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Novel tools for the diagnosis and differentiation of acute and chronic bovine besnoitiosis

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ABSTRACT

Diagnosis of acute bovine besnoitiosis is a major diagnostic problem. We developed diagnostic tests to serologically diagnose and differentiate acute and chronic cases of bovine besnoitiosis using affinity purified antigens of *Besnoitia besnoiti* tachyzoites in immunoblots and in both, a conventional ELISA and an avidity ELISA. Sera of acutely and chronically infected cattle were investigated using these tests. Acutely infected cattle initially recognised an antigen of 74 kDa relative molecular mass, followed by reactions with increasing intensity against 81 and 28 kDa antigens. In addition, faint reactions against antigens with 36, 37, 39 and 42 kDa molecular mass started soon after seroconversion and increased over time. An antigen of 45 kDa molecular mass was transiently recognised early after infection but not or only weakly in the chronic stage. At least two antigens, the 39 and the 42 kDa antigens, seem to be located on the surface of *B. besnoiti* tachyzoites as determined by biotinylation. Affinity purified antigen was used to establish an APure-BbELISA which showed excellent sensitivity (100%) relative to a serological reference system in naturally, most likely chronically, infected cattle. Specificity was also high (99.8%) as determined in cattle from herds with *Neospora caninum*-associated abortions. The antibody levels in APure-BbELISA were correlated with the parasite load in the skin or the mucous membrane of the vestibulum vaginae as determined by real-time PCR. In acute cases of bovine besnoitiosis (confirmed by the detection of low avidity IgG in the APure-BbELISA) first specific antibodies were detected by ELISA in all animals except one, at the same time or earlier than in the serological reference system. The detection of parasite DNA in skin by real-time PCR was clearly superior to serological analysis in detecting infected cattle during acute besnoitiosis.

1. Introduction

Besnoitia besnoiti – the cause of bovine besnoitiosis – is a cyst forming apicomplexan parasite closely related to *Toxoplasma gondii* and *Neospora caninum* (Ellis et al., 2000). In acutely infected cattle, bovine besnoitiosis is characterised by pyrexia, nasal and ocular discharge, salivation, stiff gait and – in severe cases – clinically apparent subcutaneous edema. In chronically infected cattle, the skin may become severely lichenified and alopecic. Bulls can develop orchitis, which may result in infertility (Kumi-Diaka et al., 1981; Bigalke and Prozesky, 2004). Fortunately few cattle in affected herds develop typical clinical signs, while most animals remain subclinically infected (Bigalke, 1968; Jacquiet et al., 2010).

The life cycle of *B. besnoiti* is not known but it can be transmitted mechanically by biting flies (Bigalke, 1968). A carnivorous definitive host is suspected but has not been identified (Diesing et al., 1988; Basso et al., 2011). Introduction of infected cattle into naive herds seems to play a major role in the transmission of the infection among herds (Bigalke, 1968). Therefore, early detection of infected cattle is important to prevent the spread of bovine besnoitiosis, locally, nationally and internationally. Before the development of PCR assays, early phases of infection were diagnosed by the examination of blood and lymph node smears for parasites (Pols, 1960). Recently PCR techniques have been developed (Cortes et al., 2006b, 2007; Schares et al., 2011b) and may be more sensitive in the detection of acute cases than other techniques. However, PCR assays are not routinely used in many diagnostic laboratories.

A number of serological techniques, including IFAT, ELISA and immunoblots have been reported for the diagnosis of bovine

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infection with *B. besnoiti* (Neuman, 1972; Janitschke et al., 1984; Shkap et al., 1984; Cortes et al., 2006a; Fernandez-Garcia et al., 2009, 2010; Schares et al., 2010, 2011a; Garcia-Lunar et al., 2012). A recent multicenter study showed that the sensitivity was low in cases of early, edematous *B. besnoiti* infection in almost all studied serological tests (Garcia-Lunar et al., 2012). In addition, some of the available tests had a reduced sensitivity when animals had been sampled after a climatic season with low or no insect activity, i.e. after phases in which mechanical transmission by insects was low or even absent (Liénard et al., 2011; Schares et al., 2011a). Diagnostic problems result also from suboptimal specificity of the serological tests which may cause high proportions of false-positive reactions under certain conditions (Nasir et al., 2012). Cross-reactions with related protozoan parasites, especially *N. caninum*, have been shown by immunoblotting (Shkap et al., 2002; Cortes et al., 2006a; Schares et al., 2010).

The objective of this study was to investigate the use of diagnostic antigens of *B. besnoiti* tachyzoites enriched by immune affinity chromatography for the differentiation and confirmation of acute and chronic cases of bovine besnoitiosis.

2. Materials and methods

2.1. Sera

2.1.1. Sera from cattle experimentally infected with related protozoans

Sera from 27 cattle, experimentally infected with *N. caninum* ($n = 2$; 0, 14, 27, 57, 91 days p.i.), *T. gondii* ($n = 12$; 20 to 75 days p.i.), *Sarcocystis cruzi* ($n = 9$; 35 to 50 days p.i.), *Sarcocystis hominis* ($n = 2$; 43 days p.i.), or *Sarcocystis hirsuta* ($n = 2$; 34 days p.i.) (for details, see Schares et al., 1999) were included.

