
4
B
C
D
E
F

be a specific Brucella antigen-antibody reaction since a precipitation reaction was also observed between normal whey and these antisera. This reaction was not observed when whey from infected animals was tested with antisera prepared against protein-rich Brucella fractions (B. abortus 2308 and B. abortus strain 19). Since the Brucella cells used for immunization of rabbits were grown on a medium containing bovine serum, this reaction may be due to rabbit anti-bovine immunoglobulins. However, adsorption with insolubilized normal bovine serum did not remove the reacting components.

Evaluation of the coagglutination test for Brucella antigen

Experiment 1 - Comparison of the coagglutination test, the Brucella ring test and direct culture for the diagnosis of udder infection by B. abortus The data shown in Table 4 are a summary of a comparison of the coagglutination test, the Brucella ring test and direct culture for the diagnosis of udder infection. B. abortus was consistently isolated from the same two quarters in each of the thirteen samplings (52 samples). Direct culture results ranged from an initial 5×10^3 CFU/ml with a gradual decrease to 25 CFU/ml at the sixth week postpartum (Table 13 of Appendix). B. abortus agglutinins were detected in 51 of 52 samples by the BRT. The BRT colostral titers were high in all quarters. In the culture positive quarters BRT titers remained high, fluctuating between 1280 and 5120. In the culture negative

Table 4. Comparison of coagglutination test, *Brucella* ring test and direct culture using two different reagents for use in the diagnosis of udder infection by *Brucella abortus*

	S. aureus coated with rabbit anti-*B. abortus* 2308 (whole cell) serum			
	Group 1[a]		Control 1[b]	
	Samples		Samples	
	Tested	Positive	Tested	Positive
Coagglutination test	52	52	25	25
Brucella ring test	52	51	25	0
Direct culture	52	26	25	0

[a]Group 1: all samples were collected from infected cow Y-65.

[b]Control 1: samples were collected from nonexposed cows #s 154, 215, 238, 245, and 247.

[c]Group 2: samples were collected from infected cows Y-65 and C-28.

[d]Control 2: samples were collected from nonexposed cows #s 154, 238, and 247.

S. aureus coated with rabbit anti-*B. abortus* 2308 (CSP) serum			
Group 2[c]		Control 2[d]	
Samples		Samples	
Tested	Positive	Tested	Positive
12	0	12	0
12	12	12	0
12	6	12	0

quarters, the BRT titers rapidly decreased and fluctuated between 160 and negative (Table 13 of Appendix). All 52 samples were positive by the coagglutination test. All 25 control samples were negative by direct culture and the BRT, but positive by the coagglutination test.

Results were inconclusive since rabbit anti-B. abortus 2308 (whole cell) serum-coated cells aggregated in normal whey.

Experiment 2 - Further comparison of the Brucella ring test, direct culture and the coagglutination test for the diagnosis of udder infection using a different coagglutination reagent Because of the aggregation of coated cells in Experiment 1, a further comparison of the BRT, direct culture and the coagglutination test was conducted using staphylococci coated with rabbit anti-B. abortus 2308 (CSP) serum (Table 4). B. abortus was isolated from 50% (6 of 12) of the samples tested. The number of organisms excreted in the milk ranged from 5 CFU/ml to 175 CFU/ml. Brucella agglutinins were detected in all samples. BRT titers of culture positive samples were 2560 or greater and the titers of culture negative samples varied from 40 to 160. None of the samples were positive by the coagglutination test. The normal whey samples were negative by all three tests. The coagglutination test for the detection of antigen was not a

satisfactory method for detecting udder infection by B. abortus.

A Coagglutination Test for the Detection of Brucella abortus Antibody

Test Development

Experiment 1 - Evidence for the reaction between anti-Brucella whey immunoglobulins and staphylococcal protein A

Agglutination test The ability of Brucella antibodies to react with protein A was tested by determining the agglutination titer of whey before and after adsorption with S. aureus Cowan I. Agglutination titers of whey from Brucella-infected cows were determined by the mercaptoethanol tube test and the whey tube test (Table 5). Most of the samples showed a reduction in titer by one dilution after the second adsorption as determined by both agglutination tests. Two samples showed no reduction in titer, whereas, four samples showed a reduction in titer by the mercaptoethanol test and not by the whey tube test. Reduction in the whey agglutinin titers after adsorption with Cowan I cells indicated that anti-Brucella immunoglobulins were bound to protein A.

Table 5. Binding capacity of S. aureus cells for Brucella antibodies in whey: agglutinin titers before and after adsorption with strain Cowan I

Sample[a]	Before adsorption WTT[b]	Before adsorption ME[c]	1st adsorption WTT	1st adsorption ME	2nd adsorption WTT	2nd adsorption ME
1	I800[d]	+800[e]	I800	I800	I800	I400
2	I6400	I6400	+3200	I3200	+3200	I3200
1	I800	I800	I800	I800	I800	I400
58	I400	I400	+200	I400	I400	+200
44	+800	+800	I800	+800	I800	I800
98	+1600	+1600	I1600	I1600	+800	+800
11	+800	+800	I800	I800	+800	+400
19	I400	I400	+200	I400	+200	I200
22	I1600	I1600	+800	I1600	+800	+800
47	I3200	I3200	+1600	+1600	+1600	+1600
66	+3200	I3200	I1600	+1600	I1600	+1600
28	I50	I50	I50	I50	I50	I50

[a]Composite sample.

[b]Whey tube test.

[c]Mercaptoethanol test.

[d]I: incomplete reactions.

[e]+: complete reactions.

Immunoelectrophoresis The reactivity of Brucella whey immunoglobulins and staphylococcal protein A was examined. Whey samples were subjected to immunoelectrophoresis before and after adsorption with S. aureus strains Cowan I and Wood 46 and compared (Figure 2). Immunoelectrophoretic analysis of unadsorbed whey showed formation of a precipitation line with anti-IgA, but not anti-IgM; two lines were observed with IgG antiserum. The adsorption with Wood 46 had no effect on the precipitation pattern. Brucella whey immunoglobulins of the IgA class did not readily react with protein A. Only one subclass of bovine IgG reacted with protein A. After one adsorption of the whey with Cowan I, one of the IgG components was completely removed.

