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Serological Response to In Vitro-Shed Antigen(s) of *Tritrichomonas foetus* in Cattle

R. H. BONDURANT,[1]* K. A. VAN HOOSEAR,[1] L. B. CORBEIL,[2] AND D. BERNOCO[1]†

Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, California 95616,[1] and Department of Pathology, UCSD Medical Center, University of California, San Diego, California 92103-8416[2]

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We developed a serological assay for detection of (i) an erythrocyte-adhering molecule(s) shed by the bovine venereal pathogen *Tritrichomonas foetus* and (ii) serum antibodies to this antigen(s) in exposed cattle. Sera from exposed and unexposed cattle were tested for their ability to induce complement-mediated lysis of bovine erythrocytes that had been previously incubated overnight at room temperature in pH-adjusted supernatants of *T. foetus* culture media. Eight of 180 serum specimens from six groups of presumably unexposed cows or heifers showed a positive (≥1:2) hemolytic titer (specificity = 95.6%). Thirteen of 14 females in two experimentally infected groups showed a positive hemolytic titer following infection (sensitivity = 94%). In experimentally infected heifers, there was little correlation (r^2 = 0.33) between serum hemolytic titers with respect to shed antigen and titers obtained in serum enzyme-linked immunosorbent assays in which whole *T. foetus* served as the antigen. Serum hemolytic titers rose 3 to 4 weeks sooner than did previously described vaginal mucus immunoglobulin G1 or immunoglobulin A titers with respect to whole-cell antigen or TF1.17 subunit antigen, respectively. Among 14 chronically infected bulls, only 6 (43%) showed a positive hemolytic titer. This study is the first, to our knowledge, to show a specific serological response in the host to an in vitro-shed antigen(s) of *T. foetus* and suggests a useful diagnostic test for potentially exposed herds.

Bovine trichomoniasis, caused by the sexually transmitted protozoan parasite *Tritrichomonas foetus*, is manifested by pregnancy loss (from 1 to 8 months' gestation), infertility, and occasional pyometra (1, 5). Mature males become permanent carriers, without clinical signs (11, 17). Experimental infection of virgin heifers with the parasite induces infections of variable duration (up to 32 weeks), and clearance of the organism from the urogenital tract is associated with the appearance of parasite-specific immunoglobulin G1 (IgG1) and IgA antibodies in vaginal mucus and uterine secretions (2, 10, 10a, 18, 19). Those studies detected little if any serum IgG1 or IgG2 response to infection.

Currently, the "gold standard" diagnostic test for trichomoniasis in either male or female cattle is the cultivation of live organisms from smegma or cervicovaginal mucus, respectively (17). While performing a single culture from a male specimen gives a reasonably high level of sensitivity (81 to 91% [11, 20]), culture of female cervicovaginal secretions provides only 58 to 75% sensitivity at best (17). Furthermore, the fact that most infected females clear the organism within 2 to 5 months makes a diagnosis of herd exposure to *T. foetus* particularly difficult. Attempts to develop serological tests for evidence of exposure have been generally unsuccessful. Early work by Robertson (16) suggested that serum agglutinins were formed as a response to venereal infection, but the presence of nonspecific agglutinins in uninfected females diminished the diagnostic usefulness of such an assay. Perhaps these cross-reacting antibodies were a result of exposure of the immune system to normal gut flora, including members of the family *Trichomonadidae* (3), to nonspecific binding of immunoglobulin to the parasite surface (6), or to the presence of "conglutinins" in bovine sera (12).

We report here the preliminary development of a specific serological assay for *T. foetus* exposure in cattle, using a hemolytic test for detection of serum antibodies to an in vitro-shed parasite antigen(s) that adheres to erythrocytes (RBCs) and allows complement-mediated lysis of RBCs.

MATERIALS AND METHODS

Protozoal isolates. The D-1 strain of *T. foetus*, from a cow with pyometra, was employed in the experimental infections, as reported earlier (2, 18, 19). Stabilates frozen in 10% dimethyl sulfoxide (DMSO)–trypticase-yeast-maltose (TYM) medium were thawed and passaged 6 to 10 times, at 48- to 72-h intervals, in TYM medium (7). Other isolates (600609 and EX663) were obtained from an infected cow and an infected bull, respectively.

