Short Communication

Determination of immunoreactive proteins of Babesia ovis

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Babesia ovis, an intraerythrocytic protozoan parasite transmitted by ticks, causes severe infections in sheep in tropical and subtropical regions of the world. Parasite-specific immunoreactive proteins have been used as antigen in the serological diagnosis of babesiosis. There is no study about determination of B. ovis-specific proteins in sheep. This study was planned to determine the immunoreactive proteins of B. ovis. In this study, two splenectomized lambs, and twelve seropositive sheep and five seronegative lambs for anti-B. ovis antibodies were used as materials. Infected blood samples at 5% of parasitemia from the two splenectomized lambs experimentally infected with a virulent B. ovis field strain were analyzed for B. ovis-specific proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB). B. ovis-specific five major proteins were recognized by anti-B. ovis serum but not by healthy sheep serum. They were of approximate molecular weights 154, 109, 77, 58, and 38 kDa. As the control samples, protein profiles of the blood extracts of two lambs before splenectomy operation were also blotted with the immune sera, but none of the five proteins was detected. These proteins were also immunoblotted with heterologous positive and negative sheep sera. All of twelve positive sera recognized the 109 kDa protein with 100 percent sensitivity. The 77 kDa protein reacted in 11 of 12 sera (91.6%). The sensitivities of the other 3 proteins ranged between 83.3% and 25%. The five protein bands immunoblotted with sera of the 5 negative lambs did not give any positive reaction. The results of this study revealed the presence of proteins recognized by the serum antibodies of experimentally and naturally infected sheep with B. ovis. Additional studies on the purification of these proteins and on subsequently their utilization in a serodiagnostic method are required to improve the serological diagnosis of ovine babesiosis.

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1. Introduction

Ovine babesiosis is an endemic disease threatening the sheep farming in many countries in tropical and subtropical zone (Yeruham et al., 1998; Razmi et al., 2003; Uilenberg, 2006; Ranjbar-Bahadori et al., 2012; Fakhar et al., 2012; Sevinc et al., 2013). Babesia ovis, the main causative agent of ovine babesiosis, causes clinical signs characterized by fever, hemolytic anemia, hemoglobinuria, jaundice, weakness, anorexia, and mortality (Sayin et al., 1997; Yeruham et al., 1998; Sevinc et al., 2013). The disease has considerably economic importance in terms of death cases, yield losses and the costs of treatment in the livestock industry.

The diagnosis of acute babesiosis is based on the signs of the disease and on the demonstration of the parasites in the erythrocytes in Giemsa-stained blood films by microscopy. Microscopical and clinical examinations have come up short on diagnosis of the carrier animals with subclinical
infections. Recently, the carrier animals have been determined by the immunological and molecular methods (Bose et al., 1995; Georges et al., 2001; Bock et al., 2004). For the immunodiagnosis of B. ovis, an Enzyme-linked immunosorbent assay (ELISA) has been used to detect anti-B. ovis antibodies by using a synthetically derived bovine B. ovis antigen (Duzgun et al., 1991; Emre et al., 2001; Cicik et al., 2004). Indirect fluorescent antibody test (IFAT) is also being used in a few laboratories (Wright, 1990; Sevinc et al., 2007; Ekici et al., 2012). The immunoreactive proteins of some Babesia species have commonly been used as diagnostic antigens in the diagnosis of equine, bovine and canine babesiosis (Ikadai et al., 2000; Boonchit et al., 2002; Huang et al., 2003; Zhou et al., 2007). So far, no study has been carried out to identify the B. ovis-specific proteins. The identification of B. ovis-specific protein antigens would facilitate the development of serological tests for ovine babesiosis. This study was planned to detect the immunoreactive protein components of B. ovis by WB analysis.

2. Materials and methods

2.1. The animals

Two splenectomized lambs which were negative for Babesia infections determined by microscopic and serologic examinations were inoculated with the virulent field strain of B. ovis. Infected blood at a parasitemia of 5% (3–5 days after inoculation) was collected in the heparinized tubes, and the lambs were treated with Imidocarb dipropionate (IMDP). Blood samples were taken from these animals at regular daily intervals for the homologous serum antibody collection after the treatment. Additionally, the heterologous serum samples were collected from the 12 sheep naturally infected with B. ovis three weeks after the acute stage of infection and from the 5 seronegative lambs. The Giemsa-stained thin blood smears were examined for confirmation of the infection. The positive antibody titers were determined by IFAT using an antigen preparation as described before (Sevinc et al., 2007; Wright, 1990).