Experiment 2 - Determination of the optimal Brucella antigen concentration The sensitivity of the coagglutination test was evaluated at different antigen concentrations. Seventeen (11 positive and 6 negative) BRT and WPT tested samples were selected for testing. Results are summarized in Table 6. At an antigen dilution of 1:400 only eight samples gave a positive coagglutination reaction. The eleven positive samples coagglutinated at antigen dilutions of 1:100 and 1:200. The BRT and WPT negative samples did not coagglutinate at any antigen dilution. A dilution

Figure 2. Immunoelectrophoretic analysis of whey from *Brucella*-infected cow Y-65 before and after adsorption with *S. aureus* strains Cowan I and Wood 46

U = unadsorbed whey
C1 = whey adsorbed once with Cowan I
W = whey adsorbed with Wood 46
C2 = whey adsorbed twice with Cowan I

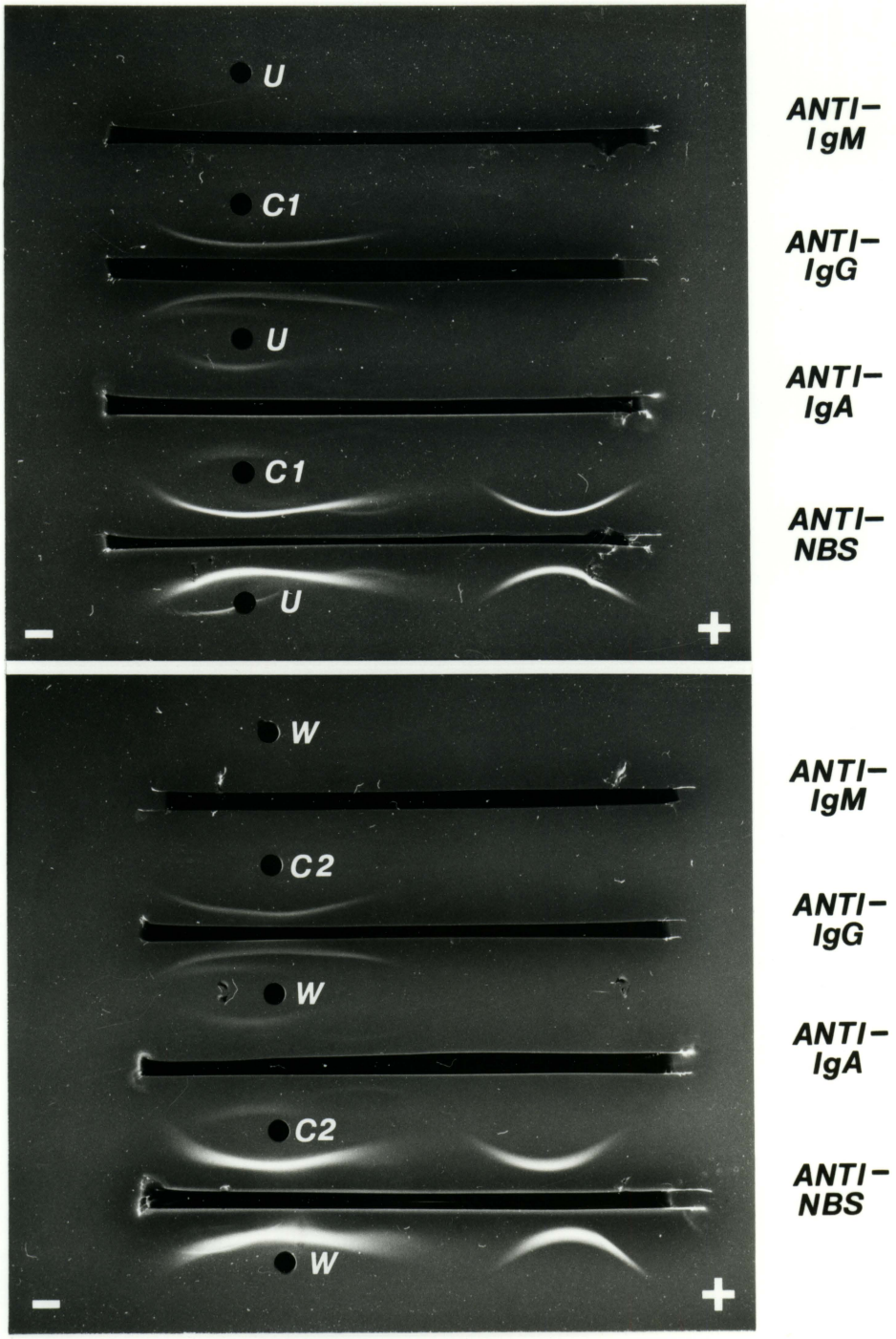

U
C1
U
C1
U
ANTI–
IgM
ANTI–
IgG
ANTI–
IgA
ANTI–
NBS
–
+
W
C2
W
C2
W
ANTI–
IgM
ANTI–
IgG
ANTI–
IgA
ANTI–
NBS
–
+

Table 6. Determination of the optimal Brucella antigen concentration in the coagglutination test

	Antigen dilution	Positive	Negative
Coagglutination reaction	1:100	11[a]	6
	1:200	11	6
	1:400	8	9
BRT		11	6
WPT		11	6

[a]Reaction results read after two minutes.

of 1:200 was determined to be the optimal antigen concentration in the coagglutination test.

Evaluation of the Coagglutination Test for Brucella Antibody

Experiment 1 - Evaluation of the coagglutination test for the detection of B. abortus antibody in the whey of experimentally infected cows Seven hundred and eight whey samples from infected cows and 127 samples from nonexposed cows were collected and tested by the BRT, WPT, and coagglutination test. Based on antibody response, the samples were divided into two categories: BRT-WPT reactors (BRT > 1:20 or WPT > 1:25) and BRT-WPT nonreactors (BRT ≤ 1:20 and

WPT ≤ 1:25). Of 375 exposed reactors, 362 (96.5%) were positive by the coagglutination test. Of 333 exposed nonreactors, 247 (74.2%) were positive by the coagglutination test, whereas only 16 of 127 (12.6%) noninfected controls were positive (Table 7). In many of the test samples (not all samples were examined), aggregation of staphylococcal cells occurred prior to the addition of *Brucella* antigen. Detailed BRT, WPT, coagglutination test, and culture results of these samples are recorded in Table 14 of the appendix.

Table 7. Evaluation of the coagglutination test for detection of *Brucella* antibody in the whey of experimentally infected cows

	Total samples tested	Coagglutination test positive
Brucella exposed,[a] reactors[b]	375	362
Brucella exposed, nonreactors[c]	333	247
Nonexposed controls	127	16

[a]*Brucella* vaccinated and challenged.

[b]BRT > 1:20 or WPT > 1:25.

[c]BRT ≤ 1:20 and WPT ≤ 1:25.

Experiment 2 - The differentiation of specific and nonspecific agglutination reactions in the coagglutination test

Part I. A heat inactivation test A heat inactivation test was conducted in an attempt to differentiate specific and nonspecific agglutination reactions. No significant difference was found when comparing the results of the coagglutination test before and after heat inactivation of the whey samples (Table 8). In a group of 16 BRT-WPT nonreactor samples, 15 (93.8%) were coagglutination positive before heat treatment and 13 (81.3%) were positive after heat

Table 8. Comparison of coagglutination test results before and after heat inactivation of whey

	Samples tested	Coagglutination test positive	
		Before treatment	After treatment
Brucella exposed, reactors[a,b]	23	23	23
Brucella exposed, nonreactors[c]	16	15	13
Nonexposed controls	32	4	7

[a] *Brucella* vaccinated and challenged.

[b] BRT > 1:20 or WPT > 1:25.

[c] BRT ≤ 1:20 and WPT ≤ 1:25.

treatment. No difference in the coagglutination test results was found in 23 BRT-WPT reactor samples after heat inactivation. Four of 32 control samples were positive before heat treatment and seven were positive after heat treatment. Heat inactivation of whey samples did not significantly alter the coagglutination test results.

Part II. Low pH coagglutination test A low pH coagglutination test was employed to distinguish between the presence or absence of nonspecific agglutinins in whey. The effect of low pH on the coagglutination test is shown in Table 9.