Antibodies. Antibodies used in these experiments included polyclonal rabbit anti-*T. foetus* serum (R+), developed for a previously reported antigen capture enzyme-linked immunosorbent assay (ELISA) (21); irrelevant rabbit antiserum (R−), a polyclonal serum raised against estradiol 17-beta; polyclonal bovine antiserum raised against *T. foetus* antigen (1276), obtained following subcutaneous inoculation of a clinically normal cow three times at 3-week intervals with 100 μg of immunoaffinity-purified TF1.17 surface antigen of *T. foetus* in adjuvant (2); irrelevant polyclonal bovine antiserum (1777), from a multiparous cow, raised against bovine major histocompatibility complex (BoLA) antigens; and polyclonal sera obtained from virgin heifers before and after experimental infection with the D-1 isolate of *T. foetus.*

Experimental animals. (i) Group A. Eight postpubertal virgin beef heifers of mixed breed, 12 to 14 months old, were estrus synchronized as previously reported (2). At estrus, each heifer was infected by the intravaginal instillation of 10^6 motile organisms in 1 ml of phosphate-buffered saline (PBS). The dose was determined by a previous study, in which 10^2, 10^4, or 10^6 organisms were introduced intravaginally (n = 8, 8, and 7 per group, respectively). All animals receiving 10^6 organisms sustained an infection for at least 6 weeks after inoculation, whereas only five of eight and four of eight animals receiving 10^4 and 10^2 organisms, respectively, sustained detectable infections (2). For 9 weeks prior to infection and for 10 weeks after infection, cervicovaginal mucus and blood were taken from each of the eight heifers. Mucus was aspirated from the anterior vagina into a plastic insemination pipette. Blood was drawn from the coccygeal vein into evacuated tubes and allowed to clot, and serum was separated by centrifugation and then frozen at −20°C until assayed.

(ii) Group B (archived sera). Sera collected and frozen 10 years previously, as

* Corresponding author. Mailing address: Dept. of (VM) Population Health and Reproduction, University of California, Davis, CA 95616. Phone: (916) 752-1358. Fax: (916) 752-4278. Electronic mail address: rhbondurant@ucdavis.edu.

† Present address: Stormont Laboratories, Inc., Woodland, CA 95776.

part of other investigators' research, was kindly supplied by D. Barrett, Caldwell, Idaho. In that previous study, serum samples were collected weekly from six beef cows that were experimentally infected with *T. foetus* at midgestation by intravaginal instillation of 10^6 *T. foetus* cells (information about the isolate used is not available). Establishment of infection in the cows was confirmed by repeated culture of vaginal mucus. Sera were kept frozen at −20°C until assayed in our laboratory.

(iii) Group C (convenience sample of heifers, presumably unexposed). Sixty-five presumed-virgin heifers, maintained for other, unrelated studies, were made available to the investigators. They had been raised in confinement, with no known exposure to trichomoniasis. All were clinically normal at the time of blood sampling.

(iv) Group D (unexposed dairy females, convenience sample). Sera were collected from 29 dairy cows and 10 dairy heifers at the Animal Science Dairy facility on the University of California, Davis, campus. This facility strictly controls animal traffic and uses artificial insemination exclusively; it is therefore presumed to be free of exposure to trichomoniasis (17).

(v) Group E ("suspect" herd). A commercial herd of beef cattle with a history of *T. foetus* infection in previous breeding seasons, and a current history of infertility following natural mating with bulls of unknown *T. foetus* status, was sampled. Sera from 20 apparently infertile (i.e., nonpregnant) adult cows and 20 randomly selected virgin heifers were harvested and stored as described above.

(vi) Group F (infected herd). Sera from 42 virgin heifers in a commercial beef herd were collected and frozen as described above. The herd had been confirmed to be infected by culture of preputial smegma of the bulls used for breeding (1, 17). The 42 samples represented all available virgin heifers in this unit.

(vii) Bulls. Fourteen adult bulls were determined to be chronically infected with *T. foetus* on the basis of repeatedly positive smegma cultures obtained at weekly sampling intervals over a 12-month period. A serum sample was obtained from each of these bulls for use in the hemolytic assay. Sera were frozen as described above.