2.1.2. Sera from unaffected cattle

Sera of 478 cattle from five German herds with no history of besnoitiosis were also used. These animals were sampled after *N. caninum*-associated abortions had been confirmed in these herds. All sera were examined for antibodies against *B. besnoiti* and *N. caninum* by methods previously published (Schares et al., 2010).

In addition, 30 sera from a previous study (Nasir et al., 2012) were used. Ten of these sera had shown false positive, another 10 borderline and the remaining 10 negative results in a commercial ELISA (PrioCHECK Besnoitia Ab 2.0, Prionics AG, Switzerland).

2.1.3. Sera from infected cattle

All sera used to evaluate the sensitivity of the ELISA were from cattle from a herd (“Herd-BbGer1”) in Germany where clinical bovine besnoitiosis was first diagnosed in September 2008 (Rostaher et al., 2010). All animals in this herd were clinically examined and blood-sampled from a jugular or tail vein in November 2008 and in April 2009. Two different *B. besnoiti* immunoblots and a *B. besnoiti* IFAT with an estimated specificity of 100% were employed as described (Schares et al., 2010). A serological reference standard was defined in which an animal was regarded as reference-positive if at least two of these three tests revealed a positive result in both November 2008 and April 2009 ($n = 110$; one male, 109 females). Signs of acute besnoitiosis were not observed in any of the animals during sampling and clinical examination.

On both sampling dates, animals with macroscopic visible tissue cysts in the scleral conjunctiva of one or both eyes or in the mucosal membrane of the vestibulum vaginae were recorded. On the first sampling date in November 2008, tissue samples and scrapings from the mucosal membrane of the vestibulum vaginae were examined in 27 animals with demonstrable tissues cysts by real-time PCR as previously described (Schares et al., 2011b).

For the validation of an IgG avidity ELISA, 56 sera from cattle collected in Herd-BbGer1 in December 2009 were used. At that time, all 56 cattle had tested serologically positive by the reference tests for more than 1 year and had shown tissue cysts in the scleral conjunctiva of one or both eyes or in the mucosal membrane of the vestibulum vaginae in November 2008 or April 2009.

2.1.4. Sera from acutely infected cattle

Sera from cattle that had shown fever as a sign of acute besnoitiosis or seroconversion during a cohabitation trial (Table 1) (Gollnick et al., unpublished data) were included in the study. On day 0 of this cohabitation experiment, serologically negative German Simmental heifers SA 3, 4, 6, 8 and 9 were placed together on a pasture with three non-pregnant, chronically infected Limousin cows (SA 13, 15 and 16). On trial days 3 and 51, two pregnant Limousin cows (SA 20 and 22), which were in the acute stage of bovine besnoitiosis, were introduced into the group. These acutely infected cattle derived from Herd-BbGer1. Both animals had tested seronegative for besnoitiosis prior to the pasture period of 2009 (Gollnick et al., unpublished data). SA 4 and 6 developed fever on trial days 36 and 29, respectively, and seroconverted later (i.e. became serologically reference positive on day 45 or 35 of the trial, respectively). SA 8 was not febrile but seroconverted on trial day 73. First macroscopic tissue cysts in the scleral conjunctiva or in the mucosal membrane of the vestibulum vaginae were observed in SA 3, 4, 20 and 22 on trial days 64, 67, 26 and 63, respectively (Table 1). Permission was granted by the responsible authorities (Animal Ethics Committee; Regional Government of Upper Bavaria) and the cohabitation experiment was registered under TV Az. 55.2-54-2531-83-09. Care and maintenance of animals were in accordance with government guidelines.

2.2. Cell culture, purification of tachyzoites and isolation of bradyzoites

The NC-1 strain of *N. caninum* (Dubey et al., 1988) and the Bb1Evora03 strain of *B. besnoiti* (Cortes et al., 2006b) were maintained in MARC-145 cell cultures and extracellular tachyzoites were purified as previously described (Schares et al., 2010). Purified tachyzoites were used to prepare IFAT slides or were pelleted by centrifugation at 1,300*g* for 10 min and frozen at −80 °C until used for immunoblotting or for ELISA antigen preparation.

Bradyzoites were purified from lichenified and partially alopecic skin samples collected after slaughtering *B. besnoiti*-infected cattle from Herd-BbGer1. These tissues had been frozen at −20 °C and submitted to the Friedrich-Loeffler-Institut, Wusterhausen, Germany. Purification was performed as previously described (Schares et al., 2010). Eluted parasites were concentrated by centrifugation (1,300*g*; 10 min) and frozen at −80 °C until used for immunoblotting.

2.3. Serological reference tests

Three serological reference tests were applied to determine the *B. besnoiti* serological status of reference and acutely infected animals: an IFAT and two immunoblots either based on *B. besnoiti* tachyzoite or bradyzoite antigen. These tests were performed as described (Schares et al., 2010) applying published cut-offs (Schares et al., 2010). A positive cut-off titer of 1:200 was used in IFAT, which had yielded a specificity of 100% and a sensitivity of 92% in previous experiments (Schares et al., 2010). In both immunoblots the recognition of at least four of 10 bands regarded as specific (Schares et al., 2010) was scored as a positive reaction. With this cut-off, both immunoblot tests exhibited 100% specificity and 90% sensitivity (Schares et al., 2010). Reference tests applied in the present study were recently validated in an inter-laboratory comparison and showed optimal results in comparison with other