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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

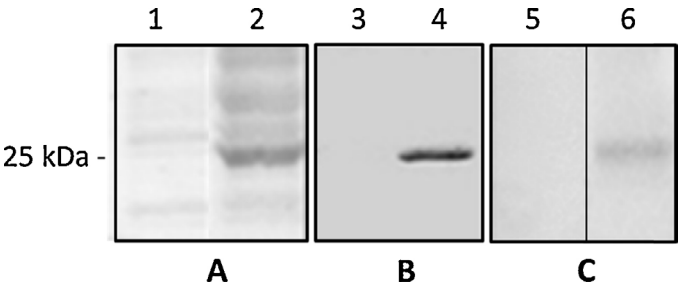

Fig. 1. Production of recombinant ovipain-2 (r-ovipain-2) and anti-r-ovipain-2 antibodies. (A) Coomassie blue-stained SDS-PAGE gel showing protein extracts of non-induced (1) and IPTG-induced (2) cultures of *E. coli* transformed with an expression vector containing *B. ovis* ovipain-2 gene complete *orf*. An expression band of 25 kDa was obtained. (B) Western blot with anti-histidine antibodies that recognized the histidine tag in r-ovipain-2 present in IPTG-induced *E. coli* cultures (4), while this band is not present in non-induced cultures (3). (C) Western blot showing the recognition of r-ovipain-2 by murine anti-r-ovipain-2 antibodies (6) and not by control murine serum (5).

transferred to nitrocellulose membranes. Blots were subsequently blocked with 3% skimmed milk in PBS-0.05% Tween-20 (PBST), and incubated with anti-histidine antibodies (Amersham), followed by alkaline phosphatase-conjugated anti-mouse IgG (Kirkegaard & Perry Laboratories) after washing with PBST. Bound Ab was then detected on washed blots by incubation with NBT-BCIP colorimetric substrate (Gibco) in the presence of 0.03% H_2O_2.

A 25 kDa expression band could be observed for all four IPTG-induced *E. coli* lysates in Coommassie Blue-stained SDS-PAGE gels, and was recognized by anti-his antibodies in Western blots (Fig. 1). Since the his-tag is added to the carboxyl terminus of the recombinant protein in the expression system used, the 25 kDa band corresponds to the ovipain-2 C-terminal region that harbors the active site. Accordingly, its molecular weight corresponds to the predicted size of the mature protease. The in-frame cloning of the complete *orf* was verified by sequencing (Macrogen, Korea), thus the observed band is indicative of proteolytic processing in the bacterial milieu.

For purification of r-ovipain-2, an induced bacterial lysate from one of the positive clones was suspended in PNB (50 mM K_2HPO_4/400 mM NaCl/100 mM KCl/10% Glycerol/10 mM Imidazole, pH 7.8), sonicated and centrifuged (14,500 × *g*, 20 min). The pellet was re-suspended in guanidine buffer (6 M guanidine-HCl/10 mM Tris–HCl/100 mM K_2HPO_4, pH 8), sonicated and centrifuged as above. The resulting supernatant was applied to a guanidine buffer-equilibrated Ni-agarose column (Invitrogen), followed by incubation at 4 °C for 30 min with shaking. The column was sequentially washed with guanidine buffer, urea buffer (8 M urea/100 mM K_2PO_4/10 mM Tris–HCl) adjusted to pH 8, urea buffer adjusted to pH 6.3, and PNB. Finally, the column bound protein was eluted with PNB containing increasing imidazol concentrations: with r-ovipain-2 starting to elute at 200 mM imidazol.

Protein concentration was estimated by the size of the 25 kDa band obtained by SDS-PAGE relative to different concentrations of bovine serum albumin standard (Pierce). Finally, r-ovipain-2 was dialyzed against distilled water, concentrated by lyophilization and re-suspended (1:1) in PBS in a final concentration of 0.4 mg/ml.

Aliquots of 100 μl (40 μg protein) of r-ovipain-2 were emulsified with 100 μl of incomplete Freund's adjuvant and sub-cutaneously injected into 3 three month-old male Balb/c mice at days 0, 15, and 30. At day 45, mice were bled and euthanized following procedures accepted by the Institutional Committee of Animal Care and Ethics (protocol no. 46/2012, CICUAE-CICVyA, INTA). The resulting antisera were separated by centrifugation, pooled and stored at −20 °C until use. A pool of the sera from three mice inoculated with adjuvant alone following identical procedures was used as a negative control. Recognition of the recombinant protein by the raised murine antibodies was verified by Western blot, using the detection system described below (Fig. 1).