ELISA. The ELISA procedure for detection of antibodies to *T. foetus* in mucus or serum and the results of those assays have been described elsewhere (2). Briefly, microtiter plates were coated with whole *T. foetus* organisms (5×10^4 per well) and incubated with dilutions of serum or genital tract secretions, and antibodies were detected with monoclonal antibodies to immunoglobulin isotypes. Wells were treated with peroxidase-labeled goat anti-mouse IgG and IgM (KPL Laboratories), and then color was developed with *O*-phenylene-diamine (Sigma). Optical densities in the ELISA were corrected for variation among plates by multiplying the mean absorbance of test day replicate wells by the ratio of the mean for all positive control wells (all assays) to the mean positive control well absorbance for that day. The positive control well used serum from a cow immunized with whole *T. foetus*. For mucus assays, postinfection corrected absorbance was normalized by dividing the corrected absorbance by the mean preinfection absorbance and multiplying by 100 (2).

RBC coating. Bovine blood was collected in acid-citrate-glucose solution by venipuncture of clinically normal adult cows with no exposure to trichomoniasis, i.e., their entire breeding history had consisted of artificial insemination only with certified specific-pathogen-free semen. RBCs were washed in PBS and packed by centrifugation. Coating antigen was generated by 24- to 48-h culture of D-1 isolates of *T. foetus* in TYM medium. Parasite numbers were counted at T_0 (time of inoculation of TYM; $n = 10^5$ organisms in total) and again at 24 and 48 h, to ensure that the organisms were in the logarithmic phase of growth. Organisms were centrifuged at approximately 1,000 × *g*, and the supernatant was transferred to sterile test tubes, where the pH was adjusted to 7.0 by the addition of 1 N NaOH. Conditioned TYM supernatants were stored at −80°C until used. Packed RBCs were inoculated at various concentrations into conditioned (antigen-coated RBCs) or nonconditioned (uncoated RBCs) TYM supernatants, and mixtures were placed on a mixing rotator for various times and at various temperatures. (See "Assay optimization," below, for specific variables.) Afterwards, RBCs were centrifuged at 1,000 × *g*, washed twice in PBS, and resuspended at various concentrations in PBS-fetal calf serum (FCS-PBS).

Agglutination assay. The agglutination test was performed at 23°C in U-shaped 96-well microtiter plates, with 25 μl of antiserum at doubling dilutions and 25 μl of a 2% suspension of washed RBCs in 1% FCS–PBS. After 30 to 120 min of incubation, trays were shaken vigorously on a plate shaker; after an additional 5 min, agglutination was evaluated by direct visual observation of clumping. Titers were reported as the highest serum dilution that yielded observable agglutination. Control wells included test sera with uncoated RBCs and coated RBCs with R− serum or preinfection cow serum.

Hemolytic assay. The hemolytic test included 25 μl of RBC suspension at various concentrations, 25 μl of antiserum at doubling dilutions, and 50 μl of complement. Antigen-antibody mixtures were incubated at 4°C for 60 min before the addition of complement. Two kinds of complement were used: guinea pig complement (Rockland Laboratories, Gilbertsville, Pa.) diluted 1:10 in various concentrations of FCS-PBS and undiluted rabbit complement (Pelfrez, Brown Deer, Wis.). The mixtures were incubated at various temperatures, shaken on a microtiter plate shaker after 1 h, and read after an additional 1.5 h of incubation. The degrees of hemolysis were estimated and expressed on a subjective 0-to-4 scale as follows: 4 = complete hemolysis; 3 ≈ 75% hemolysis; 2 ≈ 50% hemolysis; 1 ≈ 25% hemolysis; and 0 = no visible hemolysis. Control wells included test sera with uncoated RBCs and complement, coated RBCs and complement without test sera, coated RBCs and undiluted test sera without complement, and coated RBCs with cow 1777 serum (irrelevant anti-BoLA) and complement. Titers were reported as the highest serum dilution that yielded at least 50% hemolysis, i.e., a score of 2 or more as detailed above.