Table 9. The effect of low pH on the coagglutination test

	Samples tested	Coagglutination test positive	
		Diluent pH 7.3	Diluent pH 4.0
Brucella exposed,[a] reactors[b]	11	7	11
Brucella exposed, nonreactors[c]	23	17	23
Nonexposed controls	8	0	5

[a] *Brucella* vaccinated and challenged.

[b] BRT > 1:20 or WPT > 1:25.

[c] BRT ≤ 1:20 and WPT ≤ 1:25.

All eleven of the exposed reactor samples agglutinated at pH 4.0. Seventy-four percent (17 of 23) of the exposed nonreactor samples were positive at pH 7.3 compared to 100% at pH 4.0. Agglutination occurred in 63% of the control samples at low pH, but all controls were negative at normal pH. Increased aggregation of cells was observed in the reactor, nonreactor, and control groups when resuspended in low pH diluent.

Part III. Chelation of divalent cations A chelating agent (EDTA) was used to reduce nonspecific coagglutination reactions in whey. The addition of EDTA to whey did not significantly affect the coagglutination test results when compared to the untreated samples (Table 10). All 13 of the expected positive samples were positive by the coagglutination test before and after EDTA treatment. Seventeen of 19 expected negative samples were positive to the coagglutination test before and after EDTA treatment.

Experiment 3 - Coombs test for the detection of incomplete antibodies in whey The Coombs test was conducted to detect the presence of incomplete antibodies in whey. BRT-WPT nonreactor samples which gave positive coagglutination reactions were tested using the Coombs test. Incomplete antibodies were not detected in any of the 28 test samples, nor in eight control samples.

Table 10. Comparison of coagglutination test results before and after EDTA treatment

	Samples tested	Coagglutination test positive	
		Untreated	EDTA treated
Brucella exposed, reactors[b]	13	13	13
Brucella exposed, nonreactors[c]	21	17	16
Nonexposed controls	8	0	0

[a] *Brucella* vaccinated and challenged.

[b] BRT > 1:20 or WPT > 1:25.

[c] BRT ≤ 1:20 and WPT ≤ 1:25.

DISCUSSION

Although isolation and identification of Brucella abortus from milk constitutes a definitive diagnosis of brucellosis, cultural examination is time consuming. The coagglutination technique has been described as a specific and sensitive rapid slide test for the identification of bacterial antigens and was applied in the examination of milk for the presence of B. abortus antigen.

The coagglutination test proved to be a practicable method for detecting Brucella antigen in whey. Brucella antigen added to negative whey could be detected at a concentration between 10 and 100 μg/ml. Whole milk was not satisfactory for use in the coagglutination test. Titrations of B. abortus antigen using whole milk from nonexposed cows gave atypical reactions which resembled aggregation. This reaction appeared to be a sedimentation of milk solids.

Attempts to increase the sensitivity of the Brucella antigen assay were conducted by adapting the coagglutination test to a microtiter system. The microcoagglutination test proved to be an unsatisfactory method for detecting Brucella antigens since spontaneous aggregation of coated cells occurred in diluent controls.

B. abortus antigen was not detected in whey samples from

cows experimentally infected with B. abortus 2308 by the coagglutination test. These results are in agreement with those reported by Pruett (107). Pruett tested for the presence of free B. abortus antigen in serum and milk samples using a double antibody sandwich ELISA (enzyme-linked immunosorbent assay). Antigens were not detected in the milk or serum of culture positive animals by this technique.

The inability of the coagglutination test to detect B. abortus antigen in milk from infected cows may be attributed to two factors. First, the coagglutination procedure may not be sensitive enough to detect the quantity of B. abortus antigen that is eliminated in cow's milk. Between 10 and 100 μg/ml of B. abortus antigen could be detected when added to normal whey. Based on a comparison of viable counts and packed cell volumes for B. abortus strain 19 (1), it was estimated that there are approximately 3.3×10^5 cells per 100 μg of B. abortus standard tube test antigen. The number of viable organisms as determined by direct culture of the milk samples ranged from 5 to 200,000 per ml. The majority of milk samples tested contained less than 100 cells per ml, which would not be a sufficient number of organisms for detection by the coagglutination test. However, enumeration of Brucella organisms by the viable count method may not be an accurate estimation of antigen concentration since it does

not include nonviable cells.

The second factor which may contribute to the inability of the coagglutination test to detect antigen is the formation of antigen-antibody complexes. Brucella antigen may be bound to specific antibody which is already present in the whey. Antibodies attached to staphylococcal protein A are unable to bind Brucella antigen because the antigenic sites are blocked by specific antibody which is produced locally in the mammary gland. The presence of Brucella antibody in milk from infected cows was confirmed by the BRT. Work by Prichard (106) and Pruett (107) also indicated that Brucella antigen in milk was bound to specific antibody. Using the ELISA technique Pruett could not detect antigen in milk from experimentally infected cows, but could detect antigen (as low as 0.75 μg) added to negative control milk. However, when antigen was added to milk which contained Brucella antibody the results became negative. Pruett concluded that the presence of excess antibody in milk precludes the detection of added B. abortus antigen. Prichard studied the fluorescent antibody method as a means of detecting B. abortus antigen in tissues. Specific fluorescence was not observed in naturally infected milk which had a BRT titer of 1:1024. Specific fluorescence was noted in replicate tests after treatment with urea; also in tests in which known

concentrations of Brucella antigen were added to urea-treated and normal milk. Prichard concluded that urea treatment of milk caused dissociation of the antigen-antibody complex so that antigen became available for combination with the fluorescent antibody conjugate.

One problem encountered in the coagglutination test for detection of antigen was the occurrence of false positive reactions. In part, these reactions could probably be attributed to the presence of antibodies to staphylococci in the whey. The incidence of staphylococcal alpha antitoxin occurring in whey has been reviewed by Brown (13). Alpha antitoxin occurs in the whey at a much lower level than in blood. The presence of staphylococcal alpha antitoxin is indicative of a past or current exposure to S. aureus (15). Some whey samples gave positive agglutination reactions when tested with uncoated staphylococcal control. Adsorption of these samples with Cowan I cells eliminated most of the false positive reactions. This would indicate that the reaction was largely due to antibodies to staphylococcal antigens. Adsorption with Cowan I cells also eliminates false reactions by removing other IgG molecules via the Fc fragment that could potentially react with staphylococcal protein A on antibody-coated cells (103).

Another factor contributing to false positive reactions

is the tendency of some antibody-coated staphylococci to aggregate. Bovine immune serum was not suitable for use in the coagglutination test since coated staphylococcal cells aggregated. S. aureus cells sensitized with bovine anti-B. abortus 2308 (whole cell) serum and cells coated with normal bovine serum agglutinated in PBS and normal whey. This reaction may be caused by specific staphylococcal antibodies present in the bovine immune sera. Brown and Scherer reported that staphylococcic antitoxins were found in the blood of a large percentage of dairy cattle (14). S. aureus cells sensitized with purified fractions (IgG1 and IgG2) of bovine anti-B. abortus strain 19 antisera aggregated in the presence of normal whey but not PBS. Specific antibodies to S. aureus in bovine sera were either absent or present in a concentration too low to give an agglutination reaction. Another explanation may be that specific agglutinating staphylococcal antibodies are of an immunoglobulin class other than IgG. Aggregation of sensitized staphylococcal cells in normal whey and not in PBS indicated that there was a component in the whey which contributed to the aggregation of the cells. Rabbit hyperimmune sera proved to be a more suitable reagent in sensitizing staphylococcal cells for the coagglutination test. Sensitized cells did not agglutinate in PBS implying the absence of antistaphylococcal antibodies in

rabbit sera. However, aggregation of sensitized cells in normal whey did occur with all but two of the rabbit hyperimmune sera, anti-B. abortus strain 2308 (CSP) and strain 19 (CSP). Analysis by gel diffusion demonstrated a reaction between the rabbit hyperimmune sera and the bovine whey. The reacting component was not identified. This reaction was not observed when using anti-B. abortus 2308 (CSP) serum. Therefore, this antiserum was selected for the preparation of the coagglutination reagent.

