

8-30-2017

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Abstract

Tritrichomonas foetus is a flagellated protozoan parasite that causes inflammation of the reproductive tract leading to early embryonic death and abortion in cattle, thereby resulting in significant economic losses. Testing and culling infected bulls is an important strategy for parasite control. Routine testing is mainly limited to bulls that are traveling across state lines or within states that have specific control programs. Both culture and PCR detection methods are available, but they are not typically conducted as part of a yearly breeding soundness program and are not easily conducted in the field. In the present study, we developed a bead agglutination assay for detection of *T. foetus* antigens. Our experiments revealed that latex beads conjugated to *T. foetus* lipophosphoglycan-binding antibodies visibly clump in the presence of *T. foetus*. The detection limit of the assay, determined using both field and laboratory isolates of the parasite, was 0.25 µg/mL and 1.0 µg/mL total *T. foetus* antigen, respectively. Our results indicate that an antigen detection test could offer a tool for screening bulls under field conditions.

Keywords

Trichomoniasis, Cattle parasitism, Reproductive disease, Field diagnostics

Disciplines

Veterinary Infectious Diseases | Veterinary Pathology and Pathobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments

This article is published as Schaut, Robert G., Lynette B. Corbeil, Courtney N. Blake, and Matthew T. Brewer. "Development of a bead-agglutination assay for rapid detection of *Tritrichomonas foetus*." *Veterinary Parasitology* 243 (2017): 188-191. DOI: [10.1016/j.vetpar.2017.06.025](https://doi.org/10.1016/j.vetpar.2017.06.025).

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Research paper

Development of a bead-agglutination assay for rapid detection of *Tritrichomonas foetus*



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ARTICLE INFO

Keywords:

Trichomoniasis
Cattle parasitism
Reproductive disease
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ABSTRACT

Tritrichomonas foetus is a flagellated protozoan parasite that causes inflammation of the reproductive tract leading to early embryonic death and abortion in cattle, thereby resulting in significant economic losses. Testing and culling infected bulls is an important strategy for parasite control. Routine testing is mainly limited to bulls that are traveling across state lines or within states that have specific control programs. Both culture and PCR detection methods are available, but they are not typically conducted as part of a yearly breeding soundness program and are not easily conducted in the field. In the present study, we developed a bead agglutination assay for detection of *T. foetus* antigens. Our experiments revealed that latex beads conjugated to *T. foetus* lipophosphoglycan-binding antibodies visibly clump in the presence of *T. foetus*. The detection limit of the assay, determined using both field and laboratory isolates of the parasite, was 0.25 µg/mL and 1.0 µg/mL total *T. foetus* antigen, respectively. Our results indicate that an antigen detection test could offer a tool for screening bulls under field conditions.

1. Introduction

The protozoan *Tritrichomonas foetus* is an obligate parasite of the bovine reproductive tract. Trophozoites of *T. foetus* live within the prepuce of bulls which then transmit the parasite during breeding. While infection of bulls does not typically lead to clinical signs, infections result in early embryonic death and abortion in cows. *T. foetus* occurs throughout the world and causes serious economic loss in the cattle industry (Ondrak, 2016). In herds with endemic trichomoniasis, producers experience 5–35% decrease in revenue per cow (Rae, 1989).

Surveillance for trichomoniasis focuses on sampling bulls and has the potential to eradicate the disease since the parasite can only survive in the bovine reproductive tract (Yao, 2013). Detection of parasite antigens by immunohistochemistry allows for visualization of antigen at the anatomical sites of infection (Rhyan et al., 1999); however IHC requires tissue collection via biopsy and further processing which is not practical under field conditions. *T. foetus* can be detected in smegma obtained in preputial washes by culture and PCR methods (Effinger et al., 2014). The PCR technique is sensitive and specific, however, it requires a level of expertise and specialized equipment limited to diagnostic laboratory settings. Recently, it was demonstrated that PCR also amplifies DNA from *Simplicimonas*-like organisms in bovine vaginal

samples thereby confounding diagnosis (Frey et al., 2017). Importantly, *T. foetus* diagnostics focus on bulls that are traveling to shows, crossing state lines, or in states with specific control programs. In contrast, many bulls remain untested as *T. foetus* diagnostics are not typically considered part of a yearly breeding soundness exam. A diagnostic test that can be conducted in the field by veterinarians could lead to an increased number of bulls screened for *T. foetus* and aide in surveillance and eradication efforts.

