Identification and Characterization of Merozoite Antigens of a *Theileria* Species Highly Pathogenic for Small Ruminants in China

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ABSTRACT: A new pathogenic *Theileria* species transmitted by *Haemaphysalis qinghaiensis* was identified in the Northwestern part of China and was shown to be highly pathogenic for small ruminants. The present article aimed at identifying merozoite antigens that might be suitable for developing diagnostic methods and designing a potential vaccine. Absence of other theilerial or babesial infections was confirmed by reverse line blot in all antigen samples used. Extensive Western blot analyses using serum from infected and noninfected animals led to the identification of four potential merozoite immunoreactive proteins at different molecular weights. Further protein characterization using peptide mass mapping by matrix-assisted laser desorption/ionization (MALDI) followed by database searching resulted in two significant hits that identified two proteins of parasite origin, one homologous to a possible MO25-family protein from *Cryptosporidium parvum* and the other with an HSP70 from *Theileria annulata.* Another protein was also identified as a parasite protein but without significant homology. Immunization of rabbits with selected proteins produced antisera that reacted specifically on Western blots with merozoite antigens of the corresponding sizes. This article represents the first identification and characterization of potential antigenic proteins of *Theileria* sp. (China) for veterinary purposes.

KEYWORDS: *Theileria*; merozoites; Western blot; matrix-assisted laser desorption/ionization (MALDI); China

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INTRODUCTION

Theileria organisms are tick-borne protozoan parasites infecting mainly ruminants in tropical and subtropical countries. Although capable of infecting a wide range of vertebrates, theilerial parasites require both vertebrate and nonvertebrate hosts (ticks) to maintain their life cycle. Depending on the *Theileria* species a variety of ticks are responsible for their transmission including *Rhipicephalus*, *Haemaphysalis*, and *Hyalomma*. Theileriosis is still a serious disease because it is responsible for severe economic losses in the livestock industry.[1]

Due to the economic relevance of small ruminants, interest has arisen in sheep-infecting *Theileria* parasites.[2] Recently, a *Theileria* species causing a fatal disease in small ruminants has been identified in the Northwest of China.[3–8] This parasite is transmitted by *Haemaphysalis qinghaiensis* and often occurs in mixed infections with other parasites.[8–10] Depending on the geographic distribution, the rate of morbidity due to the Chinese *Theileria* parasite varies between 19% and 65%, whereas the mortality ranges from 17% to 75%. The highest mortality is observed in lambs and in imported exotic animals. In summary, from 4% to 47% of sheep in the investigated area die due to the infection.[4] The diagnostic techniques for small ruminant piroplasmosis detection are mainly based on the morphological examination of blood smears and clinical symptoms.[11–15] However, these methods require experience in microscopy and are difficult to apply on a large scale for epidemiological studies. Consequently, the development of specific and sensitive diagnostic tools for parasite surveillance, as well as immunoprophylatic means to control and prevent the spread of diseases is crucial. Thus, there is an urgent need for a laboratory diagnostic test combining specificity, sensitivity, and cost effectiveness, such as enzyme-linked immunosorbent assay (ELISA).[16,17] Furthermore, ELISA offers an attractive alternative since reading of the results can be fully automated.

Unlike the leukoproliferative *Theileria* species (e.g., *T. annulata*, *T. lestoquardi*, or *T. parva*), the schizont stage of *Theileria* sp. (China) is very short and until now it could not be cultivated *in vitro*.[18] Additionally, the merozoite stage is the most exposed stage to the host immune system. For this reason the aim of the present article was to identify and characterize merozoite antigens using sera from infected animals. This was achieved by screening lysates of infected erythrocytes as well as purified merozoites in Western blot.

MATERIALS AND METHODS

Antigen and Serum Samples

Experimental infections were conducted in the experimental animal facility of the Lanzhou Veterinary Research Institute, China, using sheep from

Theileria and other protozoa-free areas. Animals were infected by injection of stabilated blood as previously described[19] and were used for the collection of infected erythrocytes (L1, L2, and L3). For the collection of serum samples, sheep were experimentally infected with ticks as described.[7] Negative controls (Ln, Ln3, Ln4) were obtained from sheep held in a *Theileria*-free area (Germany).

