Bovine Vaginal Antibody Responses to Immunoaffinity-Purified Surface Antigen of *Tritrichomonas foetus*

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Bovine trichomoniasis is a prevalent sexually transmitted disease of cattle caused by the protozoan *Tritrichomonas foetus*. Currently, diagnosis is most often made by culture. In order to provide a faster immunodiagnostic approach, a specific enzyme-linked immunosorbent assay (ELISA) was investigated. A protective surface antigen (TF1.17 antigen) of *T. foetus* was immunoaffinity purified and used in an ELISA to detect antibodies in vaginal mucus from heifers inoculated with *T. foetus*. In preliminary studies, antibodies of the immunoglobulin A (IgA) isotype were detected in mucus from all experimentally infected heifers which were tested at 6 weeks postinoculation, whereas IgG1 and IgG2 were not. In addition, IgA responses detected in postinoculation samples were all greater than those detected in preinoculation samples, unlike those detected by a whole-cell antigen ELISA. For these two reasons, IgA antibodies appeared to be useful diagnostically. Further investigation of IgA antibodies used vaginal mucus collected weekly from heifers inoculated intravaginally with 10^2 , 10^4 , or 10^6 *T. foetus* organisms. Heifers with positive cultures for *T. foetus* had similar IgA responses to TF1.17 antigen over the 10 weeks of infection regardless of the initial inoculum dose. This indicates that if the dose is sufficient to establish infection, the magnitude and duration of the immune response are no longer dependent on dose. All of the infected animals receiving all dosages responded with high absorbance values in the IgA anti-TF1.17 antigen ELISA by 6 weeks postinoculation, and all absorbance values remained high at 10 weeks. To determine the duration of the IgA response, four other heifers inoculated with 7×10^6 *T. foetus* organisms were studied through 24 weeks postinoculation. IgA antibody responses to TF1.17 antigen were still high at 24 weeks, even though some heifers cleared the infection several weeks earlier. These results indicate that experimentally infected heifers produced detectable long-lasting IgA responses to TF1.17 antigen in vaginal mucus, which may be useful in a herd test for the diagnosis of bovine trichomoniasis.

Bovine trichomoniasis is a sexually transmitted disease of cattle caused by the flagellated protozoan *Tritrichomonas foetus* (19). Infected cows usually develop transient vaginitis and endometritis resulting in transient infertility. More rarely, later-term abortion or pyometra occurs. Cows may also become asymptomatic carriers, while bulls are nearly always asymptomatic. Trichomoniasis is common in areas of the world where natural breeding is practiced, such as the western United States (3, 15), Argentina (6), and Costa Rica (17). In California, for example, approximately 16% of beef herds had at least one infected bull and 4% of bulls examined were infected (3). Economic losses owing to trichomoniasis are great. For example, the loss in one California dairy herd that had an average of 940 cows and that used natural breeding practices was estimated to be just under \$70,000 during an 18-month period ending in June 1985 (8). In 1991, losses to the United States beef industry were estimated at \$650 million annually (24).

The diagnosis of bovine trichomoniasis is important for decreasing reproductive losses because of this disease. Currently, diagnosis is based on history and culture and/or direct microscopic examination of secretions (2). The sensitivities of culture methods were reported to be 58.7% with vaginal mucus from cows in a dairy herd (8) and 81.6% with preputial smegma samples from bulls in a beef herd (22). A commer-

cially available in vitro culturing system has been developed, and it has several advantages for use in the field (1, 25). However, culture results are not available for 1 to 7 days after inoculation of the media. Also, to increase the speed of diagnosis, an assay has been developed for the detection of *T. foetus* DNA by hybridization to oligonucleotide probes (24). The sensitivity of this assay has been controversial since a subsequent study reported that of 26 culture-positive bulls only 10 or 14 animals were identified as being infected by the DNA probe assay (1). More recently, using PCR amplification of repetitive genomic sequences of DNA, Ho and coworkers (11) were able to identify 90% of infected samples from naturally infected bulls, while uninfected bulls were all classified as negative with the same PCR probe. This is promising. An antigen assay would be another method for detection of the parasite. For example, an enzyme immunoassay was developed to detect *T. foetus* antigens in cervicovaginal mucus (26). However, the sensitivity of that test was low, possibly owing to cross-reactivity with the bovine secretions.