In the absence of a positive culture, a presumptive diagnosis of brucellosis is made based on the detection of Brucella antibody. The ability of the coagglutination test to detect B. abortus antibody in whey is dependent on the binding capacity of staphylococcal adsorbent. Kronvall measured the capacity of staphylococci to adsorb IgG by use of ^{125}I-labeled normal human IgG. Formaldehyde-treated S. aureus Cowan I cells showed a maximal uptake of approximately 2 mg of IgG for every 1 ml of a 10% suspension (64). In this study, no quantitative determinations of the binding capacity of staphylococci was made. However, immunoelectrophoretic analysis of whey (BRT-WPT positive) from Brucella-infected cows before and after adsorption with S. aureus Cowan I indicated that whey immunoglobulins of the IgG class reacted with staphylococcal protein A. One of the IgG components

was completely removed after one adsorption. The binding capacity was also tested by determining the agglutination titer of whey before and after adsorption by the mercaptoethanol and whey tube tests. Most samples showed a reduction in titer by one dilution after the second adsorption as measured by both tests. Four samples showed a reduction in titer by the mercaptoethanol test and not by the whey tube test. These results would be expected since theoretically, the mercaptoethanol test detects IgG and the ability of immunoglobulin to react with protein A is limited to IgG. The reduction in the whey agglutinin titers after adsorption with Cowan I cells indicated that Brucella antibodies in whey were bound to protein A.

Examination of whey samples for the presence of Brucella antibodies by the coagglutination test showed positive reactions occurring in 96.5% of the Brucella exposed, reactor group (BRT or WPT positive) compared to 74.2% in the Brucella exposed, nonreactor group (BRT and WPT negative). Twelve percent of the nonexposed control herd reacted positively by the coagglutination test. The discrepancy of results between the standard milk tests (BRT and WPT) and the coagglutination test was examined. In order to eliminate the possible involvement of nonspecific antibodies in the coagglutination test, several procedures were applied for differentiating

specific and nonspecific reactions. Neither heat inactivation of whey nor addition of a chelating agent (EDTA) to the whey, significantly altered the coagglutination test results. The use of low pH antigen reduces the nonspecific agglutination reaction in the Brucella plate test by reversing the electrostatic charge of the agglutinins (113). However, application of this principal to the coagglutination test increases the number of positive reactions. False positive coagglutination reactions caused by nonspecific agglutinins in whey from Brucella-infected animals could, therefore, be excluded. Evaluation of Brucella exposed, nonreactor samples by the Coombs test demonstrated that incomplete antibodies were not present. In many of the positive coagglutination reactions, aggregation of cells was observed prior to the addition of Brucella antigen. Since this aggregation was not observed in the control samples, it was concluded that the reaction was specific. This specific reaction could be directed against staphylococcal antigenic components. To eliminate this possibility, all whey samples were adsorbed with S. aureus strain Wood 46 to remove staphylococcal antibodies. At least ten of the seventeen control animals had a known S. aureus infection at the time of sampling. The positive coagglutination reactions occurring in the control group may be attributed to the presence of staphylococcal antibodies

in the whey. One adsorption with S. aureus Wood 46 cells may not have been sufficient to remove all staphylococcal antibodies that were present. Since strains Wood 46 and Cowan I are not antigenically identical it is possible, however, that antibodies specific to strain Cowan I would not be removed by adsorption with Wood 46 cells. Because this strain lacks protein A on its cell wall, any antibodies specific to protein A would not be removed.

To the extent that some of the coagglutination reactions may be attributed to the presence of staphylococcal antibodies in the whey, the percentage of such reactions should be similar in all groups. However, coagglutination reactions occurred six times more frequently in the Brucella exposed, nonreactor group than in the control group. Therefore, the high number of coagglutination reactions in this group cannot be attributed to the presence of staphylococcal antibodies in the whey.

Another explanation for the discrepancy of results between the standard milk tests and the coagglutination test may be that the BRT and WPT do not detect the same classes of antibodies that are detected by the coagglutination test. Beh (5) and Collin (18) concluded that milk immunoglobulins IgM and IgA reacted in the BRT, whereas IgG1 and IgG2 did not. The ability of immunoglobulins to react with

staphylococcal protein A is limited to IgG.

Although Brucella antibodies could not be detected in the whey of exposed, nonreactor cows by conventional milk assays, specific antibodies were detected in the serum of these animals. Fifty-seven of the 63 nonreactor cows were serologically positive (standard tube test ≥ I25) at the time of sampling and 55 were positive by the coagglutination test (Table 11). Positive coagglutination reactions when the BRT results were negative would indicate that only Brucella antibodies of the IgG class were present in the whey.

Table 11. Comparison of the coagglutination test, standard tube test and direct culture between reactor[a] and nonreactor[b] cows

	Reaction	Reactors	Nonreactors	Total
Coagglutination test (milk)	Positive	30	55	85
	Negative	0	8	8
Standard tube test (serum)	Positive	30	57	87
	Negative	0	6	6
Culture at necropsy (tissues)	Positive	24	9	33
	Negative	6	54	60

[a]BRT > 1:20 or WPT > 1:25.

[b]BRT ≤ 1:20 and WPT ≤ 1:25.

Since aggregation of staphylococcal cells occurred before the addition of Brucella antigen, this would indicate that specific antigen was already present in the whey. Antigen was demonstrated in nine of the nonreactor animals by culture at necropsy. The presence of soluble Brucella antigen in whey cannot be eliminated. Fluorescent studies conducted by Biegeleisen, et al., (10) demonstrated Brucella soluble antigen in tissues. These antigens appear to be bound to Brucella antibodies in the whey as an immune complex. Therefore, the addition of Brucella antigen was not required to aggregate the staphylococcal cells.

The standard milk tests would not be able to detect antibodies if bound to antigen in an immune complex. Positive results would be obtained with the coagglutination test and the standard milk tests if Brucella antibodies are present in excess as was observed in the exposed reactor cows. Antibodies present as an immune complex have a stronger affinity for staphylococcal protein A than noncomplexed antibodies (75).

The presence of B. abortus-anti-B. abortus immune complex activity in Brucella infected cows has been reported (107). Using the ELISA, Pruett detected immune complex activity in paired serum and milk samples from an animal positive to Brucella by culture and serology. High levels

of immune complex activity were detected in the serum, but not in the milk of a serologically negative-culture positive animal.

Several investigators have described the use of staphylococcal protein A as an immune complex adsorbent (4, 56, 75, 91, 101). Further research in the use of staphylococcal protein A as an adsorbent for B. abortus-anti-B. abortus immune complex is necessitated.

