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Research paper

In vitro production of *Trypanosoma equiperdum* antigen and its evaluation for use in serodiagnosis of dourine

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ABSTRACT

A modified Baltz's *in vitro* cultivation system for the propagation of *Trypanosoma equiperdum* strain OVI was established to develop a replacement for the conventional production procedure of dourine diagnostic antigen in rats. To increase trypanosome yields we designed an optimized culture medium by addition of supplemental compounds. Trypanosomes were adapted to this medium by two succeeding cultivation steps which led to a substantial proliferation rate and an increased cell density tolerance, respectively. As a result, adapted parasites could be propagated to maximum cell densities of $>2 \times 10^6$ cells/ml, facilitating *in vitro* antigen production in preparative quantities comparable to the conventional method. A panel of 180 horse field sera, previously sent for testing to the German National Reference Laboratory for Dourine, was tested by complement fixation test using culture-derived as well as conventionally produced dourine antigen. Cohen's kappa values for results obtained with two batches of culture-derived antigen as compared to conventional antigen were 0.91 (95% confidence interval [CI]: 82.2–99.7) and 0.83 (95% CI: 70.3–95.3), respectively. Performance of antigens for diagnostic purposes was characterized in an inter-laboratory comparative study deploying 14 sera from horses with defined dourine statuses. Complement fixation test results from 15 participating European laboratories showed a diagnostic sensitivity of 94.1% (95% CI: 89.4–98.7) and a diagnostic specificity of 96.2% (95% CI: 92.5–99.9) for conventional antigen and a slightly higher diagnostic sensitivity of 96.0% (95% CI: 92.2–99.8) and a diagnostic specificity of 97.1% (95% CI: 94.0–100) for culture-derived antigen. We conclude that our novel approach for dourine antigen production from *in vitro*-grown trypanosomes described and evaluated herein meets the requirements for the prospective purpose in quantitative and qualitative terms and should be considered by the competent authorities as an alternative for the animal experiment currently prescribed by international standards.

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1. Introduction

Trypanosoma equiperdum is the causative agent of dourine or covering sickness, a disease of horses and other members of the family Equidae. Rats, mice, rabbits and dogs can be infected experimentally as well. *T. equiperdum* belongs to the subgenus *Trypanozoon* that also includes *T. brucei* and *T. evansi*, and recent studies have argued, that *T. equiperdum* along with *T. evansi* should be classified as a subspecies of *T. brucei* (Carnes et al., 2015; Jensen et al., 2008; Lun et al., 2010). Due to the low number of trypanosomes normally present in infected tissues and because of the short-lasting and mild parasitemia direct detection of the parasite is difficult to accomplish (Zablotskij et al., 2003). Consequently, diagnosis of dourine is based on clinical signs and/or an epidemiological link to a confirmed case, together with serological evidence, mainly from complement fixation tests (CFTs) (OIE, 2013; Pascucci et al., 2013; Zablotskij et al., 2003). The CFT prescribed by the World Organization for Animal Health (OIE) is a reliable serological test for the diagnosis of dourine (Cauchard et al., 2014; Zablotskij et al., 2003). In Germany, CFT antigen is prepared from *T. equiperdum* strain OVI (Onderstepoort Veterinary Institute) according to the OIE-recommended method (referred to as "conventional" throughout this manuscript), which includes an animal experiment to propagate the trypanosomes in rats (OIE, 2013). Based on interpretation of recent genome and phylogenetic analyses (ANSES, 2013; Schnaufer, 2010), *T. equiperdum* OVI was selected by agreement of the European dourine reference laboratory leaders including the

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European Union Reference Laboratory (EU-RL) at their meeting in 2012 as reference strain for antigen production within the EU to harmonize the diagnostic methods (ANSES, 2013).

To efficiently produce dourine antigen from *T. equiperdum* OVI *in vitro* culture, the parasites should reach high cell densities. Several methods to grow bloodstream forms of *T. brucei* in axenic culture *in vitro* have been developed (Baltz et al., 1985; Duszenko et al., 1985; Duszenko et al., 1992; Hirumi et al., 1977; Hirumi and Hirumi, 1989). *T. evansi* and *T. equiperdum* in particular were reported to grow in a minimum essential medium-based standard medium (Baltz et al., 1985). We hypothesized that further supplementation of this medium and application of a certain passaging regime may decrease generation times and increase maximum achievable cell density, respectively, and made efforts to optimize the *T. equiperdum* OVI culture protocol. To evaluate the resulting dourine antigen, different antigen batches were compared in CFTs utilizing different serum panels. Cohen's kappa values as well as diagnostic specificity and sensitivity were determined.

2. Material and methods

2.1. Trypanosome

T. equiperdum strain OVI (ITMAS 241199C) was isolated in 1975 from a horse in South Africa. Cryostabilates were kept at −80 °C for up to three years or deposited in liquid nitrogen for long-term storage. Unless stated otherwise trypanosomes were centrifuged with 1300 x *g* at 4 °C for 10 min. Cell densities were determined using a hemocytometer with a Neubauer-improved chamber counting grid and a depth of 0.02 mm (Hecht Glaswarenfabrik, Sondheim vor der Rhoen, Germany, product code 441/72_0,02).