A variety of diagnostic tests have utilized immobilized antibody arrays which utilize chromogenic compounds to visualize test results in veterinary clinics (Atkins, 2003). However, no such tests are available for *T. foetus*. Previous studies have revealed the utility of anti-*T. foetus* monoclonal antibodies TF1.15 and TF1.17 for detection of the parasite by immunofluorescence assay (Corbeil et al., 2008). These antibodies target the parasite lipophosphoglycan (LPG). LPG is a glycolipid which coats the surface of the parasite and is comprised of a phospholipid anchor, polysaccharide core and branching oligosaccharides (Roychoudhury et al., 2015). These unique features of the LPG glycolipid are a specific trait of protozoan parasite species which makes LPG an ideal parasite-specific target (Dos-Santos et al., 2016; Menezes et al., 2016). However, the antibodies are IgM isotype and these pentamers are more bulky and less amenable to an immunochromatography

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format. However, the pentamer structure of IgM makes it an especially good agglutinin. We hypothesized that anti-*T. foetus* antibodies TF 1.15 and TF1.17 could be utilized to detect parasite antigens in a bead agglutination diagnostic assay that could be conducted under field conditions. In previous studies, bead agglutination has been used to detect a related parasite, *Trichomonas vaginalis* (Darani et al., 2010). In the present study, we assessed agglutination of anti-*T. foetus* antibodies conjugated to latex beads as a method for detecting *T. foetus* antigens.

2. Materials and methods

2.1. Antibodies

Monoclonal antibodies, TF 1.15 and TF 1.17 were harvested from hybridomas and prepared as previously described (Hodgson et al., 1990). These mAbs were both of the IgM class, which made them especially suitable for development of a latex bead agglutination assay. Mouse total IgG and mouse anti-ovalbumin (Fisher Scientific) were used as control antibodies.

2.2. Preparation of latex beads

Commercial latex microspheres (1 μm , Bangs Laboratories) were conjugated to anti-LPG antibodies. 1 mL of microspheres was washed by suspension in 10 mL of MES activation buffer (Thermo Scientific), followed by centrifugation at 3220 $\times g$ for 20 min at 4 °C. Beads were then resuspended in 5 mL of MES activation buffer, vortexed, and sonicated by inserting a remote sonication probe into the tube for 2 min (Fisher Sonic Dismembrator Model 100). 100 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Fisher Scientific) was added and the beads were allowed to react for 5 min at room temperature (21 °C) with continuous mixing. Monoclonal antibody supernatants were diluted in MES buffer (100 $\mu\text{g}/\text{mL}$ antibody) and mixed with the activated bead suspension at room temperature for 4 h with constant mixing. Reacted microspheres were washed and resuspended in 10 mL of quenching solution (30 mM glycine in 1% BSA) and mixed gently for 30 min at room temperature. Linked beads were washed and resuspended in storage buffer (PBS + 0.1% BSA) to storage concentration (10 mg/mL) and stored at 4 °C.

2.3. Parasite antigens

Trichomonas foetus (BP-4 Beltsville strain, ATCC[®] 30003™ and field strain IA.1) were cultured in trypticase-yeast-maltose (TYM) medium as described previously (Bader et al., 2016). Cultures were maintained at 32 °C and regularly sub-cultured. When cells reached concentrations of 5×10^5 trophozoites/mL, whole cell lysates were harvested by re-suspending parasites in an SDS-containing lysis buffer followed by 3 freeze-thaw cycles. Antigens were stabilized with Halt Protease Inhibitors (1X) (Thermo Scientific). *Leishmania major* antigens were prepared from promastigotes grown in Grace's medium by a similar method. The protein concentration was determined by BCA assay according to the manufacturer (Thermo Scientific). Antigen preparations from 5×10^5 trophozoites/mL yielded 1.5–1.8 mg/mL soluble protein. Bovine serum albumin and ovalbumin (Fisher Scientific) were used as antigen controls to assess non-specific binding.

2.4. Agglutination assay

Antibody conjugated-beads (50 μL) were added to flat-bottom 96 well microtiter plates and co-incubated with dilutions of parasite antigen (100 μL final volume). Experiments were repeated a minimum of two times in duplicate for each experimental condition. Bead-antigen mixtures were allowed to incubate at room temperature for 2 h. After incubation, plates were microscopically (10X magnification) evaluated for determination of agglutination. Agglutination was recorded on an

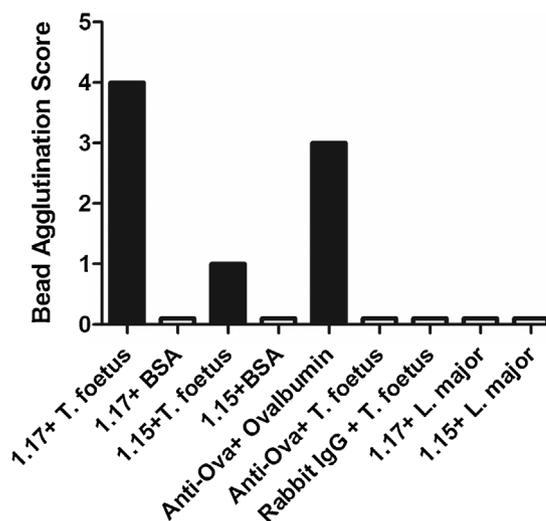


Fig. 1. Agglutination of latex beads conjugated to antibodies in the presence of 50 $\mu\text{g}/\text{mL}$ antigen. Antibody and antigen pairs are shown on the X axis. 1.17 and 1.15 represent *T. foetus*-specific antibodies. Black bars represent antibody-conjugated beads paired with target antigens and open bars represent negative antigen controls.

arbitrary scale of 0–4 with 0 as no visual agglutination and 4 as very strong agglutination (Mahat et al., 2014).

