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Production, purification and therapeutic potential of egg yolk antibodies for treating *Trypanosoma evansi* infection

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ABSTRACT

The use of avian antibodies has aroused interest in biomedical research due to the numerous advantages compared to mammal's antibodies. Our study aimed to produce and purify IgY immunoglobulins in order to use as an alternative therapy against *Trypanosoma evansi*. Every 14 days, four New Hampshire chickens were immunized with trypomastigotes of *T. evansi*, totaling five inoculations. Eggs were collected during 70 days and the extraction of IgY was performed by precipitation through the PEG-6000 method. Characterization and purification of IgY anti-*T. evansi* were carried out by SDS–PAGE and Western blot, where heavy and light chains were detected. The production of IgY was noted during the whole period, and the average production was 2.87 ± 0.14 at the end of this study. Sample's titration allowed the quantification of specific IgY anti-*T. evansi*, with antibodies produced showing high avidity indexes. The results indicated that *T. evansi* is able to generate an immune response in poultry, resulting in a production of specific antibodies. In vivo test showed that IgY treatment resulted in increase of prepatent period, longevity and survival of infected animals, when compared with the positive control, demonstrating an initial, but no curative, trypanocidal activity.

1. Introduction

Trypanosoma evansi (*T. evansi*) is the etiologic agent of the disease known in Brazil as trypanosomosis, "*Mal das Cadeiras*" or "*Surra*", which affects horses of all breeds and has worldwide distribution (Darling, 1910; Hoare, 1972). Cattle, sheep, goats, donkeys, cats and pigs are also susceptible hosts. Canines, capybaras (*Hydrochaeris hydrochaeris*), coatis (*Nasua nasua*) and vampire bats (*Desmodus rotundus*) are reservoirs and occasionally may manifest clinical symptoms; additionally, bats are considered vectors of this disease (Nunes et al., 1993; Silva et al., 2002). The transmission of the parasite is essentially mechanical, since trypomastigotes are transferred from one host to another through blood-sucking insects (flies of the families

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Tabanidae and Muscidae) or artificially by needles contaminated with infected blood (Silva et al., 2002). Clinical signs include weight loss, pale mucous membranes, intermittent fever, cough, swelling in the lower parts of the body, superficial lymph nodes increased, muscle atrophy, incoordination, hindquart's paresis, difficulty to get up and proprioceptive deficits (Levine, 1973; Aquino et al., 1999; Herrera et al., 2005). Chronic infections can last for years (Brun et al., 1998), usually occurring at this stage the worsening of the clinical signs and cachexia (Brandão et al., 2002; Silva et al., 2002; Rodrigues et al., 2005).

In Brazil, the most commonly drug used in trypanosomosis treatment in domestic animals is the diminazene aceturate, which is able to provide an elimination of the trypanosomes in bloodstream, just few hours after its administration (Peregrine and Mamman, 1993). However, it do not presents curative efficacy, sometimes occurring parasitemia relapses, mainly because most of the trypanocidal drugs do not cross the blood brain barrier (Lonsdale-Eccles and Grab, 2002; Masocha et al., 2007).

The production of polyclonal antibodies, by poultry immunization, has aroused interest in scientific community. In addition to the numerous advantages described in literature (Olovsson and Larsson, 1993; Svendsen et al., 1996; Contreras et al., 2005; Schade et al., 2005) for antibodies produced in mammals, the IgY technology is an ethical experimental procedure by replacing the bleeding for collecting eggs. The process consists in the immunization of chickens at regular intervals, with subsequent production of serum immunoglobulin and transfers these proteins to the egg yolk. The amount of IgY in the yolk is dependent upon its concentration in serum (Carlander et al., 2002; Tini et al., 2002). The time between immunization and detection of antibodies in yolks is variable, depending on the biological assay performed (Patterson et al., 1962; Schade et al., 2005). The extraction and purification of it from yolk involves two mechanisms: delipidation and aqueous extract purification (Staak et al., 2001).