TABLE 1. Optimization of agglutination test for in vitro-shed *T. foetus* antigen(s)

Factor	Titer for: R+ (antiserum from a rabbit hyperimmunized with *T. foetus*)	Titer for: R− (irrelevant rabbit antiserum, polyclonal antiestrogen)
TYM supernatant		
6 h	0	0
12 h	0	0
24 h	0	0
48 h	1:64	1:2
72 h	1:64	1:2
Coating		
37°C	1:32	1:2
Room temp	1:16	1:2
4°C	1:8	1:2
6 h	1:4	0
24 h	1:8	1:2
Single	1:8	0
Double	1:8	1:2
Reused supernatant	1:8	0

Assay optimization. In order to optimize the procedures, the following factors were varied for the agglutination assay (Table 1): interval from TYM inoculation to harvesting of supernatant (6, 12, 24, 48, or 72 h), temperature for coating of RBCs with supernatant (37°C, room temperature, or 4°C), duration of coating of RBCs (6 or 24 h), number of coatings (single versus double coating) of RBCs in fresh supernatants, and number of times (once versus twice) a supernatant was used to coat the same RBCs.

For the hemolytic assay, the following were varied and/or investigated (Table 2): interval from TYM inoculation to harvesting of supernatant (6, 24, 48, 72, or 96 h), complement source (guinea pig or rabbit), RBC-coating temperature (37°C, room temperature, or 4°C), heat inactivation of the supernatant (56°C, 30 min); the use of proteinase inhibitors [a combination of 1 mM EDTA, 10 μg of *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E64; Sigma Chemical Co., St. Louis, Mo.) per ml, and 2 μg of leupeptin (Sigma) per ml], concentration of FCS in PBS (0, 1, or 2.5%), concentration of RBCs (suspensions of 1 or 2% added to wells to make a final concentration of 0.25 or 0.5% in the hemolytic test), concentration of RBCs incubated in TYM supernatant (2, 9, 15, or 30%), and ability to exhaust antigen in the supernatant by a single absorption with an equal volume of packed RBCs and supernatant.

RESULTS

Agglutination assay. The results of the optimization of the agglutination assay, using rabbit sera, are summarized in Table 1. Briefly, supernatant from TYM conditioned for 48 to 72 h with *T. foetus* provided the maximum amount of antigen, as evidenced by the 1:64 agglutination titers of R+ rabbit antiserum, versus the moderate agglutination (titer = 1:2) of the R− antiserum. Uncoated RBCs, used as a control, were not agglutinated by either rabbit antiserum (data not shown). Coating of RBCs at either room temperature or 37°C yielded higher agglutination titers than did coating at 4°C, although there were only small titer differences within temperature groups when coating occurred over 6 to 24 h. Likewise, a double incubation of the same RBCs in two consecutive fresh supernatant samples did not raise the titer in the agglutination assay. When the same conditioned TYM was reused to incubate additional RBCs, the agglutination titers remained unchanged. When cow serum was used as the antibody source, we did not observe strong specific agglutination as seen with spe-

TABLE 2. Optimization of hemolytic test for antibodies to in vitro-shed *T. foetus* antigen(s)

Factor	Titer for[a]:			
	1276	1777	Cow 4.5/4.6	Cow 9.5
TYM supernatant				
6 h	0	0	0	0
24 h	0	0	0	0
48 h	1:64	0	1:32	1:32
72 h	1:64*	0*	1:64*	1:32*
96 h	1:128*	0*	1:64*	1:32*
Guinea pig complement, 1:10 (final concn, 1:20)				
Coating of RBCs at 37°C	1:128	0	1:64	1:64
Coating of RBCs at room temp	1:64	0	1:64	1:64
Coating of RBCs at 4°C	0	0	0	0
Rabbit complement, undiluted				
Coating of RBCs at 37°C	>1:128	0	1:64	1:64
Coating of RBCs at room temp	>1:128	0	1:32	1:32
Coating of RBCs at 4°C	0	0	0	0
Inactivation				
56°C, − inhibitors	1:64	ND[b]	ND	ND
56°C, + inhibitors	1:64	ND	ND	ND
None, − inhibitors	1:64	ND	ND	ND
None, + inhibitors	1:64	ND	ND	ND
% FCS in PBS				
0	1:32	0	1:64	1:64
1	1:64	0	1:64	1:64
2.5	1:64	0	1:32	1:32
Final RBC concn (%)				
In test				
0.25	1:64	0	1:64	1:64
0.5	1:16	0	1:32	1:16
In TYM				
2	1:64	ND	1:64	1:32
9	1:32	ND	1:32	1:32
15	1:16	ND	1:64	1:32
30	1:16	ND	1:64	1:32
Antigen exhaustion[c]	0	ND	0	0
Control	1:64	ND	1:128	1:32