2.2. Animal infections

Trypanosomes were propagated *in vivo* using male Wistar rats purchased from Charles River GmbH (Sulzfeld, Germany) and raised to a body weight of 500 g. All animal experiments were conducted according to the rules laid down in the German Animal Protection Act and approved by the competent authority (Thuringian State Office for Consumer Protection, reg. no. 04-101/12). Rats were injected intraperitoneally with 1×10^7 trypanosomes from a freshly thawed blood cryostabilate re-suspended in 0.5 ml citrate-glucose anticoagulant (100 mM sodium citrate, 40 mM glucose, pH 7.7) and the infection was monitored by counting parasites in tail blood. In logarithmic growth phase, the generation time of *T. equiperdum OVI* was 4.5 h. After 2.5 – 3 days, parasitemia reached $0.5–1 \times 10^9$ trypanosomes per ml of blood. The rats were anesthetized with isoflurane and blood was drawn by cardiac puncture using heparinized syringes and cannulas. The harvested blood was either utilized to produce new blood cryostabilates or to purify trypanosomes from blood components for further use. To produce new blood cryostabilates the harvested blood was diluted in ice cold citrate-glucose anticoagulant to adjust 5×10^8 trypanosomes per ml. The cell suspension was mixed 1:2 with cryopreservation buffer, portioned to 1 ml aliquots and stored at −80 °C or in liquid nitrogen. Cryopreservation buffer consisted of a mix of 77 parts trypanosome dilution buffer (18 mM Na_2HPO_4, 2 mM NaH_2PO_4, 80 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 20 mM D-glucose; pH 7.7) and 23 parts glycerol (87%).

2.3. Culture media

Trypanosomes were grown *in vitro* in basal and optimized medium, respectively. All media components, unless otherwise stated, were purchased from Sigma-Aldrich GmbH, Taufkirchen, Germany. Media were adjusted to pH 7.4, sterile filtered and supplemented with heat-inactivated newborn calf serum (NCS). Freshly mixed ready-to-use media were stored at 4 °C, left for one day to degas, and used within 8 weeks. Basal medium was composed of a modified minimum essential medium (MEM) with Earle's salts (product code M0268) and 1% (v/v) MEM non-essential amino acid solution (product code M7145) supplemented with 26.2 mM sodium bicarbonate (Merck, Darmstadt, Germany, product code 6329), 25 mM HEPES (product code H3375), 5.55 mM D-glucose (product code G8270), 2.0 mM sodium pyruvate (product code P2256), 0.2 mM 2-mercaptoethanol (Merck, Darmstadt, Germany, product code 15433), antibiotic-antimycotic solution (penicillin 50 U/ml, streptomycin, 50 μg/ml and amphotericin B 0.125 μg/ml; product code A5955) and 10% (v/v) heat-inactivated NCS (product code N4762). The medium contained 0.16 mM L-cysteine derived from the MEM non-essential amino acid solution and 11.1 mM D-glucose in total (5.55 mM glucose from modified minimum essential medium with Earle's salts and 5.55 mM from added glucose). Optimized medium was additionally supplemented with 1.5 mM L-cysteine (product code 168149), 0.05 mM bathocuproine disulfonic acid (product code B1125), 0.05 mM adenosine (product code A9251), 0.1 mM hypoxanthine (product code H9636), 0.016 mM thymidine (product code T1895), 0.3 mM L-ornithine (product code O2375) and a total of 15% (v/v) heat-inactivated NCS.

2.4. In vitro propagation

Generally, trypanosomes were grown in liquid medium in the presence of 5% CO_2 at 37 °C in vented flasks (Thermo Fisher Scientific, Braunschweig, Germany, Nunc cell culture treated flasks with filter caps). Culture vessels with a surface to volume ratio of about 4 cm^2 per ml were used to ensure sufficient oxygen supply to culture. Shaking of the culture vessels was avoided. When necessary, a daily culture medium exchange was performed. To this end, trypanosomes were centrifuged, more than 92.5% of depleted medium was exchanged with fresh pre-warmed (37 °C) medium and the cell pellet was carefully re-suspended.

Axenic cultures of *T. equiperdum* OVI were initiated by inoculating basal or optimized medium with trypanosomes from a blood cryostabilate. A culture with 2×10^3 trypanosomes/ml reached its maximum cell density after four days. In order to adapt trypanosomes to reach higher cell densities a continuous culture (7 ml) of trypanosomes in optimized medium was grown in logarithmic growth phase for nine weeks by keeping its cell density between 1×10^3 and 2.5×10^5 cells/ml. Before the culture grew up to 2.5×10^5 cells/ml an appropriate volume was taken and diluted in fresh medium to form a new 7 ml culture at a cell density of not less than 1×10^3 cell/ml. In a secondary adaptation step, a 7 ml culture of the aforementioned trypanosomes in optimized medium was allowed to grow for four weeks without manipulation of the trypanosome numbers except replacing culture medium every 24 h involving a centrifugation step.

Culture cryostabilates were produced from a logarithmically growing culture. Trypanosomes were centrifuged, re-suspended at 1×10^8 cells/ml in fresh culture medium, mixed 1:2 with cryopreservation buffer, portioned to 1 ml aliquots and stored at −80 °C or in liquid nitrogen.

To start a culture cycle, a cryostabilate was rapidly thawed and immediately suspended in 15 ml of ice cold culture medium. The trypanosomes were centrifuged and carefully re-suspended in 10 ml fresh culture medium pre-warmed to 37 °C by pipetting up and down. Following cell counting, the starter culture with 2×10^5 cells/ml was initiated. After 16–20 h trypanosomes were counted and the main culture at the desired number of trypanosomes was started.

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