2.5. Detection of ovipain-2 in B. ovis merozoites by immunoblot

A suspension of *B. ovis*-infected erythrocytes was pelleted, re-suspended in a solution of 0.5 M NaOH and incubated overnight at 4 °C. After addition of 1 mM pepstatin (Thermo Scientific) and protease inhibitor cocktail (EASYpack, Roche), the suspension was centrifuged, re-suspended in PBS/1 mM $CaCl_2$, pH 8, and layered on to an Easycoll (Gibco) discontinuous gradient (50–100%) in PBS. After centrifugation (500 × *g*, 30 min, 4 °C), merozoites concentrated in the upper portion of the gradient were collected, centrifuged at 15,700 × *g* and the resulting pellet lysed by freezing in liquid nitrogen. The lysate was re-suspended by vortexing in 10% trichloroacetic acid/acetone/60 mM dithiotreitol and incubated overnight at −20 °C. Precipitated proteins were collected by centrifugation at 16,000 × *g* for 10 min, washed with cold 80% acetone, dried and dissolved in sample buffer (7 M urea/2 M thiourea/4% (w/v) 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS detergent)/40 mM Tris base). A suspension containing an identical number of non-infected sheep erythrocytes was treated in the same way and used as a control. Aliquots containing 6 μg protein were electrophoresed using SDS-PAGE minigels. A lane with MagicMark™ XP Western protein standard (Invitrogen) was included in each gel. Proteins were transferred to Immobilon-P PVDF membrane (Millipore), which were then blocked with 1% Roche blocking reagent in PBS for 1 h, and incubated overnight at 4 °C with murine control or anti-r-ovipain-2 sera diluted 1:500 in 0.5% blocking buffer (0.5% Roche blocking reagent in 10 mM Tris–HCl/150 mM NaCl, pH 7.6 (TBS)). The membrane was then washed three times with TTBS (0.05% Tween 20 in TBS), and incubated with horse radish peroxidase (HRP)-conjugated anti-mouse IgG (R & D Systems) diluted 1:2000 in 0.5% blocking buffer for 2 h. After three washes, bound Ab was detected using Western Lightning Plus ECL substrate (PerkinElmer), and documented using Acquire Image equipment with Chemidoc software. The molecular weight (MW) of the detected band in *B. ovis* lysates was extrapolated from a plot of R_f (relative mobility) *vs* log MW of the marker proteins.

2.6. Detection of ovipain-2 in B. ovis merozoites by immunofluorescence

Aliquots of a *B. ovis in vitro* culture containing 5% infected sheep erythrocytes or non-infected sheep erythrocytes were centrifuged, re-suspended in PBS/fetal calf serum (1:1, v/v) and smeared on to immunofluorescence slides that, upon drying, were stored at −80 °C until use. Before use, slides were thawed for 20 min at room temperature, fixed with cold methanol for 10 min and washed with PBS for 5 min. Slides were then blocked with 1% BSA in PBS for 1 h at room temperature, with shaking; different wells were then incubated with PBS, or 1:50 dilutions of control mouse serum or anti-r-ovipain-2 mouse serum for 30 min at 37 °C in a humid chamber. This particular serum concentration was found to be optimal in preliminary experiments where serial dilutions (1:25 to 1:400, v/v) were tested. Slides were washed twice with PBS/0.05% Tween-20 for 5 min and then with double distilled water, and dried. A secondary goat anti-mouse IgG antibody, conjugated to fluorescein isothiocyanate (Sigma), was then applied to the slides at a 1:500 dilution and incubated in the dark for 30 min. Finally, slides were washed as before, dried and cover slips mounted with glycerol/PBS (1:1, v/v). Fluorescence was observed at 1000× magnification with a Zeiss AxioImager Upright Microscope and images obtained using Axiocam MRmAxioVisionRel 4.8.2 software.

2.7. Seroneutralization assay

The assay was carried out in 96-well culture plates in a final volume of 250 μl. First, 210 μl aliquots of complete medium containing 5 μl complement-inactivated mouse serum (control or anti-r-ovipain-2) or an equal volume of PBS were added to wells in triplicate, and the plate incubated at 37 °C for 1 h in a 5% CO_2 atmosphere. Then, each well received a 40 μl aliquot of a *B. ovis* (Portugal strain)-infected erythrocyte suspension in culture medium. Final concentrations in each well were 10% (v/v) erythrocytes, an initial approximate percentage of *B. ovis* infected erythrocytes of 0.5% (v/v) and 2% (v/v) mouse serum. Plates were incubated for four days in a low O_2 atmosphere as described before (Horta et al., 2014). A 100 μl aliquot of the culture supernatant was removed from each well on a daily basis and the same amount of fresh medium containing 2 μl of either mouse serum or PBS was added. At 0, 24, 48, 72 and 96 h of culture, 2 μl packed erythrocytes were collected from the bottom of each well and smeared onto glass slides, which were then Giemsa-stained and microscopically analyzed. Percentages of *B. ovis*-infected erythrocytes were calculated after examining 5000 erythrocytes per slide. The statistical significance of the differences in average values was calculated using Student's *t* test.