For herd testing, an assay for antibody would be faster than culture but simpler than PCR or antigen enzyme-linked immunosorbent assay (ELISA). However, no reliable immunological assays are currently available. An indirect fluorescent-antibody test for the detection of antibodies to whole-cell antigens was recently reported for measuring immunoglobulin G (IgG) antibody titers in serum in response to vaccination (9). Since the response to infection is primarily in reproductive secretions (4, 20), this test would need to be adapted to mucus for diagnosis. One problem with immunologic tests for the detection of antibody is their use of crude preparations con-

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FIG. 1. Whole-cell antigen (a) and TF1.17 antigen (b) ELISA detection of vaginal IgG1 antibodies. Vaginal mucus was collected from eight heifers 2 weeks before (open bars) and 6 weeks after (hatched bars) intravaginal inoculation with 10^6 *T. foetus* organisms. Each value for A_{492} is the mean of two measurements.

taining several antigens that cross-react with other microorganisms. A purified diagnostic antigen which was not cross-reactive would be ideal. More than 55 proteins ranging between 15 and 225 kDa in molecular mass have been reported in *T. foetus* extracts, as resolved by polyacrylamide gel electrophoresis (10, 13). Bovine antiserum prepared against *T. foetus* reacted with approximately 35 to 38 of those components on immunoblots. Burgess (5) identified with monoclonal antibodies a 150-kDa surface antigen expressed by different strains of *T. foetus* from diverse geographical areas. One or more of these antigens may be useful in an improved immunodiagnostic assay.

We have investigated a surface antigen of *T. foetus* (TF1.17 antigen) originally identified with two monoclonal antibodies (12). These monoclonal antibodies immobilized, agglutinated, and mediated complement killing of live organisms, as well as prevented the adherence of *T. foetus* to bovine vaginal epithelial cells. Immunization of heifers with the immunoaffinity-purified TF1.17 antigen enhanced the clearance of *T. foetus* from the reproductive tract (4). Importantly, TF1.17 antigen was shown to be conserved among isolates of *T. foetus* cultured from cattle in Argentina, Costa Rica, and the United States (4, 14). Thus, TF1.17 antigen may be useful as a subunit vaccine, a diagnostic antigen, or both.

The purpose of the study described here was to determine by ELISA the vaginal antibody responses to TF1.17 antigen in heifers experimentally infected with *T. foetus*. The vaginal response was studied because little systemic response occurs during infection but the vaginal antibody response is readily detectable (4). Also, bovine vaginal mucus is already used for culture, so collection of mucus is routine. Isotypic antibody responses to TF1.17 antigen were compared with responses to whole-cell antigens in ELISA. The kinetics and duration of the antibody responses during infection were determined to assess

the potential of a TF1.17 antigen ELISA for use in immunodiagnosis.

MATERIALS AND METHODS

Organisms. *T. foetus* D1 was originally cultured from a cow with pyometra (20, 21). *T. foetus* cells were grown in Trypticase-yeast extract-iron serum medium (7) at 37°C for 48 h to the late logarithmic phase of growth (approximately 5×10^6 organisms per ml).

Infection of heifers. Thirty-three virgin heifers were inoculated intravaginally with *T. foetus* at the time of estrus in studies already reported (4, 20, 21). In brief, the estrous cycles were synchronized by use of progestin implants as described previously (4). Washed logarithmic-phase *T. foetus* cells were resuspended in phosphate-buffered saline (PBS; pH 7.2) at a cell concentration of 1×10^2 , 1×10^4 , 1×10^6 , or 7×10^6 organisms per ml. One milliliter of the cell suspension was introduced into the anterior vagina with an artificial insemination pipette at week 0. Vaginal mucus was collected with an insemination pipette at weekly intervals before and after inoculation with *T. foetus*. Samples were examined for *T. foetus* by direct microscopic examination and by culture of secretions in Trypticase-yeast extract maltose medium with 10% serum (4). Medium with penicillin (1,000 U/ml) and streptomycin (1 mg/ml) was used for initial isolation only.

