Veterinary Immunology and Immunopathology 151 (2013) 140–146

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

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

Research paper

IgG antibodies from dourine infected horses identify a distinctive *Trypanosoma equiperdum* antigenic pattern of low molecular weight molecules

M. Luciani[a], C. Di Pancrazio[a], T. Di Febo[a], M. Tittarelli[a], M. Podaliri Vulpiani[a], M.O. Puglielli[a], J. Naessens[b], F. Sacchini[a,*]

[a] *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Via Campo Boario, 64100 Teramo, Italy*
[b] *International Livestock Research Institute, Old Naivasha Road, PO Box 30709, 00100 Nairobi, Kenya*

ARTICLE INFO

Article history:
Received 15 June 2012
Received in revised form 6 November 2012
Accepted 7 November 2012

Keywords:
Trypanosoma equiperdum
Dourine
Immunoblotting
Humoral response
IgG

ABSTRACT

Diagnosis and control of dourine is strongly based on serological evidence, but knowledge of the humoral response of horses during infection is limited. In this study we developed a chemiluminescent immunoblotting (cIB) assay to characterise the *Trypanosoma equiperdum* antigen pattern recognised by IgGs from naturally or experimentally dourine-infected horses and analyse the kinetics of IgG humoral response following the infection. One compounding factor is that sera from uninfected animals often cross-react with *T. equiperdum* antigens. Development of the cIB assay was based on the hypothesis that serum IgGs from healthy and infected animals recognise different *T. equiperdum* antigen patterns. We used sera from 8 naturally infected horses which had recovered from Italian outbreaks and 2 experimentally infected mares. In addition, sera from 10 healthy control animals, eight of which were CFT positive but IFA negative for dourine, were collected from disease free regions. Sera were compared by the complement fixation test (CFT), indirect immune fluorescence (IFA) and the cIB assay.

cIB analysis revealed that IgGs from infected horses, in contrast to IgGs from healhty horses, specifically recognise a *T. equiperdum* antigenic profile with low molecular weight bands ranging between 16 and 35 kDa. A time course experiment indicated that IgGs specific for the 16–35 kDa parasite protein fraction appear 17 days post-infection. The cIB assay confirmed all ten infected animals as positive and all controls as negative. This study demonstrated that analysis of IgGs by cIB can provide clear confirmation of trypanosome infection in horses, suggesting that this technique can be applied as a confirmatory serological test for dourine infection.

1. Introduction

Dourine is a contagious disease of equids caused by the protozoan parasite *Trypanosoma equiperdum*. It is directly transmitted from animal to animal during coitus and often has a chronic clinical outcome (OIE, 2008). Outbreaks must be notified to the World Organisation for Animal Health (OIE) and movement and trade restrictions on breeding equids or semen are imposed on infected countries (OIE Code, 2011).

The disease was first eradicated in Italy in the 1940s, but a serious epidemic reoccurred between the 1970s and 1980s (Caporale et al., 1980). After sporadic reports at the end of the 1990s (OIE, 2011), there was a new outbreak in May 2011 (Scacchia et al., 2011). Clinical findings, laboratory and epidemiological investigations suggested that all

* Corresponding author. Tel.: +39 0861 332432; fax: +39 0861332251.
E-mail address: f.sacchini@izs.it (F. Sacchini).

0165-2427/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetimm.2012.11.004

recent Italian outbreaks were caused by *T. equiperdum* and were related to sexual transmission (Scacchia et al., 2011; Calistri et al., 2012; Podaliri Vulpiani et al., 2012). The parasite was never detected by microscopy in the blood stream of infected animals over a period of weeks or months. All the attempts to adapt the field strain to mice or rats failed while evidences of parasite replication were recorded in rabbit scrotum. These diagnostic peculiarities are reported to be characteristic of *T. equiperdum* (Zablotskij et al., 2003). Moreover, no seroconversion or PCR positivity were observed in healthy horses stabled in close contact with infected animals and in the presence of insect vectors after 6 months exposure (Podaliri Vulpiani et al., 2012). Epidemological analyses of the outbreaks based on features such as prevalence, age, reproductive activity and relationship between the affected animals indicated that the infection is transmitted directly from animal to animal during coitus. This supports the thesis that the disease was caused by *T. equiperdum* and not *Trypanosoma evansi* that is transmitted by mechanical vectors (Calistri et al., 2012).