2.8. Recognition of r-ovipain-2 by serum from B. ovis-infected sheep

Whole blood and serum samples were obtained from 36 adult sheep from *R. bursa*-infested regions of Portugal. Samples were stored at −20 °C until use. DNA was extracted from whole blood samples and used as template to diagnose the presence of *B. ovis* DNA by semi-nested PCR, as described by Horta et al. (2014).

Ten μl aliquots of r-ovipain-2 (0.26 mg/ml) were separated by SDS-PAGE in preparative 4-12% Nupage BisTris Precast Gels (Invitrogen). Proteins were then transferred to Immobilon P membranes, which were cut into strips. Strips were blocked with 1% blocking buffer (as above) for 30 min at room temperature, and then separately incubated overnight at 4 °C with different serum samples, diluted 1:500 in 0.5% blocking buffer. As a positive control, one of the strips was incubated with anti-his serum and processed as described before. After two washes with TTBS, strips were incubated with HRP-conjugated anti-sheep IgG (R & D Systems) diluted 1:2000 in 0.5% blocking buffer, at room temperature for 120 min. After two washes as above, and one wash with TBS, reactions were detected by chemiluminescence. Separate lanes were used for SeeBlue® plus 2 pre-stained protein standard (Invitrogen) and MagicMark™ XP Western protein standard.

3. Results and discussion

3.1. The Babesia ovis genome contains four predicted C1A cysteine protease genes.

Due to their likely relevance in host/pathogen relationships, papain-like cysteine proteases were investigated in the hemoparasite *Babesia ovis*. A total of four C1A cysteine protease peptide sequences were retrieved from the draft *B. ovis* genome assembly using an exhaustive search strategy. The phylogenetic relationship of *B. ovis* sequences with C1A cysteine proteases of *B. bovis* and the related piroplasmids *Theileria annulata* and *T. parva* was determined by Neighbor-Joining analysis (Fig. 2). Each *B. ovis* protease sequence grouped in one of the four ortholog groups generated by the analysis. As described in previous work (Mesplet et al., 2010), and consistent with the results of Martins et al. (2011), ortholog groups 1, 3 and 4 consisted of one sequence for each parasite, while ortholog group 2 contained one protease each from *B. ovis* and *B. bovis*, but 6 and 7 sequences from *T. parva* and *T. annulata*, respectively. Previous synteny studies between *B. bovis*, *T. annulata* and *T. parva* showed that several gene duplication events took place within the ancestral gene locus in *Theileria* parasites, probably associated with their more complex life-cycle as compared to *Babesia* species (Mesplet et al., 2010; Martins et al., 2011).

In addition to the phylogenetic analysis demonstrating that the *B. ovis* sequence BoCP_ort2 (KR819159) is the ortholog of *B. bovis* bovipain-2 (XP_001610695; Mesplet et al., 2010), a Reciprocal Best hits test verified that it exhibits an orthologous relationship to *P. falciparum* falcipain-2b (XP_001347832). BoCP_ort2 was thus named "ovipain-2". BLASTp searches in the *P. falciparum* genome for the other three C1A cysteine proteases yielded maximum scores for falcipain-2b (BoCP_ort1), and a pre-procathepsin c precursor (XP_001350862; BoCP_ort3 and BoCP_ort4).

Since falcipain-2b has been shown to be involved in essential mechanisms at the host–pathogen interface (Rosenthal, 2011), this study focused on its ortholog in *B. ovis*, ovipain-2, for further characterization.

3.2. B. ovis ovipain-2 has a similar predicted structure to falcipain-2 and bovipain-2

In silico analysis revealed that ovipain-2 has the typical preproprotein conformation of C1A cysteine proteases, as illustrated in Fig. 3. It contains an internal inhibitor domain which, when cleaved, releases the mature active enzyme harboring the catalytic site, of a predicted approximate size of 25 kDa. The active enzyme contains three thiol protease segments, where residues Q(250), C(256), H(386),and N(408), necessary for the conformation of the catalytic pocket, are present. This active site is the most conserved region between ovipain-2 and homologous cysteine proteases of other apicomplexans (Fig. 3). With the exception of a short 42 aa cytosolic N-terminal region, followed by a 20 aa transmembrane region (aa 43–63), the remainder of ovipain-2 is mainly hydrophilic. The first 42 amino acids are predicted to be cytosolic, while aa 64–448 are predicted as oriented either to the extracellular milieu or to the lumen of membranous vesicles. Signal peptide predictions gave negative results.