[a] 1276, bovine antisera from a cow immunized with affinity-purified TF1.17 antigen; 1777, irrelevant antiserum (polyspecific and BoLA alloantiserum); cow 4.5/4.6, experimentally infected heifer 4, at 5 or 6 weeks postinfection; cow 9.5, experimentally infected heifer 9, at 5 weeks postinfection. *, complement controls showed 25 to 50% hemolysis.

[b] ND, not done.

[c] Sequential incubation of 50 and 10% RBC in same TYM supernatant.

cific rabbit antiserum, although nonspecific agglutination was occasionally seen to occur in control sera raised against coated and/or uncoated RBCs (data not shown).

Hemolytic assay. Results of optimization of the hemolytic assay are summarized in Table 2, which shows data for serum from the cow immunized with purified TF1.17 antigen (cow 1276), the irrelevant bovine serum (cow 1777), and sera for two representative cows from the experimental infection study (cow no. 4, 5 or 6 weeks postinfection, and cow no. 9, 5 weeks postinfection). Incubation of uncoated RBCs with test and positive control sera consistently yielded hemolytic titers of less than 1:2 (data not shown in Table 2). As in the agglutination assay, 48- to 72-h supernatants yielded the highest hemolytic titers without affecting the controls. Regardless of the complement source (guinea pig or rabbit), coating at room temperature and at 37°C yielded similar hemolytic titers, but RBCs coated at 4°C did not show hemolysis at any serum dilution. Neither heat inactivation nor combined proteinase treatment of supernatants had an effect on hemolytic titers. For the different concentrations of FCS tested in the diluent, there was no clear difference in hemolytic titers. Increasing the concentration of RBCs in the hemolytic assay had a slight negative effect on titers, and titers were only slightly lowered in some but not all cow sera when the coating concentration (i.e., the concentration of RBCs incubated in TYM) was increased from 2 to 30%. However, antigen in TYM could be exhausted, as evidenced by the finding that incubation of 50% RBCs in TYM, followed by reuse of the same supernatant to coat 10% RBCs, reduced hemolytic titers from 1:64 to less than 1:2. The R+ antiserum also showed hemolytic titers of over 1:128, whereas the R− serum reacted at a titer of 1:2 to 1:4 (data not shown). When supernatant was used without adjustment of pH to 7.0, titers were inconsistent, and frequently the control wells showed hemolysis, especially at temperatures above 23°C.

