Veterinary Parasitology 170 (2010) 323–326

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Short communication

An indirect ELISA for detection of *Theileria sergenti* antibodies in water buffalo using a recombinant major piroplasm surface protein

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ARTICLE INFO

Article history:
Received 6 October 2009
Received in revised form 4 February 2010
Accepted 10 February 2010

Keywords:
Theileria sergenti
ELISA
33-kDa major piroplasma surface protein
Theileriosis

ABSTRACT

In this study we investigated the prevalence and enzootic potential of *Theileria* spp. in water buffalo in the Hubei province in China. An indirect ELISA based on a recombinant major piroplasma surface protein was developed. The complete ORF of the 33-kDa major piroplasma surface protein (p33) was obtained from *Theileria sergenti* genomic DNA by PCR, cloned into the pET-28(a) vector and expressed in *E. coli* as a His-fusion protein. Then the recombinant p33 (rp33) was purified and used as the antigen to develop an iELISA. Specificity test showed that there was no cross-reaction with *Babesia orientalis*, *Schistosoma japonicum*, *Anaplasma marginale* and *Toxoplasma gondii*. 178 water buffaloes raised in different locations in Hubei province in China were detected by this iELISA, all samples were also examined by PCR and microscopy at the same time. The iELISA result showed a higher positive rate (27.5%) than PCR (22.5%) and microscopy (12.9%). This result indicated that the iELISA is a suitable method for the diagnosis of *T. sergenti* infection and could be used in serological surveys to map out the prevalence of the disease.

1. Introduction

Theileria spp. is a tick-borne, intraerythrocytic protozoan parasite of worldwide economic and veterinary importance in ruminants (Aktas et al., 2007). Bovine theileriosis caused by *Theileria sergenti* is a major tick-borne protozoan parasite of grazing cattle in Japan and Korea (Hagiwara et al., 2005; Ko et al., 2008; Shiono et al., 2003), and it is also one of the most important diseases of water buffalo with clinical manifestations of apathy, fever, anaemia, lymphadenectasis and in some cases death in China (Jin et al., 2007).

To date, benign bovine *Theileria* parasites, known as either *Theileria buffeli*, *Theileria orientalis* and *T. sergenti* are recognized as of the *T. sergenti/T. buffeli/T. orientalis* group, and have a worldwide distribution. Their nomenclature is usually based upon the geographic origin of the parasite (Gubbels et al., 2000; Stewart et al., 1996). In terms of pathogenicity, benign *Theileria* spp. unlike malignant *Theileria* species, the schizont does not play a major role. The erythrocytic stage is more significant in pathogenicity since anaemia as a clinical symptom is caused by intraerythocytic parasite growth (Jeong et al., 2005; Kim et al., 1998; Shiono et al., 2004).

Even though *T. sergenti* infection in ruminants has extensively been studied, the diagnosis of theileriosis is based on the observation of the parasite in Giemsa-stained blood films, the clinical manifestations in a later phase of the disease or the detection of serum antibodies to piroplasm antigens by an indirect immunofluorescent antibody assay (Jeong et al., 2005; Miranda et al., 2006). Recently, a TaqMan polymerase chain reaction (TaqMan PCR) was found to be a promising diagnostic method with high sensitivity and specificity to detect the parasite, and a serological method

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doi:10.1016/j.vetpar.2010.02.009

such as latex agglutination test could be used to monitor antibodies against *T. sergenti* (Jeong et al., 2003; Jeong et al., 2005). Another serological method, namely enzyme-linked immunosorbent assay (ELISA), with advantages such as easy performance, inexpensive and reproducible, was developed to monitor theileriosis caused by *Theileria annulata*, resulting in facilitating the study of its epidemiology (Salih et al., 2005). An ELISA method has been described by Katende et al. (1998) and Bakheit et al. (2004) for detection of *Theileria parva* and *T. annulata*, respectively. However, there is no report to detect the antibodies of *T. sergenti* by an ELISA method in water buffaloes.

In this study, an indirect ELISA (iELISA) based on a recombinant major piroplasm surface protein (rp33) was developed to diagnosis the infection of *T. sergenti* in water buffalo and as such it was used in the epidemiological survey of theileriosis in water buffaloes in the Hubei province of China.