ELISA. The vaginal mucus of the heifers was tested for antibody to *T. foetus* whole-cell antigens or TF1.17 antigen by ELISA by a procedure modified from our previously reported work, with *T. foetus* cells used as antigen (4). Microtiter plates (half well; Costar, Cambridge, Mass.) were coated with whole-cell antigens by incubating each well with 50 μ l of a suspension of *T. foetus* organisms that were washed four times in PBS and resuspended to a concentration of 10^6 cells per ml of PBS. Microtiter plates were coated with TF1.17 antigen by incubating each well with 50 μ l of TF1.17 antigen immunoaffinity purified as described previously (4) and diluted in PBS to a concentration of approximately 3 μ g/ml. After incubating the plates for 4 h at room temperature, the wells were forcefully emptied and treated with 95% ethanol for 2 min. The wells were emptied again, and 50 μ l of 95% ethanol was added to each well. Plates were air dried overnight at room temperature and were then stored at -20°C for up to 3 months before use in the immunoassay. The wells were blocked with 100 μ l of PBS containing 0.05% Tween 20 and 0.2% gelatin (PBS-TW-G) for 30 min at 37°C. The wells were emptied and washed three times with PBS containing 0.05% Tween 20 (PBS-TW). Vaginal mucus was diluted 1:50 in PBS-TW-G, and 50 μ l was added to each well. After incubating for 30 min at 37°C, the wells were washed with PBS-TW as described above, and 50 μ l of mouse monoclonal antibodies to

FIG. 2. Whole-cell antigen (a) and TF1.17 antigen (b) ELISA detection of vaginal IgG2 antibodies. Vaginal mucus was collected from eight heifers 2 weeks before (open bars) and 6 weeks after (hatched bars) intravaginal inoculation with 10^6 *T. foetus* organisms. Each value for A_{492} is the mean of two measurements.

bovine IgA, IgG1, or IgG2 (provided by A. Guidry, U.S. Department of Agriculture, Beltsville, Md.) diluted 1:4,000, 1:8,000, or 1:2,000 in PBS-TW-G, respectively, was added to the appropriate well. After incubating for another 30 min at 37°C, the wells were washed with PBS-TW and treated with 50 μ l of peroxidase-conjugated goat anti-mouse IgG and IgM (heavy and light chains; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:1,000 in PBS-TW-G. The plates were incubated for 30 min at 37°C, emptied forcefully, and washed with PBS-TW, and each well was developed in 50 μ l of a solution of 0.043% (wt/vol) *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) in a citrate buffer (34 mM citric acid, 34 mM sodium citrate) with 0.01% (vol/vol) hydrogen peroxide. After incubating for 1 h at room temperature, the A_{492} was read on an ELISA reader (Titertek Multiskan; Flow Laboratories Inc., Ingleswood, Calif.). Controls for blanking consisted of wells containing antigen, mouse monoclonal antibodies to bovine immunoglobulin isotypes, and peroxidase-conjugated goat anti-mouse immunoglobulins. Samples were analyzed in duplicate (to whole-cell antigens) or triplicate (to TF1.17 antigen), and the mean values are reported.

RESULTS

TF1.17 antigen and whole-cell antigens were compared in separate ELISAs to detect the IgG1, IgG2, and IgA antibody responses of eight heifers infected with *T. foetus* (Fig. 1 to 3). IgM responses were not investigated because previous studies showed that IgM titers varied little during infection (4, 20). Preinoculation mucus samples were collected 2 weeks before *T. foetus* was given intravaginally, and postinoculation mucus samples were collected 6 weeks after the introduction of 10^6 live *T. foetus* D1 organisms. With the whole *T. foetus* cells used as antigen, some preinoculation responses were greater than some of the postinoculation responses whether the isotype was IgG1 (Fig. 1a), IgG2 (Fig. 2a), or IgA (Fig. 3a). With TF 1.17, very weak or no IgG1 (Fig. 1b) or IgG2 (Fig. 2b) antibody responses were detected in two of the eight infected heifers (heifers 4 and 22). However, an IgA response was detected by the TF1.17 antigen ELISA in all of the heifers infected with *T. foetus* (Fig. 3b), including the two heifers with weak or no IgG responses (heifers 4 and 22). In addition, postinoculation IgA

responses to TF1.17 antigen were greater than preinoculation values in all cases (Fig. 3b).

