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Serodiagnosis of bovine trypanosomosis caused by non-tsetse transmitted *Trypanosoma* (Duttonella) *vivax* parasites using the soluble form of a Trypanozoon variant surface glycoprotein antigen

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ABSTRACT

Previous studies have shown that a 64-kDa antigen (p64) that was purified from the Venezuelan TeAp-N/D1 isolate of *Trypanosoma* (Trypanozoon) *equiperdum* corresponds to the soluble form of its predominant variant surface glycoprotein (VSG), and exhibited cross-reactivity with *Trypanosoma* (Duttonella) *vivax*. The course of experimental acute infections of bovines with *T. vivax* were followed by measuring whole anti-p64 antibodies and specific anti-p64 IgG and IgM antibodies in animal sera by indirect enzyme-linked immunosorbent assay (ELISA). The value of p64 to diagnose bovine trypanosomosis was also examined using 350 sera from healthy and *T. vivax*-infected cows living in a trypanosomosis-endemic and enzootic stable area, and 48 sera obtained during a trypanosomosis outbreak. Serological assays showed that ~70–80% of the infected sera contained anti-p64 antibodies, based on the comparative immunodetection of the *T. equiperdum* clarified antigenic fraction used as a reference test. In the absence of a gold standard, Bayesian analysis for multiple testing estimated a sensitivity and specificity of 71.6% and 98.8%, respectively, for the indirect ELISA using p64 as antigen. An apparent prevalence of 37.7% for bovine trypanosomosis infection was also estimated with a Bayesian approach when the p64 ELISA test was used. Employing blood from acute infected cows, the indirect ELISA response against p64 was contrasted with the microhematocrit centrifuge method and analyses by polymerase chain reaction (PCR) using specific primers targeting the inter-specific length variation of the internal transcribed spacer 1 region of the 18S ribosomal gene. The efficiency of p64 for the detection of anti-trypanosome antibodies in acute infected bovines was also corroborated serologically by comparing its response to that of the Indonesian *Trypanosoma evansi* Rode Trypanozoon antigen type (RoTat) 1.2 VSG, which possesses high specificity and sensitivity. As expected, PCR was the best method to detect parasites and diagnose bovine trypanosomosis; however, a substantial level of concordance (Cohen's $\kappa = 0.667$) was obtained when serological tests using p64 and RoTat 1.2 VSG were compared. Additionally, an agglutination assay was designed using p64 covalently coupled to carboxylate-modified latex microparticles, which was proven here to be suitable for a fast qualitative diagnosis of bovine trypanosomosis.

Abbreviations: VSG, variant surface glycoprotein; VAT, variant antigen type; RoTat, RodeTrypanozoon antigen type; ELISA, enzyme-linked immunosorbent assay; MHC, microhematocrit centrifuge; PCR, polymerase chain reaction; ITS1, internal transcribed spacer 1 region; MPP, microparticles; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; PCV, packed cell volume; HAT, human african trypanosomiasis; DME, direct microscopic examination; IIF, indirect immunofluorescence.

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1. Introduction

In Venezuela, *Trypanosoma* (Duttonella) *vivax* is the major causative agent of trypanosomosis in cattle (Rivera, 1996). *T. vivax* is a non-tsetse transmitted trypanosome in this region, and is spread mechanically by bloodsucking flies such as *Tabanidae* and *Stomoxys* species (Desquesnes, 2004; Osório et al., 2008). Bovine trypanosomosis causes severe anemia, edema, immunosuppression, and various neurological disorders, which may eventually produce the death of the affected animals (Gonzatti et al., 2014). Hence, bovine trypanosomosis generates significant economic losses to the farmers in terms of morbidity, mortality, abortion, infertility, reduced milk yield, and costs for trypanocides.

Salivarian parasites evade the adaptive immune system of the host using an antigenic variation strategy (Horn, 2014; Cnops et al., 2015). The surface of salivarian trypanosomes is covered with a densely packed layer of dimers of one type of variant surface glycoprotein (VSG). These VSGs are strong immunogens, but the parasite avoids elimination by the host immune system by changing the variant antigen type (VAT) of its VSG coat. Each parasite genome contains a large repertoire of several hundred to thousand *VSG* genes, supplemented by recombination and gene conversion events; however, only one is predominantly expressed at a time (Berriman et al., 2005; McCulloch and Horn, 2009). Switching the expression of one *VSG* gene to another results in a trypanosome bearing a different VAT, that may escape immune destruction as long as the infected host do not possess antibodies against this particular VAT (Barry and McCulloch, 2001). This antigenic variation strategy makes it difficult to develop a VSG-based vaccine against animal trypanosomosis, and vaccine design strategies have been focused on invariant trypanosome molecules that mediate pathogenesis.