Reverse Line Blot

In order to confirm that the biological material used in this study consisted of *Theileria* sp. (China), the samples were analyzed by reverse line blot (RLB) as described previously.[20,21]

Merozoite Antigen Preparation

Merozoite antigenic material was generated by a method previously described[22] with some modifications. In summary, fresh blood from infected sheep was diluted 1:1 (v/v) in Alsever solution (Sigma-Aldrich, St. Louis, MO) and centrifuged at 900*g* for 10 min at 4°C. The supernatant was discarded; then the pellet was washed three times in Tris–HCl buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl), and was centrifuged at 500*g* for 10 min at 4°C. After that, the pellet was diluted 1:5 (v/v) in Tris–HCl, erythrocytes, separated from leukocytes by using a cellulose–powder column, (Whatman cellulose CF11; Millipore, Schwallbach, Germany), and lyzed with α−hemolysin (Sigma) at 37°C for 30 min. Merozoite material was generated by a Percoll gradient centrifugation (40% and 60%; Amersham, Munich, Germany) where the merozoite-containing band was localized between the Percoll layers. After collection, merozoites were washed three times at 5000*g* for 10 min at 4°C, resuspended in Tris–HCl buffer, lyzed by 5 freeze/thaw cycles, and stored at –70°C.

Sample Fractionation and Triton X-100 Extraction

Fractionation was performed based on the method of Nagamatsu *et al*.[23] with some modifications. Briefly, 500 μL of erythrocytes obtained from cellulose powder column separation were homogenized in 5 mL of homogenization buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). Cell debris was removed by centrifugation at 900*g* for 10 min at 4°C and the supernatants were collected. The antigen was then ultracentrifuged at 110,000*g* for 75 min at 4°C. The supernatant fraction (sob) was removed and the membrane-containing pellet was solubilized with solubilization buffer (10 mM Tris–HCl, pH 7.4,

1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) for 1 h at 4°C. The insoluble material was removed by centrifugation at 14,000*g* for 10 min at 4°C resulting in a pellet (insoluble fraction, Fi) and a supernatant (soluble fraction, Fs). The insoluble fraction was resuspended in PBS, pH 7.4 and the soluble fraction was concentrated by ultrafiltration using Millipore Centricon ultrafiltration columns with a cutoff molecular weight of 10 kDa. All samples were stored at –70°C.

SDS-PAGE and Western-Blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 8–12% polyacrylamide gels under nonreducing conditions. Proteins were transferred onto PVDF (polyvinylfluoride, Immobilon-P; Millipore) for antigen screening or nitrocellulose membrane (Schleicher & Schuell/Whatman, Dassel, Germany) for immunoblotting with rabbit antisera. For the screening of antigen samples, sheep antisera were diluted to 1:400 in the dilution buffer (3% skim milk powder, 0.05% Tween-20 in TBS) and immunoreactivity was detected by an indirect immunoperoxidase method (donkey anti-goat IgG HRP (Santa Cruz, Santa Cruz, CA) with chemiluminescence detection (WestPico reagent, Pierce, Perbio Science, Bonn, Germany). In addition, fractionated samples were immunoblotted against rabbit antisera, which were raised against the individual protein band (see below). In this case, immunodetection was achieved with goat anti-rabbit alkaline phophatase conjugated antibody (Jackson, West Grove, PA) with BCIP/NBT for chromogenic detection.

MALDI MS Peptide Mapping

Protein SDS-gel bands were excised and polypeptides were subjected to reduction, alkylation, and digestion with modified sequencing-grade trypsin (Promega, VWR International Material de Laboratorio, Lisbon, Portugal) according to Pandey *et al*.[24] Sample peptides were assayed for peptide mass fingerprint (PMF) in a Voyager-DE STR (Applied Biosystems, Foster City, CA) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. Peptide crystallization was achieved by using 0.5 L of samples on a plate and adding on top with an equal volume of recrystalized matrix α-cyano-4-hydroxycinnamic acid (CHCA) (10 mg/ml) prepared in acetonitrile (50%, v/v) with trifluoracetic acid (0.1%, v/v). The mixture was allowed to air dry. Monoisotopic peptide masses were used to search for homology and protein identification with Peptide Mass Fingerprint of Mascot (http: //www.matrixscience.com). Searches were done in MSDB database. It was considered a mass accuracy of 50–100 ppm for external calibrations and

Cys carbamidomethylation and Met oxidation as fixed and variable amino acid modifications, respectively. The criteria used to accept the identification were significant homology score achieved in Mascot.[25] Additionally, a minimum of four peptides match, and at least 10% sequence coverage criteria were also considered.

