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Original article

Characterization of a papain-like cysteine protease essential for the survival of *Babesia ovis* merozoites

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ABSTRACT

Babesia ovis, a tick-transmitted intraerythrocytic protozoan parasite, causes severe infections in small ruminants from Southern Europe, Middle East, and Northern Africa. With the aim of finding potential targets for the development of control methods against this parasite, sequence analysis of its genome led to the identification of four putative cysteine proteases of the C1A family. Orthology between *B. ovis*, *B. bovis*, *T. annulata*, and *T. parva* sequences showed that each *B. ovis* C1A peptidase sequence clustered within one of the four ortholog groups previously reported for these piroplasmids. The ortholog of bovipain-2 of *B. bovis* and falcipain-2 of *Plasmodium falciparum*, respectively, was designated "ovipain-2" and further characterized. *In silico* analysis showed that ovipain-2 has the typical topology of papain-like cysteine peptidases and a highly similar predicted three dimensional structure to bovipain-2 and falcipain-2, suggesting susceptibility to similar inhibitors. Immunoblotting using antibodies raised against a recombinant form of ovipain-2 (r-ovipain-2) demonstrated expression of ovipain-2 in *in vitro* cultured *B. ovis* merozoites. By immunofluorescence, these antibodies reacted with merozoites and stained the cytoplasm of infected erythrocytes. This suggests that ovipain-2 is secreted by the parasite and could be involved in intra- and extracellular digestion of hemoglobin and/or cleavage of erythrocyte proteins facilitating parasite egress. A significant reduction in the percentage of parasitized erythrocytes was obtained upon incubation of *B. ovis in vitro* cultures with anti-r-ovipain-2 antibodies, indicating an important functional role for ovipain-2 in the intra erythrocytic development cycle of this parasite. Finally, studies of the reactivity of sera from *B. ovis*-positive and negative sheep against r-ovipain-2 showed that this protease is expressed *in vivo*, and can be recognized by host antibodies. The results of this study suggest that ovipain-2 constitutes a potential target for immunotherapies and drug development against ovine babesiosis.

1. Introduction

Babesia ovis is the main causative agent of ovine piroplasmosis, a tick-borne disease affecting small ruminants in southern Europe (Mediterranean region), the Middle East and North Africa (Yeruham et al., 1998; Altay et al., 2007; Esmaeilnejad et al., 2014; Ranjbar-Bahadori et al., 2012; Ros-García et al., 2013; Rjeibi et al., 2014; Horta et al., 2014). Within sheep and goat erythrocytes, the *B. ovis* parasite reproduces asexually forming two pear-shaped merozoites. Sexual reproduction takes place in an Ixodid tick, with *Rhipicephalus bursa* and *R. turanicus* described as important vectors of *B. ovis* (Yeruham et al., 1998; Rjeibi et al., 2014). Phylogenetically, *B. ovis* belongs to the *sensu stricto Babesia* group, and is closely related to the cattle-infecting species *Babesia bovis* (Nagore et al., 2004; Schnittger et al., 2012).

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While *B. ovis* infections of young animals are not normally accompanied with clinical signs, primary exposure of adult sheep and goats to this parasite may lead to hemolytic anemia, hemoglobinuria, jaundice, fever, and is often fatal if untreated (Yeruham et al., 1998). Indeed, the deleterious effect of this parasite in naïve adult animals was highlighted in a recent report of a *B. ovis* outbreak with high mortality in a sheep herd that had been transferred from a tick-free region in Spain to a *R. bursa*-infested grazing region in the Basque country (Hurtado et al., 2015).

No vaccine against ovine babesiosis is available, so imidocarb dipropionate is normally used to control clinical signs (McHardy et al., 1986). Although efficacious, imidocarb is known to leave residues in sheep and goat milk (Belloli et al., 2006). Moreover, this drug was shown to be recombinogenic in *Aspergillus nidulans*, in a test that detects carcinogenic substances, highlighting the need for safer drugs in the treatment of ovine and other types of babesiosis (Santos et al., 2012).

Characterization of parasite molecules that act at the host–pathogen and/or vector–pathogen interface may lead to the development of novel therapeutic interventions. Molecules at this interface include papain-like cysteine proteases, which have been implicated in vital functions in various parasitic protozoa, including degradation of host proteins, stage differentiation, cell cycle progression, and host cell invasion and egress. Furthermore, they have been shown to modulate the host immune response, and are considered virulence factors for some parasitic protozoa (Klemba and Goldberg, 2002).

Falcipains, papain-like cysteine proteases of *P. falciparum* have been proposed as prominent antimalarial drug targets due to their specific features (Rosenthal, 2004, 2011; Dhawan et al., 2003; Teixeira et al., 2011; Marco and Coteron, 2012). Among them, the most abundant and best investigated are falcipain-2 and falcipain-2b, which are codified by almost identical and closely-located genes, and are responsible for most of the cysteine protease activity in the food vacuole, the lysosome-like structure of the intraerythrocytic parasite (Marco and Coteron, 2012). Additionally, these enzymes have been shown to cleave the erythrocyte cytoskeletal proteins 4.1 and/or ankyrin, in a process postulated to cause membrane instability and facilitate parasite release (Dhawan et al., 2003; Rosenthal, 2004, 2011).