3. Results

3.1. Beads conjugated to anti-*T. foetus* antibodies clump in the presence of *T. foetus* antigen

We evaluated two anti-*T. foetus* monoclonal antibodies (TF1.15 and TF1.17) for the ability to detect *T. foetus* antigens in a bead agglutination test. Antibody-conjugated latex beads (1 μm , 10 mg/mL) were co-incubated with whole cell *T. foetus* lysates in 96 well plates for two hours at room temperature prior to observation.

TF1.17-conjugated beads exhibited strong agglutination when co-incubated with *T. foetus* antigens (Fig. 1). This agglutination reaction was similar to or greater than the agglutination seen in the assay positive control (ovalbumin causing clumping of beads conjugated to commercial IgG raised against ovalbumin). In comparison, TF1.15-conjugated beads had a low level of agglutination. This reaction was greater than the non-specific total IgG negative control, but more difficult to detect by microscopy, despite attempts to optimize this reaction.

3.2. Bead agglutination detected low levels of antigen for both a laboratory and a field strain of *T. foetus*

Binding of antigen by TF1.17-conjugated beads was specific for *T. foetus*. Co-incubation of TF1.17 conjugated beads with 50 $\mu\text{g}/\text{mL}$ bovine serum albumin or ovalbumin indicated minimal non-specific clumping whereas co-incubation with *T. foetus* antigen resulted in strong clumping (Fig. 2).

In order to determine the detection limit of the assay, serial dilutions of antigens derived from a laboratory and a field strain of *T. foetus* were assessed. The test was more sensitive for an Iowa field strain than for a laboratory strain (limit of detection = 0.25 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$, respectively, Table 1). This concentration of antigen corresponds to 100–300 trophozoites/mL. However, agglutination was not observed when antibody-conjugated beads were co-incubated with antigens from *Leishmania major*, another protozoan organism with substantial lipophosphoglycan content (Osanya et al., 2011; Roychoudhury et al., 2015).

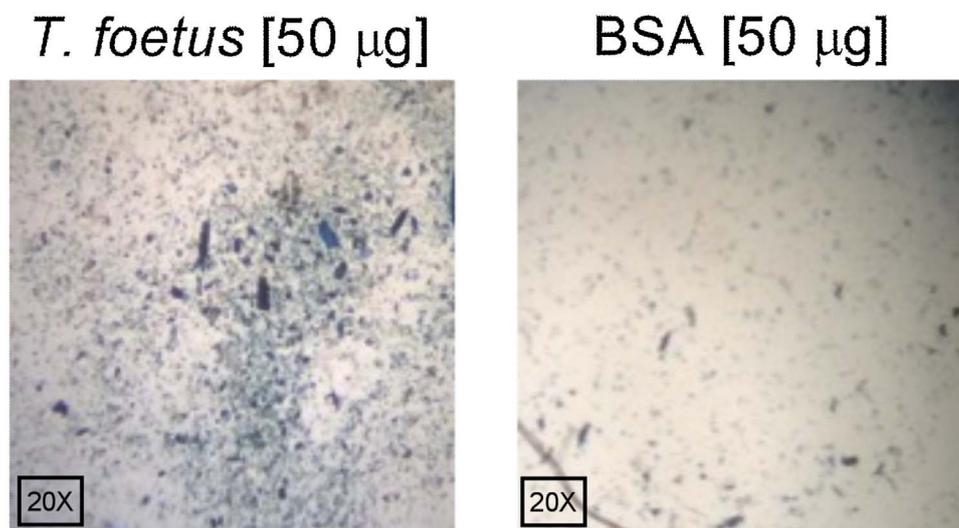


Fig. 2. Representative photomicrograph of strong positive agglutination of latex beads conjugated to monoclonal antibody TF1.17 in the presence of parasite antigens compared to bovine serum albumin.

4. Discussion

T. foetus is a sexually transmitted disease with a significant impact on livestock production. Although the United States does not have a national eradication program, many states will only allow bulls to enter if they have a negative trichomoniasis test. For many states, PCR is considered the gold standard and is likely to remain as the preferred diagnostic test due to its sensitivity and specificity. However, there is a substantial population of bulls that remain untested. The aim of this study was to develop a technique that could be used as a screening tool and would be amenable to use in the field by veterinarians.