Despite the switching strategy of *VSG* genes, various reports have shown that native and recombinant VSG antigens, VSG peptides and VSG mimotopes can be used for the diagnosis of salivarian trypanosomes (Bajyana Songa and Hamers, 1988; Ngaira et al., 2004; Penchenier et al., 2003; Sengupta et al., 2012; Van Nieuwenhove et al., 2012, 2013). Particularly, serological and PCR tests based on the VSG of *Trypanosoma evansi* Rode Trypanozoon antigen type (RoTat) 1.2, a VAT derived from an Indonesian stock of *T. evansi* isolated from a buffalo in 1982, have shown high specificity and sensitivity (Bajyana Songa and Hamers, 1988; Claes et al., 2004; Urakawa et al., 2001; Verloo et al., 2000). We have purified to homogeneity a 64-kDa glycosylated antigen (p64) from the Venezuelan TeAp-N/D1 strain of *Trypanosoma* (Trypanozoon) *equiperdum* (*aka* TEVA1), which corresponded to the soluble form of its predominant VSG and appeared to be very sensitive for diagnostic purposes (Uzcanga et al., 2004). Interestingly, p64 was also recognized by anti-*T. vivax* bovine antibodies (Uzcanga et al., 2002, 2004). TeAp-N/D1 was previously considered as a *T. evansi* isolate (Espinoza et al., 1997; Uzcanga et al., 2002, 2004; Camargo et al., 2004; Velásquez et al., 2014); however, Sánchez et al. (2015) have recently demonstrated that TeAp-N/D1 belongs to the *T. equiperdum* species by its molecular characterization using microsatellite markers and kinetoplast maxicircle genes. We have also purified and characterized the soluble forms of six additional VSGs from Venezuelan animal trypanosomes (Camargo et al., 2015). Like p64, all purified soluble VSGs exhibited cross-reactivity with *T. vivax* and were able to be used as diagnostic reagents for bovine trypanosomosis (Camargo et al., 2015). The purpose of this study was to evaluate the efficacy of p64 for the serodiagnosis of cattle experimentally or naturally infected with *T. vivax*.

2. Materials and methods

2.1. Materials

Reagents were purchased from the following sources: anti-bovine IgG (whole molecule) horseradish peroxidase conjugate, anti-bovine IgG (whole molecule) alkaline phosphatase conjugate, fluorescein-conjugated anti-bovine IgG (whole molecule), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, Sigma; horseradish peroxidase labeled anti-bovine IgG (γ) polyclonal secondary antibody, horseradish peroxidase labeled anti-bovine IgM (μ) polyclonal secondary antibody, KPL; Wizard® DNA extraction Kit, 5-bromo-4-chloro-3 indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), Promega; SYBR® Safe DNA gel stain, Invitrogen; bicinchoninic acid BCA™ Protein Assay Kit, nitrocellulose (0.45 μm pore size), Pierce; 96-well polypropylene plates (PolySorp or MaxySorp), Nunc; Opti-Link carboxylate-modified polystyrene (latex) microparticles (MPP), Seradyn; 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), Sigma or Roche; ABTS-buffer (phosphate-citrate-sodium perborate solution, pH 4.6), Roche. All other chemicals were of the highest quality grade available.

2.2. Preparation of the clarified antigenic fraction from the TeAp-N/D1 T. equiperdum isolate

T. equiperdum parasites ($\sim 10^9$) from the Venezuelan TeAp-N/D1 isolate were extracted on ice by sonication using 2 ml of a 5 mM Tris–HCl buffer (pH 7.2) containing 1 mM benzamidine, 1 mM phenyl methyl sulfonyl fluoride, 5 mM EDTA, and 1 mM iodoacetamide. The resulting homogenate was centrifuged at 15000 × *g* for 30 min, at 4 °C, to obtain the supernatant and pellet fractions. The supernatant fraction was defined as the clarified antigenic fraction from *T. equiperdum*, and was used as the source of parasite antigens for both indirect ELISA and Western blot analyses.

2.3. Purification of p64 from the TeAp-N/D1 T. equiperdum isolate

The p64 antigen was purified from the TeAp-N/D1 *T. equiperdum* isolate following the procedure described by Uzcanga et al. (2002, 2004).

2.4. Blood samples from field animals

For the diagnosis of bovine trypanosomosis, blood samples were collected from the jugular vein of 350 cows from a group of beef production farms located in the Monagas Municipality, Guárico State, which is a trypanosomosis-endemic and enzootic stable area in Venezuela. These 350 bovines were asymptomatic and possessed normal blood parameters, which suggested that they were apparently healthy. Sera from 48 hybrid Brahman cows were also obtained from a farm located near Caicara del Orinoco, Bolívar State, Venezuela, during a trypanosomosis outbreak. These 48 bovines contained numerous *T. vivax* parasites in their blood and showed clinical symptoms such as neurological signs, atypical weight, moderate or severe anemia, and decreased milk production.

Consent was obtained from all owners of the animals analyzed in this study, and all precautions were taken to minimize any contamination and suffering.

2.5. Experimental infection of animals

The *T. vivax* LIEM-176 isolate employed here was acquired from a naturally infected bovine from the Trujillo State, Venezuela (Gómez-Piñeres et al., 2014). Inoculating cryopreserved bovine