Three dimensional (3D) homology modeling of ovipain-2 and bovipain-2 mature enzymes was carried out using the X-ray-determined 3D structure of falcipain-2b as a template (Fig. 4). The identity of the mature ovipain-2 and bovipain-2 enzymes compared to falcipain-2 was found to be 40.5 and 39.8%, respectively. The three enzymes are composed of 6–8 alpha helices interspersed by 9–12 beta sheets. Even though their secondary structural elements are different in number, they display a highly similar overall structure of two hemispheres between which a catalytic pocket is formed. The four catalytic amino acids are also positioned in a similar fashion in the three proteins, as can be observed in Fig. 4D, E and F. These results strongly suggest that bovipain-2 and ovipain-2 may be susceptible to the same inhibitors as falcipain-2, an observation that is highly relevant for the selection or design of new and safer drugs against bovine and ovine piroplasmosis. Recently, 3D comparative modeling of babesipain, the bovipain-2 ortholog in *B. bigemina*, that included the docked binding of the cysteine protease inhibitors HEDICINs and HECINs, was carried out (Pérez et al., 2013). The results indicated that these drugs might effectively block babesipain activity and thus are candidates for therapeutic use against *B. bigemina*. Given the high sequence similarity between ovipain-2 and babesipain-2 (67/47% similarity/identity), these drugs may be able to inhibit ovipain-2 activity and should be tested in their efficiency against ovine piroplasmosis in further research.