Diagnosis of dourine is a challenge, due to limited knowledge about the parasite and host-parasite interaction following infection. The incubation period may vary from few weeks to several years and some of the clinical signs, which include 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 (Luckins et al., 2004; Claes et al., 2005). Moreover, most of the clinical signs of dourine are not disease-specific and laboratory diagnosis is necessary to confirm or exclude trypanosome infection. Direct diagnosis based on molecular techniques can be highly sensitive for parasite detection in body fluids such as blood (Becker et al., 2004). However, this approach is difficult to apply for mass screening and negative results do not exclude the possibility of infection. In fact *T. equiperdum* multiplies predominantly in extracellular tissue spaces and is seldom found in peripheral blood (Theis and Bolton, 1980). Diagnosis of *T. equiperdum* infection is thus still strongly based on serological evidence.

Despite the development of an indirect fluorescent antibody (IFA) test and enzyme-linked immunosorbent assays for *T. equiperdum*, the complement fixation test (CFT) remains the only recognised test for international trade purposes, and is widely used in disease eradication plans. However, it does not distinguish among *T. equiperdum*, *T. evansi* and *Trypanosoma brucei* (Zablotskij et al., 2003; Claes et al., 2005; OIE, 2008). Nevertheless, CFT can provide a specific diagnosis of *T. equiperdum* infection in countries in which other members of the Trypanozoon subgenus are not present, as was the case in Italy.

The major drawbacks of CFT are the need for careful continuous titration of numerous labile reagents and the anti-complement effect of sera frequently observed in uninfected equids, particularly donkeys and mules, but also reported in horses, which results in inconsistent or nonspecific reactions (Zablotskij et al., 2003; OIE, 2008). IFA test is often used to confirm or exclude doubtful or inconclusive CFT results, but earlier studies did not confirm the concordance between CFT and IFA (Caporale et al., 1981; Wassall et al., 1991). 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. We assessed the use of a chemiluminescent immunoblotting assay (cIB) as an indirect test for the diagnosis of dourine, based on the hypothesis that different *T. equiperdum* antigen patterns are recognised by IgGs from healthy and infected animals. An immunoblotting method based on colorimetric detection has previously been suggested (Katz et al., 1999).

This study describes the development of a cIB assay to characterise the antigen patterns recognised by IgGs from naturally and experimentally infected horses and provides additional information on the kinetics of IgG humoral response in horses following dourine infection. It also supports the use of cIB as a confirmatory test in serologically doubtful cases.

2. Material and methods

2.1. Sera

Sera were collected from 8 naturally infected horses (two stallions: #1 and #2; and 6 mares: #3, #4, #5, #6, #7 and #8), which had recovered from Italian outbreaks (Istituto Zooprofilattico Sperimentale Abruzzo & Molise, 2012) and from 2 experimentally infected mares (#9 and #10). Experimental infection was carried out by transfusion of blood collected from infected horses during parasitaemia detected by PCR, to investigate the evolution of the disease and the kinetics of humoral response over time. Animal experimentation was carried out in compliance with Italian national law (Legislative Decree 116/92) implementing Directive 86/609/EEC of the Council of the European Communities on the protection of animals used for experimental and other scientific purposes. In addition, sera from 10 healthy animals were collected from the field (C1–C10), in disease free regions. Eight of the controls were selected because resulted CFT positive but IFA negative for dourine.

All sera were analysed for dourine by CFT and IFA performed according to the OIE Manual of Diagnostic Tests and Vaccines (OIE, 2008). The tests were performed using the Onderstepoort Veterinary Institute strain of Trypanosoma equiperdum (OVI T.e.) as antigen, prepared according to the OIE manual (OIE, 2008). For CFT, sera were screened at 1:5 dilution and those showing a fixation level > 50% were considered positive and analysed again to end point using two fold dilutions. Samples testing positive on CFT were then analysed with IFA using a twofold dilution (from 1:80 to endpoint). Sera showing strong fluorescence were considered positive.

2.2. Trypanosoma antigen purification

A two-step protocol was optimised according to González et al. (2005) to obtain a purified OVI T.e. antigen. Briefly, 40 ml of blood was collected in sodium citrate tubes from rats infected intraperitoneally 48–72 h earlier with a cryopreserved stock of OVI T.e. strain adapted to rats. The rats were bled at the time of peak parasitaemia. The parasite concentration in whole blood was estimated to be