SUMMARY

A coagglutination test for the detection of Brucella antigen and antibody in whey was developed. B. abortus antigen added to whey could be detected at a concentration between 10 and 100 μg/ml. However, antigen could not be detected in the whey of cows experimentally infected with B. abortus strain 2308 even though they were culture positive. Failure to detect Brucella antigen may be due to the low number of organisms or to the presence of immune complexes in the whey. The antigenic sites may already be blocked by Brucella antibody present in the whey.

Brucella antibodies were detected in the whey from experimentally exposed animals by the coagglutination test. A higher percentage of positive reactions were observed by the coagglutination test (86%) than the BRT and WPT (53%). This difference in reactivity may be attributed to the fact that the coagglutination test is the only test employed that is capable of detecting antibodies when present as an immune complex. Of 93 Brucella exposed cows, 87 were serologically positive whereas, 85 were positive by the coagglutination test. Although the coagglutination test is more sensitive than the standard milk tests, the test is time consuming and requires a large quantity of staphylococcal cells for adsorption.

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APPENDIX

Table 12. Bacteriological and clinical mastitis history of control animals

Monthly examination[a]	Cow number and quarter sample															
	21				93				119				154			
	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR
Aug 1978	-	-	SA	SA	-	-	-	-	-	-	-	-	-	-	-	-
Sep	-	-	SA	SA	-	-	-	-	-	-	-	-	-	-	-	-
Oct					-	-	-	Co	SA	Sp	-	-	-	-	-	-
Nov					-	-	-	-					-	-	-	-
Dec													-	-	-	-
Jan 1979	-	-	SA	SA	-	-	-	-	-	-	-	-				
Feb					SA	SA	SA	SA					-	-	-	-
Mar					SA	-	-	SA					-	-	-	-
Apr					-	SA	SA	SA	-	-	-	-	-	-	-	-
May					-	SA	SA	SA								
Jun	-	-	SA	SA	-	SA	SA	SA	-	-	-	-	-	-	-	-
Jul					-	SA	SA	SA					-	-	-	-
Aug	-	-	SA	SA	-	SA	SA	SA	-	-	-	-	-	-	-	-
Sep					-	SA	SA	SA	-	-	-	-				
Oct	-	-	SA	SA	-	-	SA	-	-	-	-	-				
Nov									-	-	-	-				
Dec	-	-	SA	-	B	-	-	-								
Jan 1980	-	-	SA	SA					-	-	SE	-				
Feb	-	-	SA	SA	-	-	SA	-	-	-	-	-				
Mar	Sp	Sp	SA	SA	Sp	Sp	SA	Sp	-	-	-	-				

[a] B = Bacillus cereus; C = contaminated; Co = coliform G = gram positive rod; H = small hemolytic colonies; Pr = Proteus; Ps = Pseudomonas; S = staphylococcus (species not identified); SA = Staphylococcus aureus; SE = Staphylococcus epidermis; Sp = streptococci; Y = yeast; blank = not sampled; - = sampled and found clean.

Cow number and quarter sample																				
215				220				237				238				245				
RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	
				SE	-	-	-	-	-	-	-	-	SA	-	-	-	-	-	-	
-	-	-	-	SE	-	-	-	-	-	-	-	-	SA	-	-	-	-	-	-	
H	SA	-	SA									SA	SA	-	SA	SA	SA	-	Sp	
				SE	-	-	-	-	-	-	-									
-	-	-	-	SE	-	-	-	-	-	-	-	-	SA	-	-	-	-	SA	-	
				SE	SA	SA	SA	Sp	-	-	-									
-	-	-	-					-	-	-	-									
				SE	SA	SA	SA									SA	-	-	-	
-	-	-	-	SE	SA	-	-	-	-	-	-					SA	SA	SA	SA	
								-	-	-	G					-	SA	-	-	
-	-	-	-	-	SA	-	-	-	-	-	C					-	SA	-	-	
-	-	-	-	-	SA	-	-	-	-	-	-					-	-	-	-	
-	-	-	-	-	-	-	-	-	-	-	-					-	-	-	-	
								-	-	-	-									
Ps	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Ps	-	-	-									-	-	-	-	-	-	-	-	
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Table 12. (continued)

Monthly examination	Cow number and quarter sample															
	247				249				250				258			
	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR
Aug 1978	-	-	-	-												
Sep	-	-	-	-												
Oct																
Nov																
Dec																
Jan 1979	-	-	-	-												
Feb																
Mar					-	S	-	-	SE	SE	-	SE	SE	Pr	Sp	SE
Apr	-	-	-	-	-	S	SE Sp	SE	-	-	-	-				
May																
Jun	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jul																
Aug	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oct	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nov	-	-	-	-					-	-	-	-	-	-	-	-
Dec	-	-	-	-	-	-	-	-								
Jan 1980	-	-	-	-												
Feb	-	SE	-	-	-	-	-	-	-	-	-	SE	-	-	-	-
Mar	-	SE	-	-	-	-	-	-	-	-	SE	-	-	S	-	S

Cow number and quarter sample																
265				1035				1195				1204				
RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	
-	S	-	-	-	SA	-	SE	-	SE	-	SE	-	-	-	-	
C	SE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
				-	-	SA	SA	-	SE	SA	SA					
								-	SA SE	-	-	-	-	-	-	
-	-	-	-	-	-	-	-	Y	SE	Sp	Sp	-	-	-	-	
SA	-	-	-	Sp	-	-	SA					-	-	-	-	
-	-	-	-					-	-	-	-	-	-	-	Sp	
SA	SA	SA	SA	-	-	-	-	-	-	-	-					
-	SA	-	-									SE	-	SE	Sp	
-	-	-	-	-	-	-	-	-	-	-	-	-	SA	-	-	
												-	-	-	-	
-	-	-	-	-	-	SE	-	-	-	-	-	-	-	-	-	
-	-	-	-	SE	SE	-	-	-	-	-	-	-	-	-	-	
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
								-	-	-	-					
-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	
								Y	Y	-	-					
-	Co	-	-	-	S	-	-	-	Y	-	-	-	-	-	-	
-	-	-	-	-	S	-	-	-	Y	-	-	-	-	-	-	

Table 13. Results of coagglutination test,[a] *Brucella* ring test and direct culture for individual quarter samples from infected cow Y-65

Date (1979)	Quarter sample	CoAg[b]	BRT[c]	Direct culture (CFU[d]/ml)
5-8	RR[e]	+	40,960	2,750
	RF[f]	+	20,480	0
	LR[g]	+	2,560	0
	LF[h]	+	20,480	5,000
5-12	RR	+	2,560	5,000
	RF	+	160	0
	LR	+	160	0
	LF	+	2,560	5,000
5-14	RR	+	5,120	500
	RF	+	80	0
	LR	+	160	0
	LF	+	2,560	500
5-16	RR	+	5,120	2,750
	RF	+	80	0
	LR	+	-	0
	LF	+	2,560	500
5-18	RR	+	5,120	115
	RF	+	80	0
	LR	+	160	0
	LF	+	2,560	35

[a] *S. aureus* coated with rabbit anti-*B. abortus* 2308 (whole cell) serum.