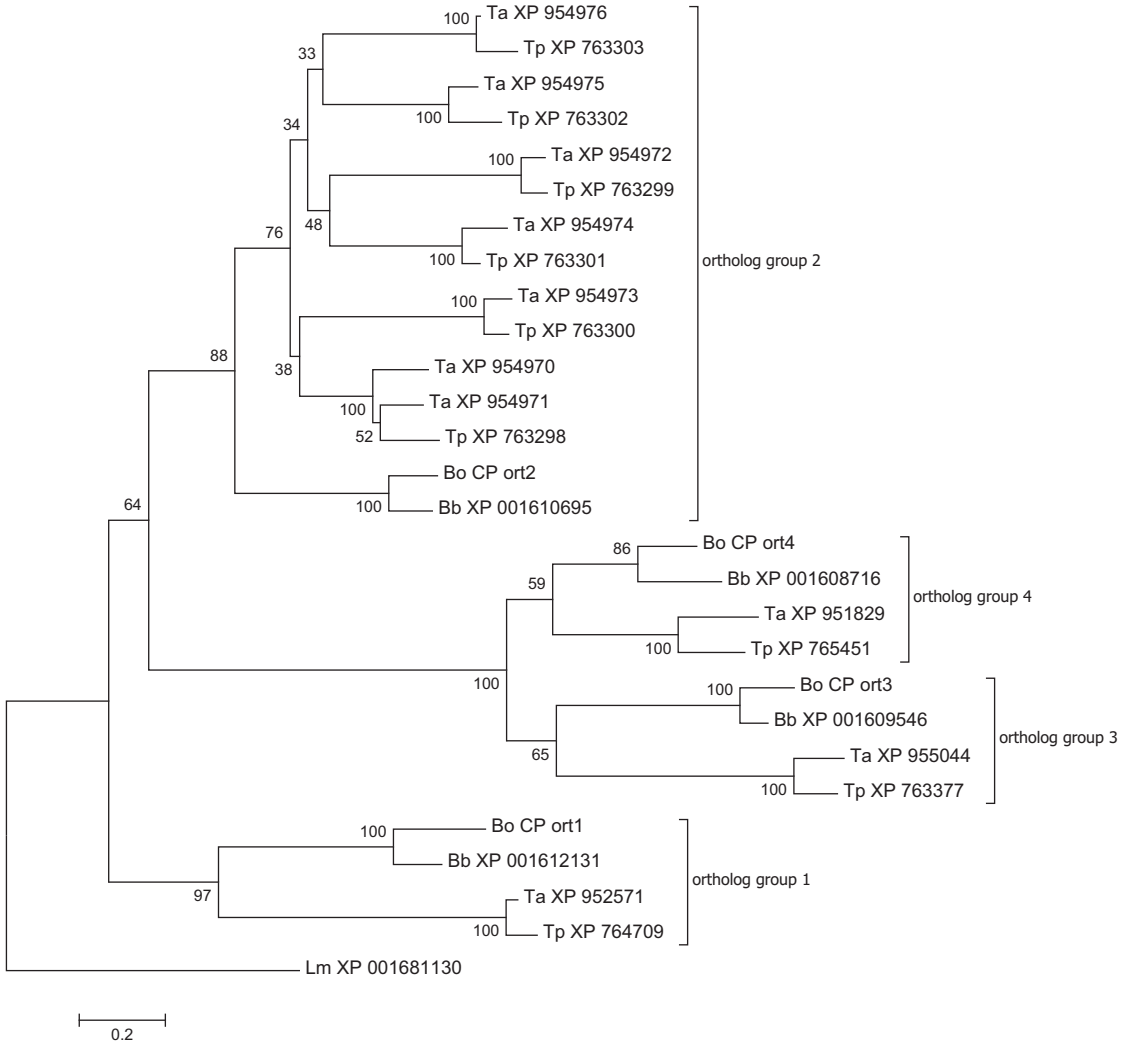

Fig. 2. Phylogenetic relationship of C1A cysteine proteases of *Babesia bovis*, *B. ovis*, *Theileria annulata* and *T. parva* as inferred by Neighbor-Joining. The Leishmania major sequence XP_001681130 was used as out-group. Percentage of bootstrap values inferred after1000 replicates are shown next to the branches. The evolutionary distances are expressed in the units of the number of amino acid substitutions per site. *B. ovis* cysteine proteases labeled as BoCP ort1–4 correspond to the sequences deposited in Genbank with accession numbers KR819160, KR819159, KR819161 and KR819162, respectively.

3.3. *Ovipain-2 is expressed in* B. ovis *merozoites and secreted to the erythrocyte cytoplasm.*

Expression of ovipain-2 in *B. ovis* merozoites was analyzed by immunoblotting and immunofluorescence. Murine antibodies against a recombinant form of ovipain-2 recognized a 70 kDa band in *B. ovis*-infected erythrocyte lysates not present in non-infected erythrocyte preparations, demonstrating its expression in the intra-erythrocytic parasite stage (Fig. 5). The discrepancy between the expected molecular weight of 50.4 kDa and the

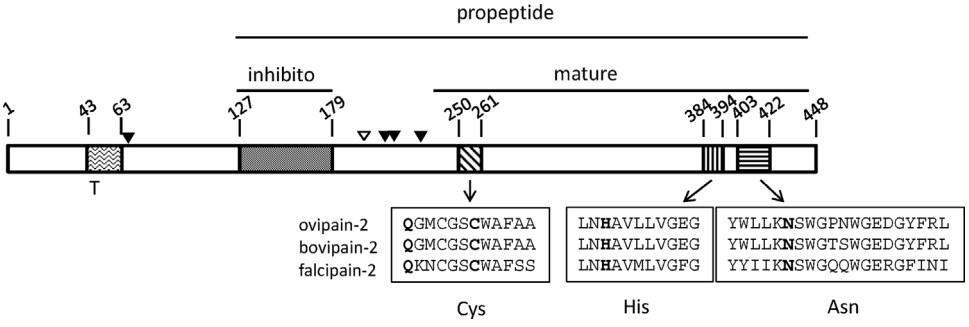

Fig. 3. Schematic representation of *B. ovis* ovipain-2 polypeptide and conserved domains. The transmembrane (TM) region, cysteine protease inhibitor domain and eukaryote thiol (cysteine) proteases cysteine (amino acids (aa) 250–261), histidine (aa 384–394) and asparagine (aa 403–422) catalytic regions are shown to scale. Sequence alignments of these three regions in ovipain-2, bovipain-2 and falcipain-2 are shown in boxes, with active site determinants Q, C, H and N in bold. The peptide segment from aa 1 to 42 was predicted to be cytoplasmic, and the segment from aa 64 to 448, extracellular. The position of putative N and O-glycosylation sites are marked with white and black inverted triangles, respectively.