In the present study, we evaluated a latex bead agglutination test based on binding of *T. foetus* antigens by mAbs TF1.15 and TF1.17. These antibodies react with epitopes of *T. foetus* lipophosphoglycan, a unique component of protozoan cell membranes that is distinct from the lipopolysaccharide of bacteria (Assis et al., 2012; Singh et al., 2001). Our results demonstrated that TF1.17-conjugated beads efficiently complexed with *T. foetus* antigens leading to visible bead agglutination (Fig. 1, Table 1). In our assays, beads coated with monoclonal antibody TF1.15 did not effectively detect *T. foetus* antigens. Modifying the concentration or distribution of this antibody on the latex beads may improve the utility of this antibody in our assay. In contrast, beads coated with non-specific antibodies did not detect *T. foetus*. Similarly, TF1.17-conjugated beads did not bind non-specific antigens such as bovine serum albumin, or *Leishmania major*, a parasite that also contains lipophosphoglycan. Although several parasites such as *Entamoeba*, *Leishmania*, and *Tritrichomonas* contain lipophosphoglycan, the spectrum of parasitic and free-living organisms with this epitope is currently unclear.

The inexpensive nature, ease of use, and field accessibility of a latex agglutination assay lend it to be a valuable tool in determining a diagnosis of *T. foetus* infection. Latex agglutination assay sensitivity and specificity against parasite antigens can vary greatly depending on the concentration of the measured antigen (Boelaert et al., 2016). Although bead agglutination assays represent a simple diagnostic test, technical issues such as conjugation stability, or *T. foetus* specific-antibody degradation (Darani et al., 2010) are areas which may cause the assay to

fail. However, strict production standards can overcome these limitations. Future studies will aim to address proper preparation of preputial samples prior to assessment in the assay.

Another potential drawback to the bead agglutination method is that the antibodies have some potential to cross react with other trichomonads that are occasionally present in the reproductive tract (Corbeil et al., 2008). Non-pathogenic trichomonads have also confounded diagnosis by culture, as well as PCR (Frey et al., 2017). In the current format, this diagnostic method is best used as a screening tool where positive reactions can be further assessed by culture and PCR. At the present time the test is unreliable for making definitive culling or quarantine decisions. The advantage of the bead agglutination method is that it can be achieved more rapidly and at a lower cost. Therefore, it is more attractive as a screening tool where no *T. foetus* testing is currently conducted and not yet suitable for making regulatory decisions on herd infection status. In humans, a bead agglutination is available to detect *Trichomonas vaginalis* (Darani et al., 2010). This approach had a high sensitivity and a low specificity, which would also suggest that the bead agglutination approach is most suitable for a screening tool but not definitive diagnosis.

Our results demonstrate that antibodies binding *T. foetus* lipophosphoglycan are capable of detecting low levels of parasite antigen. In these experiments, 1 µg/mL total protein antigen represented 100–300 trophozoites/mL, depending on the parasite isolate. Although studies of *T. foetus* antigen concentration during infection have not been performed, it is known that IgA antibodies in male and female genital secretions are generated during infection (Anderson et al., 1996; Ikeda et al., 1995; Rhyan et al., 1999). Infected animals have a strong antibody response to TF 1.17 antigen but the response to whole trichomonads is obscured by high background, probably due to cross-reacting natural antibodies. (Ikeda et al., 1995; Cobo et al., 2011). The measured immune response is detectable a few weeks after initial infection and lasts after infection is cleared (Anderson et al., 1996, (Cobo et al., 2011; Corbeil et al., 2001)) This suggests that antigen may be detectable before the antibody response can be demonstrated. Therefore, detection of parasite antigens may provide earlier detection and is a viable option for detecting infection throughout its course under field conditions.

Table 1

Limit of detection for bead agglutination assay using monoclonal antibody TF 1.17 as determined by presence or absence of agglutination on a scale of 1–4 (positive) or 0 (negative).

[Antigen](µg/mL)	50 µg	25 µg	10 µg	5.0 µg	2.5 µg	1.0 µg	0.5 µg	0.25 µg	0.15 µg	0.075 µg	0.015 µg
<i>T. foetus</i> (Iowa field isolate)	4	4	4	4	3	3	3	2	0	0	0
<i>T. foetus</i> (ATCC strain 30003)	4	4	4	3	3	3	0	0	0	0	0
<i>Leishmania major</i> (Negative Control)	0	0	0	0	0	0	0	0	0	0	0

Future studies will entail testing the sensitivity and specificity of the bead agglutination with clinical samples from infected animals.

Acknowledgements

This work was supported by start –up funds from the Iowa State University College of Veterinary Medicine, and grants from the Iowa Livestock Health Advisory Council and National Center for Veterinary Parasitology provided to M.T.B.

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