[b] Coagglutination test for detection of antigen.

[c] *Brucella* ring test.

[d] Colony forming units.

[e] RR: right rear.

[f] RF: right front.

[g] LR: left rear.

[h] LF: left front.

Table 13. (continued)

Date (1979)	Quarter sample	CoAg	BRT	Direct culture (CFU/ml)
5-21	RR	+	2,560	500
	RF	+	160	0
	LR	+	160	0
	LF	+	5,120	500
5-30	RR	+	5,120	50
	RF	+	80	0
	LR	+	160	0
	LF	+	2,560	200
6-1	RR	+	2,560	75
	RF	+	40	0
	LR	+	80	0
	LF	+	1,280	60
6-5	RR	+	2,560	160
	RF	+	80	0
	LR	+	160	0
	LF	+	2,560	70
6-6	RR	+	2,560	160
	RF	+	80	0
	LR	+	80	0
	LF	+	1,280	35
6-8	RR	+	2,560	150
	RF	+	40	0
	LR	+	80	0
	LF	+	1,280	155
6-13	RR	+	2,560	40
	RF	+	40	0
	LR	+	80	0
	LF	+	1,280	20
6-15	RR	+	2,560	25
	RF	+	40	0
	LR	+	40	0
	LF	+	2,560	20

Table 14. Results of the coagglutination test, whey plate test, Brucella ring test and direct culture from whey samples of experimentally infected cows

Animal	Date (1980)	Quarter sample	CoAb[a]	WPT[b]	BRT[c]	Direct culture (CFU[d]/ml)
1	1-21	C[e]	+	+200	10,240	10
		RR[f]	+	+200	2,560	-
		RF[g]	+	+200	2,560	-
		LR[h]	+	+200	20,480	305
		LF[i]	+	+200	2,560	-
1	1-28	C	+	+200	20,480	500
		RR	+	+200	2,560	-
		RF	+	+200	5,120	25
		LR	+	+200	81,920	1,000
		LF	+	+200	10,240	-
2	1-14	C	+	+200	20,480	150
		RR	+	+200	81,920	2,250
		RF	+	+200	10,240	-
		LR	+	+200	10,240	-
		LF	NS[j]			

[a]Coagglutination test for detection of antibody.
[b]Whey plate test.
[c]Brucella ring test.
[d]Colony forming units.
[e]C: composite sample.
[f]RR: right rear.
[g]RF: right front.
[h]LR: left rear.
[i]LF: left front.
[j]NS: no sample.

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
2	1-21	C	+	+200	20,480	1,000
		RR	+	+200	40,960	1,500
		RF	+	+200	10,240	-
		LR	+	+200	10,240	-
		LF	+	+200	5,120	-
3	5-5	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
4	4-15	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	+	-	-	-
		LF	-	-	-	-
5	4-15	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
7	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	-	-	-	-
8	5-12	C	NS			
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
9	4-8	C	NS			
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
10	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
11	2-4	C	+	+200	2,560	-
		RR	+	+200	2,560	-
		RF	+	+200	2,560	-
		LR	+	+200	2,560	-
		LF	+	+200	2,560	-
11	2-11	C	+	+200	1,280	-
		RR	+	+200	1,280	-
		RF	+	+200	1,280	-
		LR	+	+200	1,280	-
		LF	+	+200	1,280	-
12	1-21	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
13	4-15	C	+	-	10	-
		RR	+	-	10	-
		RF	+	-	-	-
		LR	+	-	10	-
		LF	+	-	10	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
14	4-29	C	-	I50[k]	20	-
		RR	+	+100	80	-
		RF	+	-	-	-
		LR	+	I100	80	-
		LF	NS			
15	4-15	C	+	I100	1,280	10
		RR	+	I50	320	-
		RF	+	I100	160	-
		LR	+	I200	2,560	11
		LF	+	I100	640	-
15	4-22	C	+	I200	2,560	325
		RR	+	I200	640	-
		RF	+	I200	320	-
		LR	+	I200	2,560	1,500
		LF	+	I200	640	-
15	4-29	C	+	I200	5,120	470
		RR	NS			
		RF	+	I200	640	-
		LR	+	I200	20,480	1,500
		LF	+	I200	640	-
16	4-15	C	+	I200	20	-
		RR	+	I100	10	-
		RF	+	+50	20	-
		LR	+	I100	20	-
		LF	+	+25	20	-
17	4-29	C	+	-	-	-
		RR	-	-	-	-
		RF	+	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-

[k] I: incomplete.

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
19	2-11	C	+	I200	640	-
		RR	+	+200	1,280	-
		RF	+	I200	320	-
		LR	+	+200	640	-
		LF	+	I200	640	-
19	2-18	C	+	I200	640	-
		RR	NS			
		RF	+	+200	1,280	-
		LR	+	+200	640	-
		LF	NS			
20	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
21	5-20	C	+	-	20	-
		RR	+	-	10	-
		RF	+	-	10	-
		LR	+	-	20	-
		LF	+	-	20	-
22	2-11	C	+	+200	640	5
		RR	+	+200	1,280	-
		RF	+	I200	640	-
		LR	+	+200	640	-
		LF	NS			
22	2-18	C	+	+200	320	Cn[1]
		RR	NS			
		RF	+	+200	320	Cn
		LR	+	+200	640	-
		LF	+	+200	640	-

[1]Cn: contaminated.