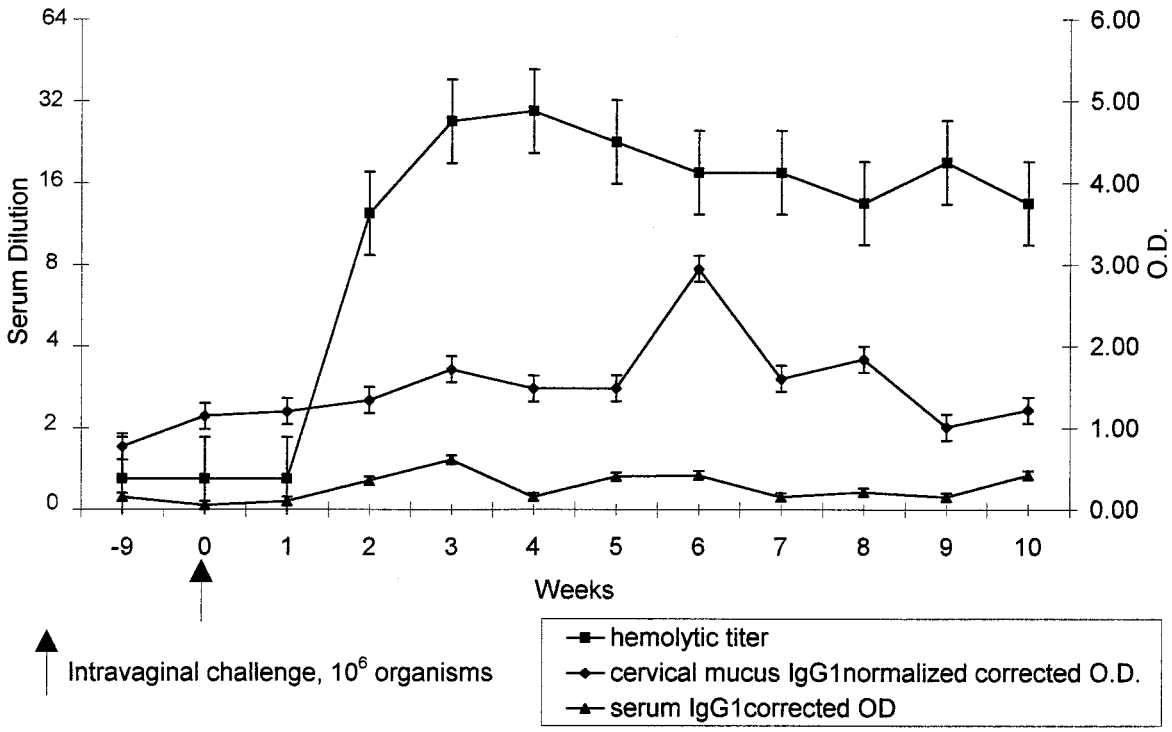

FIG. 1. Comparison of average ± standard error of the mean (error bars) serum hemolytic titer against in vitro-shed *T. foetus* antigen(s) and average ± standard error of the mean serum and cervical mucus IgG1 corrected optical densities O.D. by whole-cell ELISA, for eight experimentally infected heifers (group A). (ELISA data were reported earlier [2]).

Standard hemolytic test. From the above-described experiments, the following hemolytic assay was established. Briefly, 10% fresh bovine RBCs were added to pH-adjusted *T. foetus*-conditioned TYM and the mixture was incubated overnight at room temperature on a mixing rotator. After two washings with PBS, the RBCs were resuspended in 1% FCS–PBS at a concentration of 1% RBCs and 25 μl of suspension was added to 25 μl of doubling dilutions of serum samples. The microtiter plates were shaken and incubated at 4°C for 1 h. A 50-μl volume of guinea pig complement, diluted 1:10 in 1% FCS–PBS (final dilution of complement in the test, 1:20; final concentration of RBCs, 0.25%), was added to each well, and the plates were incubated for 1 h at 23°C, shaken, and further incubated for 1.5 h. Plates were then visually examined for hemolysis and scored. Serum from cow 1276 reacted strongly in all hemolytic assays (titer = 1:64 to 1:128), whereas serum from cow 1777 consistently yielded undetectable titers in the assay. These sera were consequently used as positive and negative controls, respectively, for the subsequent experiments.

When two different stabilates of D1 *T. foetus* were used to condition the TYM, hemolytic titers were unchanged. Likewise, the 600609 and EX663 isolates provided conditioned medium that developed titers comparable to those of D-1 supernatants. Hemolytic activity was also consistently observed when minimal essential medium was substituted for TYM (data not shown).

Group A (experimentally infected heifers). Figure 1 compares the average serum hemolytic titers with the average serum and cervicovaginal mucus IgG1 ELISA optical densities (previously reported) for eight experimentally infected heifers. In seven of these, serum hemolytic titers rose from undetectable levels prior to intravaginal infection to 1:32 to 1:64 by 3 weeks postinfection. In most cases, titers increased sharply by the second week postinfection. One animal had a preinfection serum hemolytic titer of 1:8, which rose by week 2 to 1:32, remained at this level for the next 7 weeks, and then declined to 1:16. Hemolytic titers in serum were not correlated with optical density activity obtained by whole-cell ELISA for IgG1 antibodies with sera from the same animals (r^2 = 0.33; 95% confidence interval = −0.3 to 0.9), and they peaked about 3 weeks earlier than did ELISA IgG1 activity in the vaginal mucus of these heifers (Fig. 1).