The present study aims to produce highly effective and pure antibodies by immunizing chickens with trypomastigotes of *T. evansi*; evaluate the therapeutic efficacy of these antibodies in rats experimentally infected with this protozoan; compare the therapeutical responses among rats treated only with IgY-anti *T. evansi* and rats treated with this antibody in association with diminazene aceturate and imidocarb dipropionate.

2. Material and methods

All procedures in this study were approved by the Animal Welfare Committee of Ethics in Animal Experimentation of Universidade Federal de Santa Maria (UFSM), number 018/2013.

2.1. IgY production and characterization

2.1.1. The antigen

For this experiment, it was used a strain of *T. evansi* obtained from a dog naturally infected (Colpo et al., 2005), which is kept in liquid nitrogen. Prior of each immunization, the parasites were thawed and injected into rats, intraperitoneally (Silva et al., 2003). When the rats presented high parasitemia, their blood was collected and the parasites were counted using a Neubauer chamber (Wolkmer et al., 2007).

2.1.2. Chicken immunization protocol

For inoculation, a solution containing at least 10^7 trypomastigotes of *T. evansi* was added to incomplete Freund's adjuvant (Sigma–Aldrich®) at a 1:1 ratio, with a final volume of 1.0 mL. It was injected intramuscularly at five different sites of the pectoral muscle of three 25-week-old New Hampshire chickens. The interval between immunizations was set as 14 days (days 0, 14, 28, 42 and 56), totaling five inoculations. The fourth hen was not immunized, serving as the negative control. The eggs were collected from the fourth week post-immunization (day 21) and stored at 4 °C until they were processed.

2.1.3. Extraction of immunoglobulin IgY anti-T. evansi

Extraction was performed by the method of precipitation of polyethylene glycol 6000 (PEG-6000) as described by Polson et al. (1980) and Pauly et al. (2011). After separation of white and yolk, the egg yolk was transferred to a filter paper and after to a 250 mL tube. The delipidation was performed through the emulsion of the yolk in PBS at 1:2 proportion. After this step PEG-6000 at 3.5% was added. The mixture was then centrifuged at 4 °C, 13,000 × *g* for 20 min. The supernatant obtained was transferred to a new tube, while the lipid layer was discarded. The process was repeated twice using PEG-6000 at 8.5% and 12%, respectively. After dialysis, the obtained extract was transferred into 2 mL tubes and stored at −20 °C.

2.1.4. SDS–PAGE–polyacrylamide gel electrophoresis and Western blot

Purified IgY was electrophoresed on SDS–PAGE using 10% polyacrylamide gel according to Laemmli (1970). The extracted samples were applied to the stacking gel wells in a final volume of 10 μL. The electrophoresis, subjected to an initial current of 80 V and 120 V, was stopped when the dye used in the samples reached the base of the separating gel. Once removed from the system, the gel was stained with Coomassie blue R 250 (Sigma–Aldrich®) for at least 1 h and, then, treated with a decolorizing solution (glacial acetic acid 100 mL + methanol 400 mL + distilled water 500 mL) for visualization of protein bands (Bernardo, 2009).

Next, the separated proteins were electrophoretically transferred to a nitrocellulose membrane 0.45 μm (Bio-Rad®). For the transfer it was used electroblotting mini tank (30 V), which remained in cold chambering "overnight". The membrane was blocked with 5% skim milk in PBS-T for 1 h. The membrane was washed three times with PBS-T and incubated for 1 h with rabbit anti-chicken IgY peroxidase conjugate (1:2000) (Sigma–Aldrich®, USA). Finally, the membrane was washed with PBS-T three more times. The reaction was revealed with diaminobenzidine prepared in Tris HCl + nickel sulphate + hydrogen peroxide. The membrane remained under constant stirring until visualization of the reactive bands.