Production of Rabbit Antisera

Three rabbits were immunized in the animal house facility of the Instituto Nacional de Engenharia e Tecnologia Industrial, Portugal, with selected protein bands 2, 5, and 8 (see FIG. 1). The selected gel bands were emulsified and injected four times at 3- to 4-week intervals. Antisera were collected 4 weeks after the last immunization and stored at –20°C.

RESULTS

Antigens Identification

For the identification of parasite antigens, two different types of parasite material were used: (*a*) isolated merozoites of the parasite *Theileria* sp. (China), and (*b*) *Theileria* sp. (China) infected erythrocyte lysated fractions.[26] Noninfected erythrocytes were used as controls. The fractionated erythrocyte lysates and the isolated merozoites were separated by SDS-PAGE under nonreducing conditions.

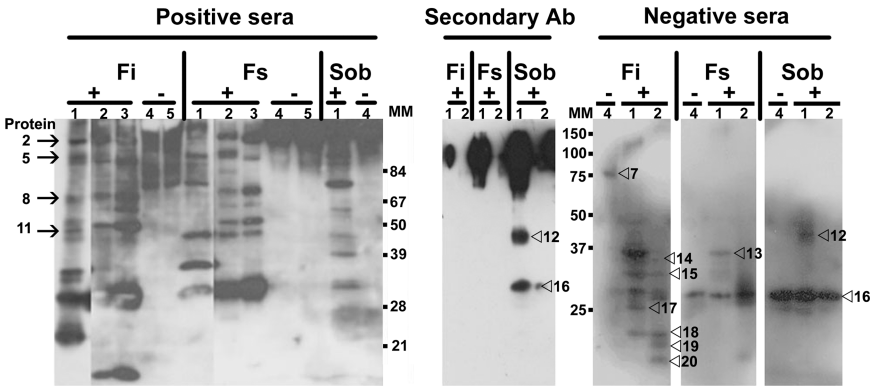

FIGURE 1. Western blots of *Theileria* infected (+; L1 (1), L2 (2), and L3 (3)) and noninfected (–; Ln3 (4) and Ln4 (5)) erythrocyte fractions (Fi, Fs, and Sob) probed with antisera from *Theileria* sp. (China) infected sheep. Protein bands subjected to further analysis are pointed out by arrows (→) and designated with the protein number as mentioned in the text. MM: molecular marker.

The comparative SDS-PAGE analysis of the different fractions revealed different protein band patterns in the higher resolution than unfractionated samples. This allowed the more precise and reproducible detection of several different immunoreactive bands when screened by immunoblotting using different sera of *Theileria* sp. (China) infected sheep. The immunodetection visualized several bands ranging from 11 to 150 kDa (FIG. 1). Despite differences in antibody titers, all the tested sera could recognize the protein bands mentioned above, indicating that these proteins were immunodominant antigens. All negative controls (secondary antibody and serum from noninfected animals) did not specifically react with the material tested (data not shown). The potential antigenic proteins were selected on the basis that they were recognized by all the positive sera tested and not by the negative controls. The serum samples exhibited a similar reaction against purified *Theileria* sp. (China) merozoite antigen samples (FIG. 2 A), resulting in the detection of the same four proteins (proteins 2, 5, 8, and 11). Thus, four potential antigens, with apparent molecular weights of 54 (protein 11), 70 (protein 8), 95 (protein 5), and 119 kDa (protein 2) specific for the merozoite infected erythrocytes fraction, were identified.

