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Research paper

Development of an indirect ELISA for the serological diagnosis of dourine

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ABSTRACT

Dourine is a parasitic venereal disease of equines caused by *T. equiperdum*. Humoral antibodies are found in infected animals, but diagnosis of dourine must include history, clinical, and pathological findings in addition to serology. Complement Fixation Test (CFT) is the Office International des Epizooties (OIE) recommended test for international trade; however, some uninfected equines may give inconsistent or nonspecific reactions in CFT due to the anticomplementary effects of their sera. In this study an Indirect Enzyme Linked Immunosorbent Assay (iELISA) was developed. This test could be used to confirm positive serological cases of dourine or to solve inconclusive results obtained by CFT, in addition to Indirect Fluorescent Antibody Test (IFAT) and a Chemiluminescent Immunoblotting Assay (cIB). Six-hundred-and-six CFT negative sera and 140 sera positive to CFT and IFAT were tested by iELISA using OVI *T. equiperdum* as antigen. Results were expressed as percentage of positivity and the optimum cut-off value determined sensitivity and specificity of 100%. All positive sera, tested by cIB, were confirmed as positive. Additionally, twenty seven sera, low-positive at CFT and negative by IFAT, were tested with iELISA and cIB. All samples resulted negative by cIB and one of them was positive in ELISA. Our results suggest that iELISA and cIB may be used as alternative or supplementary confirmatory tests whenever other recommended serological methods are inconclusive or doubtful.

1. Introduction

Dourine is a parasitic venereal disease of equines caused by the flagellate protozoan *Trypanosoma equiperdum*. Dourine has been eradicated from many countries but is still diagnosed in horses in most Asian regions, in Africa, Russia, the Middle East, South America and in Italy in 2011 (Gizaw et al., 2017). The disease is a trypanosomosis not transmitted by an invertebrate vector and the transmission is almost exclusively during coitus, facilitated by the presence of the parasite in the seminal fluid and mucous exudates of the penis and in the vaginal mucus. The incubation period may vary from 1 to 2 weeks to several years and clinical signs are highly variable in presentation and severity: genital oedema, weight loss, skin lesions known as "silver dollar" plaques and neurological signs may be absent in the early stages or during latent infections (Claes et al., 2005; Luckins et al., 2004). Diagnosis of dourine, therefore, is a challenge, due to limited knowledge about the parasite and host-parasite interaction following infection, and must include history, clinical, and pathological findings in addition to serology (Calistri et al., 2013).

As concerns the serological diagnosis, *T. equiperdum* is closely related to other Old World trypanosomes, as *T. brucei* and *T. evansi*, and share conserved cellular elements, so cross-reactions are possible, in particular in countries in which two or more *Trypanosoma* species are present (World Organisation for Animal Health, 2013). The complement fixation test (CFT) is the most commonly used OIE-prescribed serodiagnostic test developed for *T. equiperdum* and has been used successfully in eradication programs. It is still used for international trade in monitoring horses for export and import and it is applied to confirm clinical suspicions and to detect latent infection; however, some uninfected equines, in particular donkeys and mules, may give inconsistent or nonspecific reactions to CFT due to the anticomplementary effects of their sera (Clausen et al., 2003); moreover, false positive results could occur, due to the use of a crude antigen that reacts also with antibodies against *T. evansi*. Differently from surra, dourine is considered incurable, so reliable confirmatory tests are needed to avoid consequences such as slaughtering or castration of CFT-

Abbreviations: AC, accuracy; CFT, complement fixation test; IFAT, indirect fluorescent antibody test; iELISA, indirect enzyme linked immunosorbent assay; cIB, chemiluminescent immunoblotting assay; OVI *T. equiperdum*, *Trypanosoma equiperdum* isolated at Onderstepoort Veterinary Institute; kDa, kiloDalton; PP, percentage of positivity; Se, sensitivity; Sp, specificity; Ig, immunoglobulin

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positive animals (Cauchard et al., 2016). In the case of anticomplementary sera, the indirect fluorescent antibody test (IFAT) is of advantage, but earlier studies did not confirm the concordance between CFT and IFAT (Caporale et al., 1981; Wassall et al., 1991), as IFAT proved less sensitive than CFT. In these conditions confirming positive serological cases or clarifying inconclusive or discrepant cases can be challenging. A confirmatory test would thus be of great support in increasing the reliability of the results.

In this study an indirect enzyme linked immunosorbent assays (iELISA) for dourine was developed. The iELISA method has several advantages over the CFT: it can be performed in less time than the corresponding CFT procedure, it is easier, the results are objectively measured and calculated and the method is amenable to automation. The chemiluminescent immunoblotting assay (cIB) is based on different *T. equiperdum* antigen patterns recognized by immunoglobulins from healthy and infected animals (Luciani et al., 2013). These methods could be used, in addition to IFAT, to confirm positive serological cases or to solve inconclusive results obtained by CFT.

2. Materials and methods

2.1. Preparation of Trypanosoma equiperdum antigen for iELISA and cIB

The *Trypanosoma equiperdum* strain, provided by Onderstepoort Veterinary Institute, Pretoria, South Africa (OVI *T.equiperdum*) was purified according to Luciani et al. (2013). Briefly, rat blood was diluted in an equal volume of Percoll (GE Healthcare, Life Science, Uppsala, SW) solution containing sucrose and glucose and centrifuged at 17.500 × *g* for 20 min. Recovered trypanosomes were diluted at 1:3 ratio in 0.01 M phosphate-buffered saline, pH 7.4 (PBS) containing 1% glucose (PBSG, pH 7.4) and centrifuged at 4500 × *g* for 20 min. The pellet was resuspended in PBSG and purified using DEAE-cellulose chromatographic column (GE Healthcare). The eluted fraction, containing trypanosomes, was centrifuged at 6000 × *g* for 15 min, diluted in PBSG to obtain a parasite concentration of 1×10^8 trypanosomes/ml and stored in small aliquots at −80 °C until use. The concentration of trypanosomes/ml was assessed by cell count using a Bürker chamber.