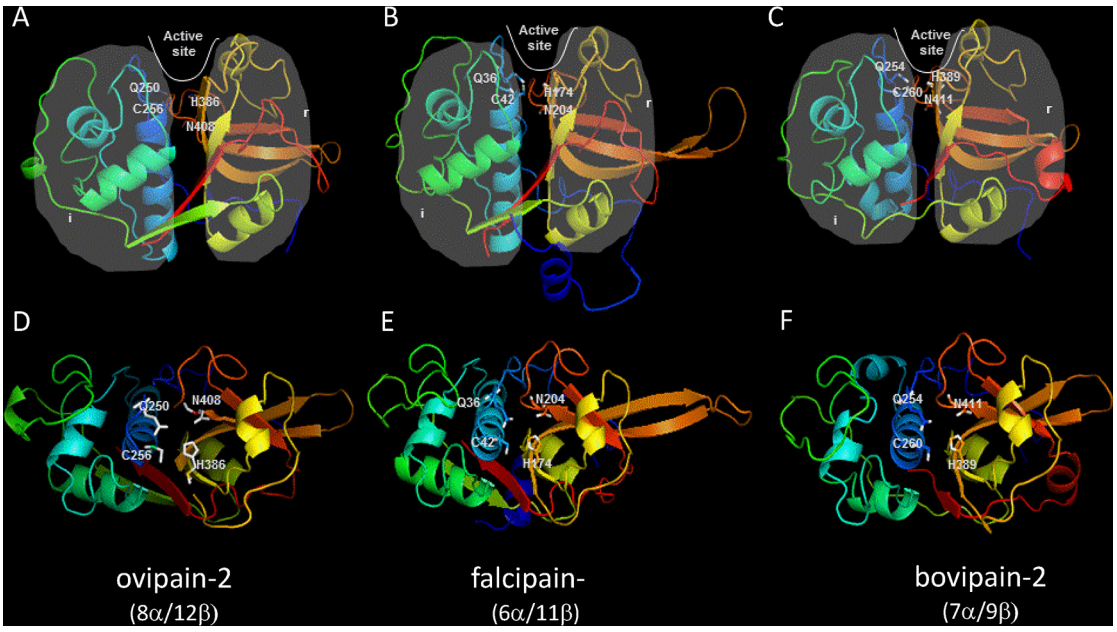

Fig. 4. Representation in ribbon format of the predicted 3D structure of ovipain-2, falcipain-2 and bovipain-2 mature peptidases. Ovipain-2 (A, D) and bovipain-2 (C, F) structures were obtained by homology modeling using falcipain-2b (B, E) as a template. The residues corresponding to the catalytic sites (ovipain-2: Q250, C256, H386, N408; bovipain-2: Q254, C260, H389, N411 and falcipain-2b: Q36, C42, H174, N204; falcipain-2b residue numbers correspond to the mature enzyme) are indicated and represented with sticks. The color spectrum varies according to the sequence, from dark blue in the N-terminal to dark red in the C-terminal. A, B and C: lateral view where the right (r) and left (l) domains and the active site are indicated; D, E and F: upper (dorsal) view showing that the location of the catalytic amino acids is very similar in the three structures. α/β: number of alpha helices and beta-sheets in each protein.

observed 70 kDa band may be due to post-translational modifications of the native protein. One N-glycosylation site and four O-GlcNAcylation sites were predicted in ovipain-2 (Fig. 3). N-glycosylation of proteins has been demonstrated in several apicomplexan parasites, including *B. bovis* (Rodriguez et al., 2012). In contrast, the presence of O-glycosylation in apicomplexan proteins has remained controversial (Macedo et al., 2010). However, recent studies have shown direct evidence of O-GlcNAcylation in *Toxoplasma gondii* and have strongly suggested that this modification of polypeptides also occurs in *P. falciparum* (Perez-Cervera et al., 2011). Examination of O-glycosylation in *Babesia* parasites remains pending. Besides the possible modifications due to N and/or O-glycosylation, other post-translational modifications may explain the observed molecular weight difference. Another possibility to explain this electrophoretic behavior could be association to cell lipids that could not be broken by the lysis buffers and protein extraction conditions employed, since the observed band corresponds to the membrane-bound form of the cysteine protease. The 25 kDa band corresponding to the mature protease was not detected in immunoblots, likely because the protein extraction method employed concentrated membrane-bound proteins while soluble ones are discarded during the process.

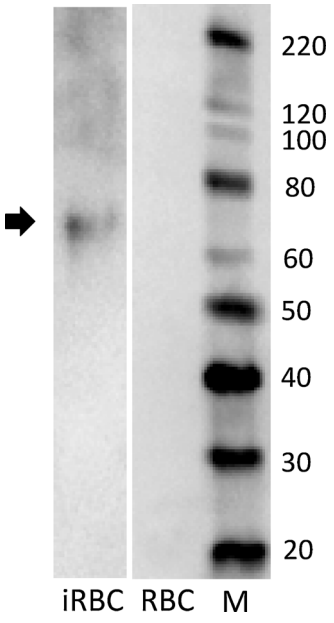

Fig. 5. Immunoblot analysis of ovipain-2 in protein extracts of *B. ovis* merozoite infected erythrocytes. Extracts of *B. ovis* (Israeli) strain-infected ovine erythrocytes (iRBC) and non-infected ovine erythrocytes (RBC) were electrophoresed on SDS-PAGE, blotted and exposed to anti-r-ovipain-2 murine antibodies. Antibody binding was detected by peroxidase-labeled anti-mouse IgG and chemiluminescence (arrow). M: MW marker. No reactivity was observed when control murine serum was used (not shown).

Indirect immunofluorescence confirmed the expression of ovipain-2 in *B. ovis* merozoites. As observed in Fig. 6, a fluorescence signal was observed inside intra-erythrocytic *B. ovis* merozoites with anti-ovipain-2 serum, but not with non-immune serum. Additionally, fluorescence was detected in the cytoplasm of *B. ovis*-infected erythrocytes, indicating a level of secretion of ovipain-2 by the parasite. Accordingly, ovipain-2 is predicted to be a "non-classically secreted protein", which corresponds to secreted proteins that do not have a signal peptide.