Falcipain-2 homologs have been described in *B. bovis* and *B. bigemina*, and shown to be expressed by the intra-erythrocytic stage and also released into the erythrocyte cytoplasm, in a similar fashion to falcipain-2 (Dhawan et al., 2003; Mesplet et al., 2010; Martins et al., 2011, 2012). So far, the only indirect evidence of the relevance of these types of enzymes for the survival of *Babesia* spp. parasites came from the observation of a hampering effect on *B. bovis* erythrocyte invasion and *in vitro* replication by cysteine protease inhibitors (Okubo et al., 2007).

The present work describes the identification and characterization of a papain-like cysteine protease of *B. ovis* and shows that it plays a vital role in parasite growth *in vitro*, highlighting this molecule as an attractive target for the development of novel therapeutic agents against ovine babesiosis.

2. Materials and methods

2.1. Babesia ovis in vitro *cultures and DNA extraction*

B. ovis merozoites of the Israel and Portuguese strains were cultured within sheep erythrocytes maintained in 20% sheep serum-containing medium, in an atmosphere of 5% CO_2/2% O_2/93% N_2 at 37 °C, as described by Horta et al. (2014). Genomic DNA was isolated from a culture containing 3% infected erythrocytes using a standard phenol/chloroform method and stored at −20 °C until further use (Sambrook and Russell, 2006). The Israeli *B. ovis* strain inoculum was kindly provided by Dr. Varda Shkap (Kimron Veterinary Institute, Israel). The Portuguese strain was obtained from a *B. ovis*-infected sheep, as described by Horta et al. (2014).

2.2. *In silico identification of* B. ovis *cysteine proteases*

Using C1A family cysteine protease sequences of *Babesia bovis* (Genbank accession numbers XP_001612131, XP_001610695, XP_001609546, and XP_001608716; Mesplet et al., 2010), a BLASTp search was performed to identify corresponding sequences in the draft genome of *B. ovis*, Israel strain, currently being annotated at the University of Glasgow, UK. Four sequences belonging to the C1A cysteine protease family as determined by Pfam (Finn et al., 2014) were identified and have been deposited at GenBank under the following accession numbers: KR819159, KR819160, KR819161 and KR819162.

For phylogenetic analysis, a total of 24 C1A cysteine proteases from the published *B. bovis, Theileria annulata* and *T. parva* genomes were retrieved and compared with those identified in *B. ovis*. Amino acid sequences were aligned using MUSCLE (Edgar, 2004) and regions containing gaps, or missing data, eliminated. Based on the estimated evolutionary model (JTT+G) and shape parameter, a Neighbor-Joining tree was constructed (Saitou and Nei, 1987). A total of 146 positions were represented in the final dataset. The analysis was carried out using MEGA6 (Tamura et al., 2013).

2.3. *In silico characterization of B. ovis ovipain-2*

Signal peptide, transmembrane regions and topology were predicted by Phobius (http://phobius.sbc.su.se/), functional domains by Pfam (Finn et al., 2014); and N- and O-glycosylation sites by NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 (www.cbs.dtu.dk/services/NetOGlyc/), respectively. Secretion and subcellular localization were predicted by SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/) and Cello v2.5 Subcellular Localization Predictor (http://cello.life.nctu.edu.tw/), respectively.

Structural modeling of the mature forms of ovipain-2 and *B. bovis* bovipain-2 (XP_001610695) was carried out using the Swiss-Model server (swissmodel.expasy.org), based on the structure of *P. falciparum* falcipain-2b (XP_001347832; PDB: 2GHU, C chain), obtained by X-ray diffraction with a 3.10 Å resolution (Hogg et al., 2006). Alignments were performed by the method of Composition-based stats (Altschul et al., 1997) and the model visualized using PyMOL (pymol.org). The predicted spatial conformation was evaluated using Verify3D (nihserver.mbi.ucla.edu/Verify_3D). Percentage similarity and identity between related sequences were calculated with MATGAT (Campanella et al., 2003).

2.4. *Production of recombinant ovipain-2 (r-ovipain-2) and antisera*

The entire ovipain-2 open reading frame (*orf*) was PCR-amplified with primers oviPet-F (5′-CACCATGGAAATACCAACTGCCACT-3′) and oviPet-R (5′-GGAAGAAATGCTGGGTTTATATGG-3′), using the *B. ovis* Israeli strain DNA as template. The resulting amplicon of 1344 bp was cloned in pET 101/D TOPO CHAMPION vector (Invitrogen). Recombinant plasmids were amplified in TOP 10 *E. coli* cells and detection of positive clones carried out by colony PCR. Plasmids were purified from four positive clones using GeneJET Plasmid Miniprep Kit (ThermoScientific) and used to transform BL21 *E. coli* cells. Positive BL21 clones detected by colony PCR were induced to express the histidine (his)-tagged recombinant protein by exposure to 0.25 mM isopropyl-1-thio-β-D-galactoside (IPTG, Invitrogen) at 37 °C with shaking. Proteins were separated by SDS-PAGE, and either analyzed by Coomassie blue staining, or