Group B (archived sera from experimentally infected pregnant cows). None of the preinfection sera from six cows showed a titer at any dilution in the hemolytic assay. In contrast, postinfection sera from five of six showed titers that peaked at 1:16 to 1:128. In three of these five cows, the peaks occurred within 2 to 3 weeks following infection, while in the remaining two, titers did not peak until 12 to 16 weeks following infection.

Group C (convenience sample heifers, presumably unexposed). Of the 65 heifers sampled, sera of 59 showed no hemolytic activity in the standard hemolytic assay described above. Sera from the remaining six showed titers ranging from 1:8 to 1:128.

Group D (unexposed dairy females). Of 39 serum samples tested, one mature cow's serum yielded a titer of 1:16, while the remainder of the sera gave negative results in the hemolytic assay.

Group E (sera from a suspect herd). Twenty serum samples from virgin (unexposed) heifers in a herd with a prior history and current suspicion of trichomoniasis showed no hemolytic titer. In contrast, sera from 6 of 20 2-year-old herd mate cows that should have been pregnant but were not showed a hemolytic titer of at least 1:2 (median titer = 1:4; mode titer = 1:16). Because of the long interval from bull exposure to blood sam-

TABLE 3. Overall specificity of hemolytic test for antibodies to in vitro-shed *T. foetus* antigen(s) in bovine females

Group[a]	No. of samples with result:		Total	Specificity (%)
	Positive	Negative		
A	1	7	8	88
B	0	6	6	100
C	6	59	65	91
D	1	38	39	97
E	0	20	20	100
F	0	42	42	100
Total	8	172	180	95.6

[a] Groups: A, preinfection sera from beef heifers infected by intravaginal instillation of *T. foetus*; B, archived preinfection sera from beef cows exposed at midgestation by intravaginal instillation of *T. foetus*; C, sera from a convenience sample of 65 presumed-unexposed beef heifers; D, sera from unexposed dairy cows and heifers; E, sera from virgin heifers in a suspect beef herd; F, sera from virgin heifers in a known infected herd.

pling (approximately 5 months), we did not attempt to ascertain culture status of these females.

Group F (sera from virgin heifers in an infected herd). None of the 42 serum samples from virgin heifers in a herd known to be infected with *T. foetus* showed a positive titer.

Specificity and sensitivity for females. Table 3 summarizes the overall specificity of the hemolytic assay for antibodies to in vitro-shed *T. foetus* antigen for the various groups of female cattle that were tested. Combining results from all groups studied showed that of 180 known or presumed nonexposed animals, 8 showed positive titers of ≥1:2 (specificity = 172 of 180 = 95.6%). For sera from experimentally infected heifers or cows (i.e., known positive infection status), 13 of 14 showed positive titers (sensitivity = 92.9%).

Chronically infected bulls. Six of 14 (43%) chronically infected bulls showed a positive hemolytic titer (median titer, 1:8; range, 1:2 to 1:128).

DISCUSSION

Tables 1 and 2 illustrate the feasibility of (i) using agglutination and/or hemolytic tests (with specific polyclonal rabbit and cow antisera) to detect an RBC-adhering molecule(s) shed by *T. foetus* in the supernatant of culture media and (ii) using such coated RBCs to detect serum antibodies in experimentally and naturally exposed cattle. Specific polyclonal rabbit antiserum to *T. foetus* showed high titers against sensitized RBCs (sensitized by overnight incubation in the presence of 48-h-conditioned TYM) in both agglutination and hemolytic tests. However, specific bovine antisera had a tendency to react more appropriately only in the hemolytic test. In fact, as others have noted (16), nonspecific agglutination, apparently due to the presence of nonspecific agglutinins, was detected in certain fresh bovine sera. Thus, we directed our attention to the hemolytic test, to study the serum immune response to an in vitro-shed antigen(s) of *T. foetus* in experimentally infected virgin heifers and cows, potentially exposed cows, and chronically infected bulls and to compare the new results with our previously reported ELISA findings.

