Veterinary Parasitology 171 (2010) 337–342

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Short communication

Development of an indirect ELISA test using an affinity purified surface antigen (P38) for sero-diagnosis of canine *Neospora caninum* infection

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ARTICLE INFO

Article history:
Received 25 February 2010
Received in revised form 26 March 2010
Accepted 1 April 2010

Keywords:
Neospora caninum
Dog
ELISA
NcSRS2
P38
Canine neosporosis

ABSTRACT

Neospora caninum is an intracellular protozoan that infects domestic and wild canids, as well as many warm blooded animals as shown by the isolation of viable parasites and the detection of parasite DNA in naturally infected hosts. *N. caninum* is described as a cause of neuromuscular disease and death in dogs. The dog is also known as a definitive host, shedding oocysts involved in the transmission of the infection to intermediate hosts. This study was conducted to develop an indirect ELISA test using an affinity purified 38 kDa *N. caninum* surface antigen (P38) for the sensitive and specific diagnosis of this infection in dog populations. To define a suitable cut-off, serum samples from 233 dogs were analyzed using an *N. caninum*-specific indirect fluorescent antibody test. All of these serum samples were subjected to the newly designed P38-ELISA. The Two-graph Receiver Operating Characteristics (TG-ROC) of the serum ELISA was determined to examine the performance of the test. TG-ROC analysis showed an area under curve (AUC) of 0.996 that indicates the test results being highly accurate. Optimal sensitivity and specificity (100% and 97.9%, respectively) were determined for SI_n cut-off point of 0.23. To examine possible cross-reactions with other parasites affecting dogs, sera of dogs positive for antibodies against *Toxoplasma gondii* ($n = 17$) and *Leishmania infantum* ($n = 11$) infections were tested. These revealed negative results when tested in the new ELISA.

1. Introduction

Neospora caninum is a protozoan of the phylum apicomplexa that was firstly isolated and described in dogs (Bjerkas et al., 1984; Dubey et al., 1988). Several species of animals are intermediate hosts for this parasite and the main clinical manifestation in these animals is abortion which occurs mainly in cattle worldwide (Dubey et al., 2006). Dogs (*Canis lupus familiaris*), coyotes (*Canis latrans*) and dingos (*Canis lupus dingo*) are definitive and intermediate hosts and shed oocysts following ingesting *N. caninum* infected tissues of intermediate hosts (Gondim et al., 2004; King et al., 2010; McAllister et al., 1998). In clinically infected dogs neurological deficits and muscular abnormalities predominate although other clinical signs also occur due to the fact that almost all tissues are infected (Dubey et al., 1988). Indications for *N. caninum* infection have been found serologically in several animal species over the world (Dubey, 1999). However, definitive proof of infection by isolation of viable parasites is only achieved in ruminants, dogs, coyotes and dingos, yet (Dubey and Schares, 2006; King et al., 2010). *N. caninum* infection was

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doi:10.1016/j.vetpar.2010.04.003

also found in dog and cattle populations in Iran, the county in which the present study was performed (Malmasi et al., 2007; Razmi et al., 2007).

Several diagnostic methods including serology, polymerase chain reaction (PCR), histology, immunohistochemistry and isolation of the organism are available to diagnose *N. caninum* infection in animals (Dubey and Schares, 2006). Among these techniques, serological methods have the benefit of the possibility to be performed in living animals, are more affordable for veterinary diagnostic centers and less time consuming. Serological tests which are mainly in use include indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assays (ELISA), immunoblotting and *N. caninum* agglutination test (Björkman and Uggla, 1999). IFAT detect antibodies to the tachyzoite surface which provides many antigens which are *N. caninum* specific (Dubey and Schares, 2006). The immunoblot analysis of serum samples of *N. caninum* infected cattle has been shown to be a valuable tool to confirm serological results obtained by other tests (Alvarez-Garcia et al., 2002; Schares et al., 1999)