2.2. Protein extraction

Protein extractions from the infected and uninfected blood samples were prepared by the method described before (Figueroa et al., 2004), with minor modifications. The anticoagulated B. ovis-infected blood samples were taken from the experimentally infected lambs at a parasitemia of 5%, and centrifuged at 120 × g for 20 min to separate the infected RBCs and the free merozoites in plasma. After the low speed centrifugation, the plasma and cell fractions of infected blood were re-suspended and washed 3 times with phosphate-buffered saline (PBS, pH 7.2) by centrifugation for 30 min at 3000 × g. In each centrifugation of the cell fractions, theuffy coat was also removed. The final pellet in each tube (infected red blood cells–iRBCs and free merozoites) was solubilized in 9 volumes of the extraction media (2% Triton X-100, 0.6 M KCl, 0.15 M NaCl, 5 mM EDTA, 0.01 M Tris–HCl pH 7.8, 1 mM PMSF, 0.1 mM TLCK) incubating at 4 °C for 1 h. After insoluble components were removed by centrifugation at 14,000 × g for 15 min at 4 °C, the supernatant was kept at –80 °C until use. The protein concentration of the supernatant was determined by using a BCA protein assay kit (Thermo Scientific, USA).

Alternatively, protein extraction protocol above was also applied after the lysis of infected blood with ammonium chloride according to the method described by Martin et al. (1971).

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting analysis

Prior to electrophoresis, each protein extract was diluted with one volume of sample loading buffer (0.06 M Tris–Cl, pH 6.8, %2 SDS, %10 glycerol, %0.25 bromphenol blue) with 5% β-mercaptoethanol, and heated at 100 °C for 5 min. 20 μl of the extracts (including 80 and 30 μg protein respectively for the extracts prepared from the RBCs and plasma samples) was loaded to per lane and the proteins were separated by electrophoresis in 12% polyacrylamide gel according to the method described by Laemmli (1970), and visualized by staining with Coomassie brilliant blue.

Size-separated proteins were electro-transferred from the unstained gel onto a nitrocellulose membrane (Whatman, Germany) using a semi-dry transfer unit (Thermo Scientific). The immunological features of these proteins were determined by the WB analysis according to the procedures described before (Kumar et al., 2002) with minor modifications. Briefly, the membranes were blocked with Tris-buffered saline (TBS, 25 mM Tris–HCl, 0.15 M NaCl, pH 7.2) containing 3% (w/v) bovine serum albumin (BSA) and 0.05% Tween 20 at room temperature (RT) for 1 h, washed three times with TBS-Tween 20 (TBST), and incubated with anti-B. ovis immune serum diluted 1:100 in TBS for 1 h at RT. After washing process, the membranes were incubated with secondary antibody peroxidase-conjugated anti-sheep/goat IgG (Sigma–Aldrich, USA) diluted 1:5000 in 3% BSA-TBS at RT for 1 h. After the final washing, immunoreactive protein bands on the membranes were visualized by treating peroxidase enzyme substrate (TMB, Sigma–Aldrich) for 5–10 min in a dark room.

Additionally, the immunoreactive proteins recognized by homologous anti-B. ovis immune serum was also tested for their antigenic activities using the heterologous serum samples from sheep, naturally infected with B. ovis, and from negative lambs. The approximate molecular weights of individual protein bands were determined by regression analysis.

3. Results

3.1. SDS-PAGE analysis

The pattern of B. ovis proteins separated by SDS-PAGE is shown in Fig. 1. The proteins extracted from infected blood were fractionated into approximately 12 polypeptide bands at molecular weights ranging from 25 to 170 kDa (Fig. 1, lanes 1, 2, 5, 6 collectively). The number of the protein bands extracted from iRBCs and plasma of the lysed and unlysed blood was almost similar. While the protein profiles of uninfected erythrocytes showed the
five noticeable major bands (Fig. 1, lane 3), no remarkable protein band in the SDS-PAGE analysis of the plasma of uninfected blood was seen (Fig. 1, lane 4).

3.2. Western blotting analysis of different antigenic fractions recognized by Babesia ovis antisera

The antigenic features of the protein fractions transferred onto nitrocellulose membrane were determined by immunoblotting using the immune serum taken from lambs which were experimentally infected with B. ovis. The protein extracts prepared from infected blood showed the five major protein bands (I: 154, II: 109, III: 77, IV: 58, V: 38 kDa) by Western blotting with homologous antisera (Fig. 2, lanes 3–6). These protein bands were not seen in the protein profiles of uninfected blood samples (Fig. 2, lanes 1 and 2).