The antigen(s) detected by this hemolytic response is apparently heat stable, since there was no lability on incubation at 56°C for 30 min. Likewise, there was no significant loss of antigen (as detected by the hemolytic test) in the absence of proteinase inhibitors, in spite of the reportedly potent extracellular proteinase activity that characterizes *T. foetus* (3).

The presence of a specific serologic response in the bovine male and female to *T. foetus*, a pathogen that is strictly limited to the reproductive tract (13, 14), is documented by this study. Previous difficulty in characterizing such a response may have been a result of interference from cross-reacting antibodies or may have been due to a relative lack of sensitivity of a given test. Using ELISA, in which the antigen was either the whole cell (19) or the purified TF1.17 antigen (10a), we have characterized a parasite-specific IgG1 and IgA antibody response in cervicovaginal mucus, following experimental infection. The former study (19) also noted that the whole-cell ELISA detected very little change in activity in the sera of these animals. This lack of detectable serum response in animals with marked vaginal mucus antibody activity was later confirmed by Gault et al. (8), who also used whole cells as antigens in ELISAs. It is not clear whether the failure to detect a systemic serological response is due to an overall lack of sensitivity of the ELISAs employed or to qualitative and/or quantitative antigenic differences between whole-cell and conditioned-medium preparations. It is possible that the antigen detected in the hemolytic assay represents a small minority of the total antigens available in whole-cell or lysate preparations but that such a shed antigen is a critical immunogen for the host.

Evidence that the antigen(s) detected in this assay may be related to a previously described surface antigen comes from the fact that serum from cow 1276, which had been immunized with immunoaffinity-purified TF1.17 antigen (a known, highly conserved surface antigen of *T. foetus* [9 and 10]) showed high titers in the hemolytic assay. We (10a) have previously suggested that detection of anti-TF1.17 IgA in vaginal secretions may be a useful diagnostic aid in herd screening. In that study (10a), we noted a significant rise in vaginal mucus antibody 6 weeks after infection, while the rise in serum hemolytic antibody in the present study occurred within 2 to 4 weeks after infection. In the current study, the duration of the hemolytic antibody response in serum was not specifically tested, although the data from the group A heifers (Fig. 1) suggest that it is sustained for at least 10 weeks following a single exposure. This compares with a 24-week-long vaginal mucus IgA response to purified surface antigen (10a).

The means by which the host is presented with antigen in this disease is not known. It has long been thought that *T. foetus* is not invasive but rather exists as a lumen dweller for the duration of infection; however, some studies (4, 15) have noted whole organisms beneath the epithelial surface of tissues from aborted conceptuses, suggesting that the organism can be invasive. If it invades maternal tissues as well, it would be expected to elicit a serologically detectable response, as reported here. But it is also possible that dendritic cells within the endometrium, endocervix, or vaginal epithelium may present *T. foetus* antigen to the host in the absence of invasion. In this respect, it is worth noting that a circulating immune response could be demonstrated in some males, in which *T. foetus* resides only on the squamous epithelial surface (13).

In summary, we developed a specific and sensitive complement-mediated hemolytic assay that detects a serological response in virgin heifers experimentally infected with *T. foetus* and in exposed cows and some chronically infected bulls. The timing of the response (2 to 4 weeks postinfection) is typical of a specific, primary immune response, and this serological response apparently precedes the peak of whole-cell-directed antibody production in reproductive tract secretions of infected heifers. While the molecular specificity of the hemolytic response is not known, the fact that host antibody is directed towards a shed parasite antigen(s) that coats host cells leaves open the possibility that the host response itself may be in-

volved in the pathogenesis of trichomoniasis, i.e., in the loss of the conceptus. The coating antigen(s) seen in this study needs to be characterized before any possible pathogenic role for it is sought. That is, we have only documented that, in vitro, *T. foetus* sheds an antigen(s) that coats host cells and that host antibody from infected females and males reacts with such shed antigen(s). The presence of these antibodies suggests but does not prove that such shedding occurs in vivo. In the meantime, the specificity and sensitivity levels of the hemolytic assay appear to be high enough to allow for further diagnostic studies at the population level. It is important to note that the detection of such antibodies indicates exposure only and not current infection status of a given individual. Further studies of the persistence and effects (if any) of these hemolytic antibodies are indicated.

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