Available ELISA tests allow a sensitive and specific diagnosis and are well suited for testing large numbers of samples. Several ELISA tests have been developed for serodiagnosis of *N. caninum* infection in animals (Dubey and Schares, 2006). Immunodominant surface antigens of apicomplexan parasites are often suitable candidates for the development of diagnostic tools (Björkman et al., 1994; Hoane et al., 2005; Hosseininejad et al., 2009; Kimbita et al., 2001; Schares et al., 2000, 2005). These antigens may be superior to antigen mixtures especially in aspects of providing a higher specificity. P38 (NcSRS2) is one of the immunodominant tachyzoite surface antigens of *N. caninum* (Dubey and Schares, 2006; Hemphill et al., 1997; Schares et al., 2000). The suitability of this antigen for the establishment of serological tests has been shown in previously studies performed in cattle populations; *N. caninum*-specific antibodies could be detected in bovine sera and milk samples using ELISA tests based on P38 (Schares et al., 2000, 2002, 2005). Although recombinant antigens could be promising with respect for the development of ELISA tests, native affinity purified antigens might be superior because inappropriate post-translational modifications and altered epitope-conformation in recombinant antigens might negatively influence their suitability as diagnostic antigens (Graille et al., 2005).

To our knowledge native P38 has not yet been used for sero-diagnosis of canine *N. caninum* infection. Hence we decided to develop an indirect ELISA test using affinity purified P38 antigen of *N. caninum*.

2. Materials and methods

2.1. Serum samples

233 serum samples were collected during 2008–2009 from dogs referred to the small animal clinics of the Veterinary Faculty of Shahrekord University and from guard dogs in Isfahan province, Iran. Animals were of both sexes, clinically healthy and were presented for annual vaccination, routine de-worming programs and checkup.

17 serum samples that were tested seropositive for *Toxoplasma gondii* in both an IFAT and an affinity purified P30(SAG1)-ELISA (Hosseininejad et al., 2009) were used to check the specificity of the P38-ELISA. 11 *Leishmania infantum* positive serum samples from infected dogs (as determined by a direct agglutination test and the isolation of viable organisms) were used to check possible cross-reactivity with this organism (Mohebali et al., 2005). These serum samples were kindly provided by Professor M. Mohebali; School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Positive and negative serum samples to *N. caninum* were used from a pool of serum samples collected in a previously done study (Malmasi et al., 2007). Positive serum samples of this pool had IFAT titers of 1:200 and more and were negative for *T. gondii* antibodies. These control sera were used for ELISA, IFAT and immunoblots performed in this study.

2.2. Parasites

N. caninum (NC1 strain) tachyzoites were grown *in vitro* using Vero cell monolayers in RPMI-1640 supplemented with 2% fetal bovine serum and a mixture of 50 U/ml penicillin and 50 mg streptomycin. The cultures were incubated at 37 °C, 5% CO_2 (Schares et al., 1998). Tachyzoites were used immediately for IFAT or frozen at −80 °C until required for further processing.

2.3. Affinity purification of P38 antigen

Affinity purification of the P38 surface antigen of *N. caninum* tachyzoites was performed by using the mouse monoclonal antibody (mAb) 4.15.15 (IgG2a) coupled to rProtein A Sepharose 4B (Pharmacia Biotec) and purity was examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and India ink staining. The process of purification was described previously (Schares et al., 2000).

2.4. IFAT

4×10^7 *N. caninum* NC1 strain tachyzoites were harvested from Vero cell cultures and fixed in 0.2% formaldehyde in 1 ml PBS. 10 well slides were coated using this suspension and were kept in the freezer at −20 °C until used. Immediately before use, slides were soaked in cold acetone for 10 min followed by 10 min washing in PBS. 20 μl of diluted serum samples (dilutions started from 1:50 in PBS) were added to each well and incubated in 37 °C incubator for 30 min. Slides were then washed using anti-fading buffer pH 9.0 (2.85 g sodium carbonate, 8.4 g sodium bicarbonate and 2.12 g natrium chloride in 1 l distilled water) and incubated in this buffer for 10 min. FITC conjugated anti-dog IgG antibodies (Jackson Immunoresearch, USA) were diluted (1:30 in PBS, 1% Evan's blue) and 20 μl of this mixture were added to each well and incubated in 37 °C for 30 min. Slides were then washed and incubated in anti-fading buffer and PBS (10 min). Slides were covered using a glass cover and examined using a fluorescent microscope (Zeiss, Axiolab, Germany). Serum