2.2. Sera

One-hundred-and-forty sera were collected at different time points after infection from 20 clinical cases of dourine occurred in Italian outbreaks in 2011. All sera were positive to CFT (titers ranging from 1:5 to 1:2560) and IFAT (titers ranging from 1:80 to 1:1280).

Six-hundred-and-six CFT negative sera were collected in 2017 during the national surveillance plan for Equine Infectious Anaemia in Italy.

Additionally, 27 low-positive CFT sera (titer ranging from 1:5 to 1:10) and IFAT negative (titers < 1:80), collected from horses without clinical symptoms during the Italian national surveillance plan for dourine (June-December 2011), were tested with iELISA and cIB.

2.3. Serological tests

CFT was performed according to the method described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (World Organisation for Animal Health, 2013), using the OVI *T. equiperdum* antigen, prepared according to the OIE Manual by differential centrifugation. Sera were screened at a dilution of 1:5, and those showing more than 50% of fixation level at this dilution were considered positive and tested again to end point using two-fold dilutions.

IFAT was performed according to the method described in the OIE Manual, using a two-fold dilution from 1:80 to 1:1280. Sera showing strong fluorescence at 1:80 dilution were considered positive.

Only samples that were positive by CFT were analyzed by IFAT.

2.4. Indirect ELISA

The purified OVI *T.equiperdum* antigen (100 µl/well), diluted 1:2000 in 0.05 M carbonate-bicarbonate buffer (pH 9.6), was coated in 96-well polystyrene plates (PolySorp, NUNC™, DK) and incubated overnight at 5 °C. The plates were washed once with PBS containing 0.05% Tween 20 (PBST) and blocked with 1% Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO, USA) in PBST at room temperature (RT) for 1 h. After four washes with PBST, 100 µl of samples were tested in duplicate at 1:100 dilution in PBST containing 0.1% BSA (dilution buffer). The positive control (PC) serum (CFT titre: 1:320, IFAT titre: 1:640) was obtained from a naturally infected horse, while the negative control (NC) serum was obtained from a healthy horse. Both positive and negative control sera were freeze-dried in bottles, each containing 1 ml. Six wells are used as controls: two wells contained 100 µl of dilution buffer, to assess the enzymatic reaction without the sample (blank), two wells contained 100 µl of PC diluted 1:100 in dilution buffer and two wells contained 100 µl of NC diluted 1:100 in dilution buffer. After incubation at 37 °C for 1 h, the microplates were washed four times with PBST and incubated with 100 µl/well of Anti-horse IgG-HRP conjugate (Sigma, A6917) diluted 1:10,000 in dilution buffer at RT for 45 min. After further washes, 100 µl of 3.3',5,5'-tetramethyl benzidine solution (TMB) (Sigma) were added into each well and the plates were incubated at RT for 30 min. The reaction was stopped by adding 50 µl/well of 0.5 N sulphuric acid and the optical density (OD) was measured at 450 nm. OD values minus the mean of blank values were used to determine sample results, expressed as percentage of positivity (PP) calculated with the following formula:

PP = (OD Sample – OD Negative Control)/(OD Positive Control – OD Negative Control)*100

The optimum cut-off value was determined using receiver operator characteristic (ROC) curve (Gardner and Greiner, 2000; Siegel and Castellan, 1988), constructed with the PP values of tested sera. Sensitivity and specificity values were calculated using a Bayesian approach (Sivia, 1996; Vose, 2000) with a Beta (s + 1, n-s + 1) probability distribution, where s was the number of samples correctly identified and n the total number of samples tested. In addition, 95% confidence intervals (CI) were calculated.

The repeatability of the method, expressed as CV%, was determined by analyzing 45 replicates of positive reference serum. To determine the Cohen's kappa value, the same repetitions were made by two operators.

2.5. Immunoblotting

The cIB was performed according to Luciani et al. (2013). Briefly, purified OVI *T.equiperdum* antigen, diluted in reducing sample buffer (Life Technologies Ltd, Paisley, UK) and heated at 70 °C for 10 min, was separated by electrophoresis using NuPage® 12% Bis-Tris pre-cast gels (Life Technologies) at 200 V, transferred onto nitrocellulose membranes, reversible stained with Ponceau S to detect protein bands and cut in strips. After blocking with PBST containing 5% skimmed milk (Biolife, Milano, IT), strips were incubated at 5 °C overnight with the sample sera diluted 1:10 in PBST containing 2.5% skimmed milk. Strips were then incubated with a monoclonal antibody anti-horse IgG–HRP conjugate (IZSAM, Teramo, IT). Antigen-antibody reaction was visualized using the Amersham™ ECL Select™ Western Blotting Detection reagent (GE Healthcare) and the Chemidoc MP (Bio-Rad Laboratories, Hercules, CA, USA), with the Image Lab Software, version 4.0 (Bio-Rad Laboratories). The molecular weight of the reacting bands was determined by comparison with the Novex® Prestained Protein Ladder (Life Technologies).