Detection of an IgA response to TF1.17 antigen in vaginal mucus might be useful diagnostically, and therefore, this response was studied in more detail. Samples from heifers inoculated at week 0 with 10^2 , 10^4 , or 10^6 *T. foetus* organisms were assayed. To establish cutoff points, vaginal IgA antibody responses to TF1.17 antigen at 8 weeks of infection were compared in culture-positive and culture-negative heifers (Table 1). Whether the initial dose was 10^2 , 10^4 , or 10^6 , animals which became infected had an average absorbance of greater than 0.7, whereas those which were culture negative throughout the study had an average absorbance of less than 0.2. Thus, absorbance readings of less than 0.2 were taken as negative. The main difference among the dosage groups was the number of animals becoming infected, as we reported previously (4). When the kinetics of these responses for culture-positive animals were studied over the 10 weeks of infection, no differences between the mean IgA responses of the heifers inoculated with different doses of *T. foetus* were detected (Fig. 4). All responses of infected animals were positive (greater than the culture-negative values of less than 0.2) by week 6 and remained high when the study was terminated at week 10.

The vaginal IgA antibody responses to TF1.17 antigen were also studied in chronically infected heifers to determine if a long-lasting response was detectable. Four heifers inoculated with 7×10^6 *T. foetus* organisms became colonized with trichomonads in the reproductive tract within the first week after inoculation, and colonization of the vagina lasted 13 to 25 weeks, as was reported previously (21). Although these heifers cleared the infection at 13 to 25 weeks, the antibody responses to TF1.17 antigen in the vaginal mucus remained high at 24 weeks postinoculation (Table 2). Interestingly, the two heifers which cleared the infection earliest (heifers 118 and 119), at 13

FIG. 3. Whole-cell antigen (a) and TF1.17 antigen (b) ELISA detection of vaginal IgA antibodies. Vaginal mucus was collected from eight heifers 2 weeks before (open bars) and 6 weeks after (hatched bars) intravaginal inoculation with 10^6 *T. foetus* organisms. Each value for A_{492} is the mean of two measurements (no preinoculation value was available from heifer 9 in the whole-cell antigen assay).

or 19 weeks, respectively, still had high ELISA values at 24 weeks, suggesting that antibody remains long after clearance.

DISCUSSION

In the present study, we showed that TF1.17 antigen is more useful diagnostically than whole-cell antigens in an ELISA for the detection of antibodies of various isotypes in the vaginal mucus of heifers infected with *T. foetus*. Preinoculation antibody levels to whole-cell antigens in vaginal mucus were high compared with the postinoculation levels (Fig. 1a, 2a, and 3a). This would obscure detection of antibodies to *T. foetus* infection, and therefore, whole-cell antigens would not be useful diagnostically. It is possible that cross-reactive antibodies present in normal bovine serum, as well as in vaginal secretions, are responsible for this reactivity. For example, it was reported that *T. foetus* antibodies cross-react with *Campylobacter fetus* (18). In contrast, preinoculation vaginal antibody values to TF1.17 antigen were low compared with the postinoculation values (Fig. 1b, 2b, and 3b). Immunoaffinity-purified TF1.17 antigen has a much lower number of epitopes and

therefore a lesser chance than whole-cell antigens of reacting with cross-reactive antibodies in the ELISA. Therefore, TF1.17 antigen is a better candidate than whole-cell antigens for use as a diagnostic antigen. In addition, TF1.17 antigen was previously shown to be conserved among different isolates of *T. foetus* (4, 14) and therefore should detect infection with other isolates in the field as well as the isolates used in the present studies.

The isotype of the antibody detected may be as important as the antigen in developing a specific ELISA. Since *T. foetus* colonizes the vagina, cervix, and uterus, it is likely that local immunity is important in the clearance of the microorganism

TABLE 1. Vaginal IgA antibody responses to TF1.17 antigen in heifers inoculated with *T. foetus*

Dose	No. of heifers	Mean absorbance at 8 wk (no. with indicated result/total no.)	
		Culture positive	Culture negative
10^2	6	0.816 (3/6)	0.011 (3/6)
10^4	8	0.787 (5/8)	0.178 (3/8)
10^6	15	0.770 (15/15)	NA ^a

^a NA, not applicable.