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
24	4-15	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
25	4-1	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
26	4-15	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
28	3-28	C	+	+200	81,920	5,000
		RR	+	+200	40,960	5,000
		RF	+	+200	5,120	-
		LR	+	+200	81,920	5,000
		LF	+	+200	2,560	-
28	4-3	C	+	+200	2,560	35
		RR	+	+200	2,560	57
		RF	+	-	40	-
		LR	+	+200	2,560	45
		LF	NS			
28	4-8	C	+	+200	2,560	48
		RR	+	I200	2,560	43
		RF	-	-	40	-
		LR	+	I200	2,560	60
		LF	-	-	40	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
28	4-11	C	+	+200	2,560	8
		RR	+	+200	2,560	75
		RF	+	-	40	-
		LR	+	+200	2,560	15
		LF	+	-	40	-
28	4-15	C	+	+200	2,560	40
		RR	+	+200	2,560	50
		RF	-	-	20	-
		LR	+	+200	2,560	40
		LF	-	-	20	-
28	4-16	C	+	I200	1,280	5
		RR	+	I200	1,280	20
		RF	-	-	20	-
		LR	+	I200	2,560	10
		LF	-	-	20	-
28	4-17	C	+	+200	1,280	-
		RR	+	I200	1,280	15
		RF	-	-	20	-
		LR	+	I200	5,120	5
		LF	-	-	20	-
28	4-18	C	-	+200	1,280	-
		RR	-	+200	1,280	20
		RF	-	-	20	-
		LR	-	+200	2,560	-
		LF	-	-	20	-
28	4-22	C	-	+200	2,560	10
		RR	+	+200	2,560	10
		RF	+	-	20	-
		LR	+	+200	2,560	5
		LF	-	-	20	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
28	4-23	C	+	I200	1,280	5
		RR	+	+200	2,560	10
		RF	-	-	40	-
		LR	+	+200	5,120	5
		LF	+	-	40	-
28	4-24	C	+	+200	5,120	5
		RR	+	+200	5,120	135
		RF	+	-	40	-
		LR	+	+200	10,240	15
		LF	+	-	40	-
28	4-25	C	+	I200	2,560	5
		RR	+	+200	2,560	5
		RF	+	-	40	-
		LR	+	+200	5,120	10
		LF	-	-	40	-
28	4-28	C	+	+200	2,560	10
		RR	+	+200	10,240	20
		RF	+	-	160	-
		LR	+	+200	10,240	5
		LF	+	I50	80	-
28	4-30	C	+	+200	5,120	33
		RR	+	+200	10,240	175
		RF	+	I200	640	-
		LR	+	+200	20,480	30
		LF	+	I200	160	-
28	5-2	C	+	+200	5,120	10
		RR	+	+200	10,240	30
		RF	+	+25	640	-
		LR	+	+200	20,480	330
		LF	+	+25	640	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
28	5-5	C	+	+200	20,480	375
		RR	+	+200	20,480	250
		RF	+	+200	1,280	-
		LR	+	+200	20,480	500
		LF	+	+200	640	-
29	4-8	C	+	-	10	Cn
		RR	+	-	-	-
		RF	+	+100	40	Cn
		LR	+	-	-	Cn
		LF	+	-	-	-
30	4-22	C	-	-	-	-
		RR	+	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
31	2-4	C	+	+200	2,560	-
		RR	+	+200	2,560	-
		RF	+	+200	2,560	-
		LR	+	+200	1,280	-
		LF	+	+200	1,280	-
31	2-11	C	+	+200	2,560	-
		RR	+	+200	1,280	-
		RF	+	+200	2,560	Cn
		LR	+	+200	1,280	-
		LF	+	+200	2,560	-
33	4-3	C	+	-	-	-
		RR	NS			
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
33	4-8	C	NS			
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
35	1-28	C	+	+200	160	-
		RR	+	+200	320	-
		RF	+	+200	160	-
		LR	+	+200	160	-
		LF	+	+200	320	-
35	2-4	C	+	+200	1,280	-
		RR	+	+200	640	-
		RF	NS			
		LR	+	+200	640	-
		LF	+	+200	2,560	-
36	4-8	C	+	-	10	-
		RR	+	-	10	Cn
		RF	+	-	10	-
		LR	+	-	10	Cn
		LF	NS			
37	4-22	C	-	-	-	-
		RR	+	-	-	-
		RF	-	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
38	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
39	4-1	C	+	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
40	4-8	C	+	-	-	-
		RR	+	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
41	2-12	C	+	I200	160	-
		RR	+	I200	80	-
		RF	+	I200	80	-
		LR	+	+200	160	-
		LF	+	+200	320	-
41	2-18	C	+	I100	80	-
		RR	+	I200	80	-
		RF	+	I200	80	-
		LR	+	I200	160	-
		LF	+	I200	160	-
43	4-8	C	NS			
		RR	+	-	10	Cn
		RF	+	-	-	-
		LR	+	-	10	Cn
		LF	+	-	-	-
44	2-4	C	+	+200	1,280	10
		RR	+	+200	640	2,500
		RF	+	+200	2,560	450
		LR	+	+200	1,280	5
		LF	+	+200	640	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
44	2-11	C	+	+200	2,560	-
		RR	+	+200	1,280	-
		RF	+	+200	2,560	-
		LR	+	+200	2,560	-
		LF	+	+200	2,560	-
45	5-12	C	+	-	-	-
		RR	NS			
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
47	2-11	C	+	+200	2,560	5,000
		RR	+	+200	20,480	5,000
		RF	+	+200	2,560	-
		LR	+	+200	2,560	-
		LF	+	+200	20,480	5,000
47	2-18	C	+	+200	20,480	1,500
		RR	+	+200	40,960	5,000
		RF	+	+200	5,120	-
		LR	+	+200	2,560	-
		LF	NS			
47	2-25	C	+	+200	40,960	5,000
		RR	+	+200	40,960	25,000
		RF	+	+200	5,120	-
		LR	+	+200	2,560	-
		LF	+	+200	40,960	3,750
47	3-3	C	NS			
		RR	+	+200	81,920	5,000
		RF	+	+200	5,120	-
		LR	+	+200	2,560	5
		LF	+	+200	40,960	5,000

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
48	5-20	C	+	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	+	-	-	-
		LF	-	-	-	-
49	1-14	C	+	+200	640	-
		RR	NS			
		RF	+	+200	640	-
		LR	NS			
		LF	+	+200	640	-
49	1-21	C	+	+200	2,560	-
		RR	+	+200	2,560	-
		RF	+	+200	2,560	-
		LR	+	+200	2,560	-
		LF	+	+200	2,560	-
50	4-8	C	+	-	-	Cn
		RR	NS			
		RF	+	-	-	Cn
		LR	+	-	-	Cn
		LF	+	-	-	Cn
52	4-8	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
53	4-15	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
54	5-20	C	+	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
55	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	-	-	-	-
56	3-18	C	+	-	10	-
		RR	+	-	10	-
		RF	+	-	10	-
		LR	+	-	10	-
		LF	+	-	10	-
56	3-25	C	+	-	10	-
		RR	+	-	10	-
		RF	+	-	10	-
		LR	+	-	10	-
		LF	-	-	10	-
56	4-22	C	+	-	40	-
		RR	+	-	40	-
		RF	+	-	40	-
		LR	+	-	40	-
		LF	+	-	40	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
57	4-29	C	+	I200	40	-
		RR	-	+200	80	-
		RF	+	I100	40	-
		LR	+	+200	80	-
		LF	+	I100	80	-
58	2-4	C	+	+200	2,560	875
		RR	NS			
		RF	+	+200	2,560	1,500
		LR	NS			
		LF	+	+200	640	10
58	2-12	C	+	+200	10,240	300
		RR	NS			
		RF	+	+200	40,960	155
		LR	+	+200	640	-
		LF	+	+200	2,560	-
59	4-8	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
60	1-7	C	+	+200	2,560	1,000
		RR	+	+200	320	-
		RF	+	+200	10,240	1,250
		LR	+	+200	320	5
		LF	+	+200	5,120	1,500
60	1-14	C	+	+200	10,240	2,500
		RR	+	+200	320	-
		RF	+	+200	40,960	2,500
		LR	+	+200	2,560	-
		LF	+	+200	20,480	2,500

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
60	1-21	C	+	+200	20,480	2,500
		RR	+	+200	640	-
		RF	+	+200	81,920	3,500
		LR	+	+200	2,560	-
		LF	+	+200	81,920	2,750
62	4-8	C	+	-	-	-
		RR	+	-	10	Cn
		RF	+	-	10	-
		LR	NS			
		LF	+	-	10	Cn
63	4-29	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
64	5-5	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	NS			
		LF	+	-	-	-
65	4-22	C	+	-	-	-
		RR	+	-	10	-
		RF	+	-	-	-
		LR	+	-	10	-
		LF	+	-	-	-
66	2-18	C	+	+200	2,560	Cn
		RR	+	+200	2,560	Cn
		RF	+	+200	2,560	Cn
		LR	+	+200	2,560	Cn
		LF	NS			