Immunoreactivities of the B. ovis-proteins recognized by homologous immune serum were tested by WB analysis using the heterologous sheep sera known their seropositivity and seronegativity status for B. ovis antibodies. The five protein fractions did not give any positive reaction with 5 negative sheep sera. All of the 12 positive sera had specific antibodies for 109 kDa protein. There were positive reactions in 11 (91.6%), 10 (83.3%), 9 (75%) and 3 (25%) of 12 positive sera for 77, 58, 154 and 38 kDa protein bands, respectively (Fig. 3).

4. Discussion

The species-specific immunoreactive proteins are valuable molecules as antigen sources in diagnosis and vaccine candidates for prevention of infectious diseases (Brown et al., 2006; Terkawi et al., 2007; Guan et al., 2010). SDS-PAGE and WB are complementary methods used for the identification of specific proteins from a complex mixture (Lodish et al., 2004). In this study, two splenectomized lambs were experimentally infected with a virulent B. ovis field strain, and the blood at 5% of parasitemia was used for analysis of B. ovis-specific proteins. The proteins in infected blood extracts were separated electrophoretically through the 12% polyacrylamide gel. Immunoreactivities of these proteins were determined by immunoblotting using the immune serum obtained from the two lambs experimentally infected with B. ovis. Approximately 12 protein bands originated from the host and/or parasite and molecular weights of which range between 25 and 170 kDa, were displayed in the polyacrylamide gel electrophoresis of the B. ovis-infected blood extracts (Fig. 1, lanes 1, 2, 5, 6). Compared with the pattern of uninfected RBCs proteins (Fig. 1, lane 3), many common protein bands were detected in the electrophoresis of the B. ovis infected blood extracts. Before the extraction of parasite-proteins, the infected blood samples were processed in two ways. While the infected blood at parasitemia of 5% was used without lysing erythrocytes in the first way, the RBCs were lysed with ammonium chloride in the second way. There were not distinctive differences between the appearances of the gel electrophoresis of the extracts processed in two different protocols. It was expected that the extract prepared after RBC-lysis would have included less host components. But it was difficult to prepare pure merozoites without contamination of host components from B. ovis-infected blood by using the RBC lysis method with ammonium chloride.

By means of Western blotting analysis, five major proteins were recognized by immune serum (Fig. 2, lanes 3–6) but not by the seronegative serum. The approximate molecular weights of immunoreactive proteins were 154, 109, 77, 58, and 38 kDa. These protein bands were not seen in the protein profiles of the uninfected blood extracts immunoblotted with the anti-B. ovis immune serum (Fig. 2, lanes 1 and 2). The results indicated that these proteins were B. ovis-specific. B. ovis-infected RBCs extracts were also immunoblotted with the 12 sera from the sheep naturally infected with B. ovis, taken three weeks after the acute stage of infection and containing anti-B. ovis antibodies at a titer of 1:5120 by IFAT. All sera recognized 109 kDa protein with 100 percent sensitivity. The 77 kDa protein
reacted in 11 of 12 sera with 91.6 percent sensitivity. The sensitivities of the other 58, 154 and 38 kDa proteins were 83.3%, 75% and 25%, respectively. These five protein fractions did not give any positive reactions with seronegative lambs’ sera (Fig. 3). The locations of these proteins were not investigated in this study. The studies related with the immunodominant proteins of apicomplexan hemoparasites such as *B. bovis*, *B. bigemina* and *B. equi* indicate that antibody-mediated host immunity against the bloodstages of hemoparasites targets the molecules located on the surface of parasite and/or infected erythrocytes, and those found in apical organelles (McElwain et al., 1987; Hines et al., 1989; Kappmeyer et al., 1993). Subsequent studies on the locations and functions of *B. ovis*-specific immunoreactive proteins are needed.

In conclusion, the presence of the five *B. ovis*-specific immunoreactive proteins was determined by WB analysis, for the first time in this study. These proteins had antigenic activities at the rates varying from 25 to 100% on the diagnosis of the natural *B. ovis* infections. Further studies with regard to the purification of these proteins and their possible usages as diagnostic proteins are required for the development of serological test for ovine babesiosis.

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References