FIG. 4. Mean vaginal IgA antibody responses from heifers with positive cultures for *T. foetus* following inoculation at week 0 with 10^2 ($\blacktriangle$; $n = 3$), 10^4 ($\circ$; $n = 5$), or 10^6 (experiment 1 [$\square$], $n = 7$; experiment 2 [$\blacksquare$], $n = 8$) *T. foetus* organisms as detected by the TF1.17 antigen ELISA and given as A_{492} values.

TABLE 2. Vaginal IgA antibody responses to TF1.17 antigen in chronically infected heifers

Heifer	Wk of last positive culture ^a	Absorbance at 24 wk
117	25	0.683
118	13	0.902
119	19	1.050
120	21	0.697

^a Vaginal cultures for *T. foetus* were done weekly before and after inoculation.

from the reproductive tract. In a previous study, Skirrow and BonDurant (20) showed that IgA, IgG1, and IgG2 antibodies to whole *T. foetus* antigens were found in the reproductive tract secretions of heifers experimentally inoculated intravaginally with *T. foetus*. Although IgG1 and IgG2 antibodies predominated in the sera of infected heifers, the levels were not much higher than preinoculation levels. Very little IgM was detected in either sera or secretions. Since serum antibody values increased only slightly, secretory antibodies of the IgA, IgG1, and IgG2 isotypes in the vaginal mucus of heifers were more likely to be diagnostic of *T. foetus* infection. With the TF1.17 antigen ELISA used in the present study, antibodies of the IgA isotype were detected in all of the infected heifers (Fig. 3b), whereas antibodies of the IgG1 and IgG2 isotypes were detected in only 75% of the infected heifers (Fig. 1b and 2b). Therefore, detection of an IgA response to TF1.17 antigen in vaginal mucus would be more useful diagnostically than detection of an IgG1 or IgG2 response.

The kinetics of the IgA response to TF1.17 antigen in heifers inoculated with different doses of *T. foetus* demonstrated no differences in responses when only culture-positive heifers were selected (Fig. 4). This probably reflects the fact that if a heifer becomes infected, the duration of infection is similar regardless of the dose (4). By 6 weeks postinoculation the average absorbance was far greater than the average preinoculation absorbance values (Fig. 3b and 4) or the average absorbance values of culture-negative heifers at 8 weeks postinoculation (Table 1). These results are in agreement with the results of other studies demonstrating vaginal IgA responses to *T. foetus* antigens 5 to 9 weeks postinoculation (20, 23). Interestingly, the period of first increase in IgA titers (6 weeks) is just before the time at which others (16) showed that endometritis became detectable (60 days). Also, Parsonson et al. (16) found no fetal loss until 63 days. Therefore, by the time that a problem is noted, a diagnosis may be made by ELISA.

In our study of the duration of the vaginal IgA antibody response in four heifers, a long-lasting response was shown by high absorbance readings at 24 weeks postinoculation (Table 2). In fact the two heifers (heifers 118 and 119) which cleared the infection the earliest had the highest absorbance values at 24 weeks, showing that vaginal IgA responses could detect trichomoniasis in a herd even after individual heifers had cleared the infection. Since trichomoniasis is a herd problem when natural breeding is used, this would be an advantage. Application of the TF1.17 antigen ELISA in monitoring the infection status of a herd would involve a random sampling of heifers. Since preinoculation (Fig. 3 and 4) and culture-negative (Table 1) samples produced absorbance values of less than 0.2, heifers tested from an uninfected herd should produce absorbance readings below this cutoff point. In an infected herd, the fact that some infected animals would not be detected during the first 4 to 6 weeks of infection would not be a problem, because the immunoassay would detect other in-

fecting animals (as well as convalescent animals) within the herd by the presence of absorbance readings above the cutoff point of 0.2. We conclude that the ELISA to TF1.17 antigen described here may be useful as a herd test for the diagnosis of bovine trichomoniasis since it detected a long-lasting IgA response from the vaginal mucus of heifers experimentally infected with *T. foetus*. Field testing with comparison with culture results would be helpful to confirm this tentative conclusion.

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