Importantly, this type of localization coincides to previous observations in *B. bovis*, *B. bigemina* and *P. falciparum* in which orthologous cysteine-proteases were also found associated to the parasite and to the erythrocyte cytoplasm (Dhawan et al., 2003; Mesplet et al., 2010), and is consistent with the postulation that *Babesia* spp. papain-like cysteine proteases perform the dual role of hemoglobin digestion and cleavage of erythrocyte cytoskeletal proteins to facilitate parasite egress, as proposed for *P. falciparum* (Dhawan et al., 2003).

Within the parasite, falcipain-2 is located in the food or digestive vacuole, which is a lysosome-like structure (Rosenthal, 2011).

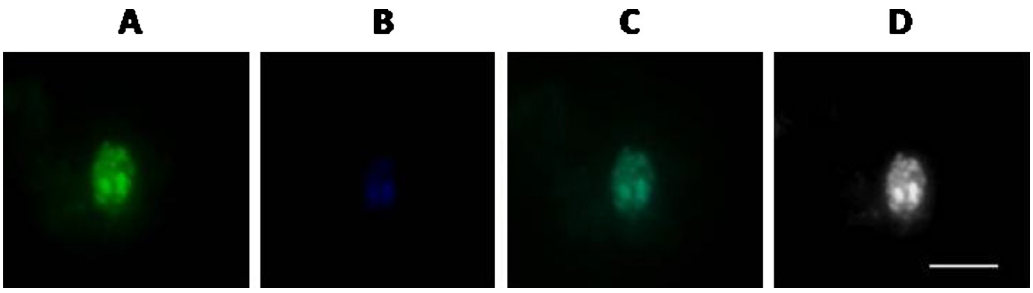

Fig. 6. Localization of ovipain-2 in *B. ovis*-infected erythrocytes. Smears of *B. ovis* (Portuguese strain)-infected ovine erythrocytes were incubated with DAPI and murine anti-r-ovipain-2 antibodies; followed by detection with FITC-labeled anti-murine IgG and observation by epifluorescence (1000× magnification), with filters to detect FITC (A) and DAPI (B). C and D show an overlay image of A and B, and the corresponding phase contrast micrograph, respectively. No fluorescence was detected when control murine serum or non-infected ovine erythrocytes were used (not shown).

Early studies indicate the presence of this type of structure in *Babesia rodhaini* and, indeed, the results obtained with ovipain-2 and homologous proteins reinforce the notion of the existence of such organelle in other *Babesia* parasites (Rudzinska and Trager, 1962). Thus, subcellular localization algorithms applied to ovipain-2 gave high scores to both lysosomal and extracellular locations. On the other hand, cysteine protease activity measurements carried out with recombinant babesipain-1, the bovipain-2 ortholog in *B. bigemina* showed an optimum at acid pH, indicating a lysosomal location, while a high level of enzymatic activity was still present at neutral pH suggesting an active role for this enzyme in the cytosol (Martins et al., 2012). Noteworthy, several acid hydrolases, including proteases, are known to be present in three locations in different free-living and parasitic protozoa: within lysosomes, secreted to the surroundings, and attached to the cell surface, and have been proposed to be involved in nutrition, immune escape and pathogenicity (Florin-Christensen et al., 1989). The results of this investigation indicate that ovipain-2 likely follows this location pattern.

3.4. *Anti-ovipain-2 antibodies significantly hamper the* in vitro *growth of* B. ovis *merozoites*

To study the biological importance of ovipain-2 during asexual multiplication of *B. ovis* merozoites, a neutralization assay was set up in infected sheep erythrocytes *in vitro*. Murine antibodies against r-ovipain-2 or normal control serum were added to *B. ovis* *in vitro* cultures and the percentage of parasitized erythrocytes (% IE) was recorded at 0, 48, 72 and 96 h. As can be observed in Fig. 7, anti-ovipain-2 hyper-immune murine serum (2%, v/v) significantly impeded merozoite growth after 72 and 96 h of exposure ($p < 0.05$ and $p < 0.001$, respectively) compared to control sera.

Babesia spp. are obligate intra-erythrocytic parasites in the vertebrate host and after erythrocyte egress, they use gliding motility to invade uninfected erythrocytes and continue asexual replication (Asada et al., 2012). Anti-ovipain-2 antibodies likely bind to the membrane-bound, surface-exposed form of ovipain-2 of free merozoites, and the strong observed neutralization effect might suggest a role of this protein in invasion, in addition to nutrition and parasite egress. Similarly, anti-bovipain-2 antibodies significantly hampered invasion and/or *in vitro* growth of *B. bovis* merozoites (Mesplet, M., unpublished results).

