Veterinary Parasitology 243 (2017) 188–191

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

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
Field diagnostics

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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http://dx.doi.org/10.1016/j.vetpar.2017.06.025
Received 27 April 2017; Received in revised form 20 June 2017; Accepted 25 June 2017
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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 centrifuged at 3220 x *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 µg/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

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

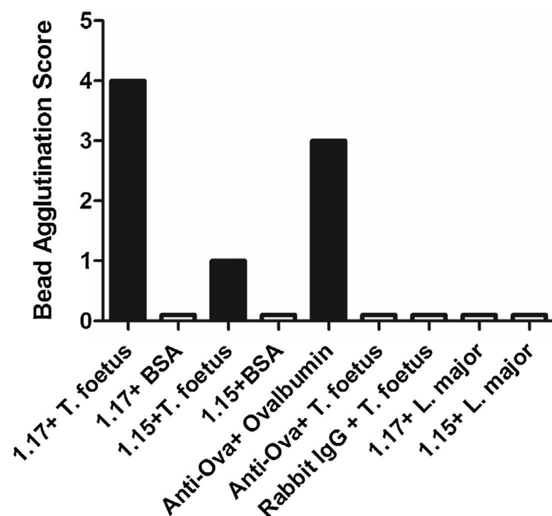

Fig. 1. Agglutination of latex beads conjugated to antibodies in the presence of 50 µg/mL antigen. Antibody and antigen pairs are shown on the X axis. 1.17 and 1.15 represent *T. foetus*-specific antibodies. Black bars represent antibody-conjugated beads paired with target antigens and open bars represent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

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 µg/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 µg/mL and 1 µg/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).

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