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
66	2-25	C	+	+200	320	Cn
		RR	+	I200	320	Cn
		RF	+	I200	160	-
		LR	+	I200	320	Cn
		LF	NS			
67	4-15	C	+	I100	20	-
		RR	+	I100	40	-
		RF	+	+100	40	-
		LR	+	I200	40	-
		LF	+	I100	40	-
68	4-29	C	+	-	10	-
		RR	+	-	-	-
		RF	+	-	10	-
		LR	+	-	-	-
		LF	+	I25	20	-
69	4-1	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
71	4-29	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
72	4-29	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
73	4-8	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	10	-
		LR	-	-	-	-
		LF	-	-	-	-
74	4-15	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
75	2-23	C	+	+200	10,240	Cn
		RR	+	+200	10,240	750
		RF	+	+200	10,240	395
		LR	+	+200	10,240	40
		LF	NS			
75	3-3	C	+	+200	5,120	-
		RR	+	+200	10,240	63
		RF	+	+200	5,120	-
		LR	+	+200	2,560	-
		LF	+	+200	5,120	-
75	3-4	C	+	+200	5,120	-
		RR	+	+200	5,120	-
		RF	+	+200	2,560	-
		LR	+	+200	2,560	-
		LF	+	+200	5,120	-
75	3-10	C	+	+200	5,120	35
		RR	+	+200	5,120	565
		RF	+	+200	2,560	-
		LR	+	+200	1,280	-
		LF	+	+200	2,560	-

Table 14 . (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
75	3-18	C	+	+200	2,560	25
		RR	+	+200	10,240	125
		RF	+	+200	5,120	-
		LR	+	+200	1,280	-
		LF	+	+200	2,560	-
76	4-15	C	+	-	-	-
		RR	+	-	-	-
		RF	NS			
		LR	+	-	-	-
		LF	+	-	-	-
77	4-15	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
78	5-5	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
79	1-7	C	+	I100	80	-
		RR	NS			
		RF	+	+50	80	-
		LR	NS			
		LF	NS			
80	4-22	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
82	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
83	3-10	C	+	I200	160	-
		RR	+	I50	40	-
		RF	+	I200	160	-
		LR	+	I200	160	-
		LF	+	I200	160	-
83	3-18	C	+	I200	160	-
		RR	NS			
		RF	+	I100	160	-
		LR	+	I100	160	-
		LF	+	I200	160	-
84	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
85	1-7	C	+	+200	320	-
		RR	+	+200	320	-
		RF	+	+200	320	-
		LR	+	+200	320	-
		LF	+	+200	320	-
85	1-14	C	+	+200	640	-
		RR	+	+200	1,280	-
		RF	+	+200	640	-
		LR	+	+200	640	-
		LF	+	+200	640	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
86	5-12	C	+	-	-	-
		RR	NS			
		RF	-	-	-	-
		LR	+	-	-	-
		LF	-	-	-	-
87	5-5	C	+	-	-	-
		RR	+	-	-	-
		RF	NS			
		LR	+	-	-	-
		LF	+	-	-	-
88	4-29	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
89	4-22	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
91	2-28	C	+	+200	10,240	432
		RR	+	+200	5,120	-
		RF	+	+200	5,120	-
		LR	+	+200	5,120	5
		LF	+	+200	20,480	1,500
91	3-3	C	+	+200	5,120	285
		RR	+	+200	5,120	-
		RF	+	+200	640	-
		LR	+	+200	2,560	-
		LF	+	+200	10,240	1,500

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
91	3-4	C	+	+200	5,120	10
		RR	+	+200	5,120	-
		RF	+	+200	1,280	-
		LR	+	+200	2,560	-
		LF	+	+200	10,240	165
91	3-10	C	+	+200	2,560	-
		RR	+	+200	160	-
		RF	+	+200	80	-
		LR	+	+200	160	-
		LF	+	+200	10,240	5
91	3-18	C	+	I200	2,560	-
		RR	+	I50	160	-
		RF	+	I25	40	-
		LR	+	I50	80	-
		LF	+	I200	5,120	-
91	4-29	C	+	+200	640	-
		RR	+	-	20	-
		RF	+	-	20	-
		LR	+	I50	40	-
		LF	+	+200	1,280	-
91	5-23	C	+	+200	1,280	-
		RR	+	-	20	-
		RF	-	-	10	-
		LR	-	-	40	-
		LF	+	+200	5,120	5
92	4-8	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
93	1-21	C	+	+200	320	-
		RR	+	+200	640	-
		RF	+	+200	320	-
		LR	+	+200	320	-
		LF	+	+200	160	5
93	1-28	C	+	I200	320	-
		RR	+	+200	640	-
		RF	+	+200	320	-
		LR	+	+200	320	-
		LF	+	+200	320	-
94	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
95	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	NS			
		LF	+	-	-	-
96	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
97	4-29	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	10	-
		LR	+	-	-	-
		LF	+	-	10	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
98	2-4	C	+	+200	2,560	1,250
		RR	+	+200	640	-
		RF	+	+200	2,560	-
		LR	+	+200	640	-
		LF	NS			
98	2-11	C	+	+200	2,560	1,000
		RR	+	+200	2,560	-
		RF	+	+200	2,560	-
		LR	+	+200	2,560	-
		LF	+	+200	10,240	2,250
98	2-19	C	+	+200	10,240	645
		RR	+	+200	2,560	-
		RF	+	+200	2,560	-
		LR	+	+200	1,280	-
		LF	+	+200	10,240	2,500
98	2-25	C	+	+200	20,480	612
		RR	+	+200	2,560	-
		RF	+	+200	2,560	-
		LR	+	+200	2,560	Cn
		LF	+	+200	81,920	5,000
98	3-3	C	NS			
		RR	+	+200	2,560	-
		RF	+	+200	5,120	-
		LR	+	+200	2,560	Cn
		LF	+	+200	81,920	3,500
99	1-1	C	+	+200	320	-
		RR	+	+100	160	-
		RF	+	+200	320	-
		LR	+	+200	320	-
		LF	NS			

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
99	1-7	C	+	I200	320	5
		RR	+	+100	160	20
		RF	+	+200	320	-
		LR	+	I200	320	-
		LF	+	+100	160	-
100	4-15	C	-	-	-	-
		RR	-	-	-	-
		RF	-	-	-	-
		LR	-	-	-	-
		LF	-	-	-	-
101	1-28	C	+	+200	1,280	-
		RR	+	+200	2,560	-
		RF	+	+200	1,280	-
		LR	+	+200	2,560	-
		LF	+	+200	320	-
101	2-4	C	+	+200	1,280	-
		RR	NS			
		RF	+	+200	2,560	-
		LR	+	+200	1,280	-
		LF	+	+200	320	-
102	3-23	C	+	-	-	-
		RR	-	-	-	-
		RF	NS			
		LR	-	-	-	-
		LF	+	-	-	-
102	4-1	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-

Table 14. (continued)

Animal	Date (1980)	Quarter sample	CoAb	WPT	BRT	Direct culture (CFU/ml)
103	4-22	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
104	5-5	C	+	-	-	Cn
		RR	+	-	-	Cn
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	Cn
105	4-15	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-
106	4-15	C	+	-	-	-
		RR	+	-	-	-
		RF	+	-	-	-
		LR	+	-	-	-
		LF	+	-	-	-