The experiments with *B. ovis* cultures show recognition of merozoite proteins of the Portuguese strain by antibodies raised against r-ovipain-2 generated from the Israeli strain gene sequence, indicating conservation of surface-exposed neutralization-sensitive B-cell epitopes among geographically distant *B. ovis* variants. These features underscore the potential usefulness of ovipain-2 in the development of subunit vaccines against ovine babesiosis since the presence of conserved and surface-exposed neutralization-sensitive B-cell epitopes has been widely perceived as an indication that an antigen is a vaccine candidate (Florin-Christensen et al., 2014).

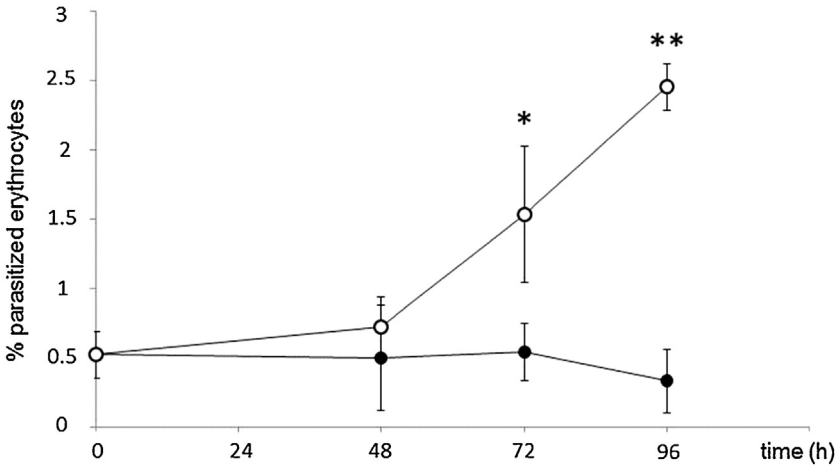

Fig. 7. Effect of anti-r-ovipain-2 antibodies on *in vitro* growth of *B. ovis*. Cultures of *B. ovis* (Portuguese strain; 0.5% infected erythrocytes) were incubated with 2% (v/v) control mouse serum (open circles) or 2% anti-r-ovipain-2 mouse serum (closed circles). The percentage of parasitized erythrocytes was determined at each sampling time by microscopic observation of Giemsa-stained smears. Means ± SD of triplicate parallel assays are shown. Statistical significances between averages of control and anti-ovipain-2 treatments at each time point are indicated by asterisks (*$p < 0.05$; **0.001).

Table 1
Detection of anti-ovipain-2 antibodies in sera of sheep with natural *B. ovis*-infection. The reaction of sera (1:500 dilution) of sheep from a *R. bursa*-infested Portuguese region against r-ovipain-2 was tested by immunoblotting. Sera were considered positive when a 25 kDa band was detected. Validation of this band as r-ovipain-2 was obtained by reaction with anti-his antibodies in a parallel blot. Molecular diagnosis of *B. ovis* was carried out by semi-nested PCR in DNA extracted from blood of the same animals.

Blood		Sera		
		Immunoblot		Total
		–	+	
PCR	–	22	0	22
	+	10	4	14
Total		32	4	36

3.5. *Ovipain-2 is expressed in* B. ovis*-infected sheep*

Finally, we analyzed expression and immunogenicity of ovipain-2 in natural *B. ovis* infections of sheep. To do this, 36 samples of blood and serum were collected from sheep herds in *Ripicephalus bursa*-infested Portugal regions. Infection by *B. ovis* was detected by an established semi-nested PCR test (Horta et al., 2014), and immunoblot reactivity of sera antibodies tested using r-ovipain-2. As shown in Table 1, sera from 4 of 14 *B. ovis*-infected sheep (28%) recognized a 25 kDa band that corresponded to r-ovipain-2 (Fig. 1), while none of the serum samples from non-infected animals showed any reaction with the recombinant protein. Recognition of r-ovipain-2 by the sera of four *B. ovis*-infected sheep confirmed that this protein is expressed during natural infections by *B. ovis*, and is exposed to the ovine immune system, eliciting a humoral response.

4. Conclusions

Our results show that ovipain-2, a papain-like cysteine protease of the tick-transmitted hemoparasite *B. ovis*, is expressed both *in vitro* and *in vivo* in the intra-erythrocytic stage of the life cycle, is released to the host erythrocyte cytoplasm, elicits a humoral response during infection of sheep and contains neutralization-sensitive B-cell epitopes. Ovipain-2 appears to represent an attractive target for drug and/or vaccine development against ovine babesiosis and consequently further studies are required to investigate these possibilities.

Acknowledgments

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