2. Materials and methods

2.1. Source of samples

A total of 178 water buffaloes were examined from eight areas, namely Wuhan, Daye, Hongan, Xiaogan, Xiantao, Zhongxiang, Macheng and Jiayu in the Hubei province in China. For this purpose, 1 mL whole blood samples were collected from the jugular vein of each buffalo using in BD Vacutainer™ tubes containing EDTA, for DNA extraction. Also 9 mL of blood were collected in Vac-*U*-Test® tubes which were then incubated at 37 °C for 2 h, followed by centrifuging at 4000 × *g* for 10 min to obtain the sera. Both DNA and sera were stored at −20 °C for further study.

2.2. Blood films and stain method

Blood films taken from the ear vein of the water buffaloes were fixed with ethanol, stained with Giemsa solution and observed with microscopy to detect piroplasm infection by counting more than 10,000 erythrocytes.

2.3. Gene clone and expression vector construction

To amplify the entire open reading frame of p33 from *T. sergenti* genomic DNA (*T. sergenti* genomic DNA were conserved in our laboratory), a pair of oligonucleotide primers (p33F:5′-CGGGATCCATGTTGTC-CAAGAGAT-3′, p33R:5′-CGCTCGAGCTARAGATAGWAGA-AWAC-3′) including the BamHI and XholI restriction enzyme sites (underline) were designed from the p33 gene (accession no. D87194) for the PCR reaction. Subsequently the PCR products were purified and cloned into pET-28(a) vector by the restriction enzyme sites of BamHI and XholI. The construct of recombined expressed plasmids was confirmed for accurate insertion by both restriction enzyme digestion and sequencing.

2.4. Expression and purification of recombinant proteins

The recombinant plasmid was transformed into *E. coli* BL21 and was expressed as a His-fusion protein according to the manufacturer's instructions. To obtain soluble protein, the washed rp33 inclusion bodies were dissolved in denaturing agents and the released protein was then refolded by gradual removal of the denaturing reagents by dilution or dialysis according to the method summarized by Sambrook et al. (2001).

2.5. Western blotting

To identify whether the rp33 had identical molecular weight and immunocompetence as predicted, positive sera of *T. sergenti* were used to analyze the rp33 by SDS-PAGE and Western blotting. The rp33 was separated on a 12% SDS-PAGE as described by Sambrook et al. (2001), and then it was transferred onto nitrocellulose filter (Millipore) with a mini trans-blot cell. The membranes were incubated with TBS containing 5% skim milk at 37 °C for 1 h and then with the serum samples (1:100 dilution with TBST containing 1% skim milk) in an incubation tray at 37 °C for 2 h. After washing with 5 ml of TBS containing 0.5% Tween-20 (TBST) for 10 min in triplicate, the membranes were left to react with anti-bovine IgG-peroxidase conjugate (1:2500 dilution) (Sigma, St. Louis, USA) at 37 °C for 1 h. The membranes were then washed again with 5 ml TBST for 10 min in triplicate. The enzyme–substrate reaction was developed using the DAB developer reagent (Sigma).

2.6. ELISA protocol

The recombinant protein rp33 was diluted to a final concentration of 10 μg/ml in bicarbonate buffer (pH 9.6) and a volume of 100 μl was used to coat 96-well polystyrene microstate ELISA plates, which were later incubated at 4 °C overnight. Subsequently the plates were washed three times with PBST. 100 μl of serum samples (diluted 1:50 with 0.1% BSA) were added to each well followed by an incubation at 37 °C for 1.5 h. The plates were rewashed as before, then horseradish peroxidase-conjugated to goat anti-cattle IgG (Cappel, Durham, N.C.) was used as secondary antibody, the plates were incubated at 37 °C for 2 h. After rewashed in triplicate with PBST, the color reaction was developed by the TMB substrate buffer solution. The OD value was read at 630 nm, using an automatic ELISA reader (Bio-TEK instruments Inc., Winooski, USA). Positive and negative serum controls were included in each plate.

2.7. Specificity of the iELISA

The sera collected from infected water buffalo with parasites other than *T. sergenti*, including *Babesia orientalis*, *Schistosomiasis japonica*, *Anaplasma marginale* and *Toxoplasma gondii* were analyzed by iELISA.

2.8. DNA extraction and PCR detection of field samples

Total genomic DNA of all samples were extracted from 200 μl of blood using the TIANamp Genomic DNA Kit (TIANGEN BIOTECH BEIJING CO., LTD, China) following the manufacturer's introductions, the DNA was eluted in 200 μl elution buffer and stored at −20 °C for further