Antigen Characterization by Peptide Mass Fingerprint

The results of the peptide mass fingerprint analysis are summarized in TABLE 1. Regarding protein 2, it showed a significant score of 79 to a possible MO25-family protein from *Cryptosporidium parvum,* where protein scores greater than 61 are significant ($P < 0.05$). The spectra results obtained from

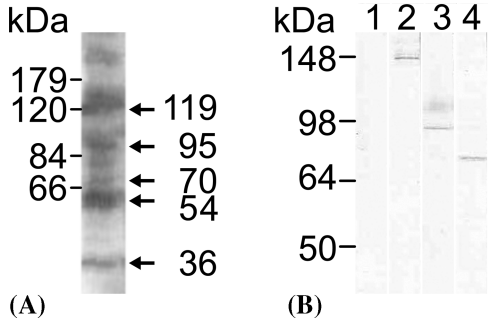

FIGURE 2. (A) Western blot analysis of *Theileria* sp. (China) isolated merozoites probed with serum from a *Theileria* sp. (China) infected sheep. Corresponding protein bands are indicated on the right. MM: Molecular marker. **(B)** Immunoblot analyses of rabbit antisera produced against proteins from *Theileria* sp. (China), P2, P5, and P8. Rabbit antisera were diluted 1:200 and probed against purified merozoite samples: Lane (1) Rabbit preimmune serum; Lane (2) Rabbit antiserum raised against protein 2; Lane (3) Rabbit antiserum raised against protein 5; Lane (4) Rabbit antiserum raised against protein 8.

TABLE 1. Summary of the peptide mass fingerprint analysis for proteins 2, 5, 8, and 11. Scores greater than 61 are considered significant

Protein	MASCOT score	Coverage (%)	Peptides matched	Highest similarity
2	79	26	14	*C. parvum* possible MO25-family protein (Accession Q7YYL6)
5	39	24	8	Hypothetical *P. chabaudi* protein (Accession Q4XUW9˙PLACH)
8	62	16	8	*T. annulata* 70 kDa heat shock protein (HSP 70.1) (Accession J04653)
11	113	30	11	Actin

protein 5 were quite clear although no significant homologies were found in the database. Protein 8 on the other hand showed some homologies with a HSP70 from *T. annulata*[27] with a significant score when a taxonomic restriction to *Alveolata* species was considered. Finally, protein 11 showed good spectra and was identified as being an actin-like protein.

Specificity of Rabbit Antisera

The rabbit antisera were then characterized for reactivity against the respective proteins and immunoblotting analysis was performed with purified *Theileria* sp. (China) merozoites (FIG. 2 B). Antisera from immunized rabbits, but not preimmune sera, recognized parasite-specific bands that could not be observed in the uninfected erythrocytes fraction. The sizes of detected protein bands corresponded to the appropriate molecular weights of 119, 95, and 70 kDa of proteins used for respective immunization.

DISCUSSION

The present study reports on the identification of antigens of a newly characterized highly pathogenic *Theileria* species of small ruminants in China. As a possible contamination with other *Theileria* or *Babesia* parasites was excluded by RLB, all the potential antigens identified in the present work were of *Theileria* sp. (China) origin. Altogether protein bands with apparent molecular weights of 119 (protein 2), 95 (protein 5), 70 (protein 8), and 54 kDa (protein 11) were identified.

For further antigen identification and characterization, mass spectrometry analysis (peptide mass fingerprint) was performed. Protein 2 had a high homology (with a confidence grade of $>95\%$) to a possible MO25-family protein from *Cryptosporidium parvum* and protein 8 to HSP70 of *T. annulata*. Protein 5 spectra presented no significant homology results, the highest score being

obtained with a *Plasmodium chabaudi* conserved hypothetical protein. This finding suggests that protein 5 may be a new nonidentified protein. Protein 11 on the other hand was identified as actin, and was not considered further for diagnostics development due to potential cross reactivity with the highly identical host actin.

Further verification of the parasite origin of the identified antigens was made by using rabbit antisera raised against the respective proteins 2, 5, and 8. These antisera recognized proteins of the respective molecular weights by immunoblots of infected erythrocyte fractions and purified merozoite extracts. These specific rabbit antisera will be employed to screen a *Theileria* sp. (China) merozoite expression library that is under construction to isolate recombinant proteins. The most promising protein will then be used for the establishment of a diagnostic ELISA. In addition, these proteins will be checked for their immunogenic potency and their possible application for perspective candidate vaccine antigens.

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