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Kinetic characterization of a novel cysteine peptidase from the protozoan *Babesia bovis*, a potential target for drug design

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1 **Kinetic characterization of a novel cysteine peptidase from the protozoan *Babesia*
2 *bovis*, a potential target for drug design**

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28 **Abstract**

29 C1A cysteine peptidases have been shown to play an important role during
30 apicomplexan invasion and egress of host red blood cells (RBCs) and therefore have
31 been exploited as targets for drug development, in which peptidase specificity is
32 deterministic. *Babesia bovis* genome is currently available and from the 17 putative
33 cysteine peptidases annotated four belong to the C1A subfamily. In this study, we
34 describe the biochemical characterization of a C1A cysteine peptidase, named here
35 BbCp (*B. bovis* cysteine peptidase) and evaluate its possible participation in the
36 parasite asexual cycle in host RBCs. The recombinant protein was obtained in bacterial
37 inclusion bodies and after a refolding process, presented typical kinetic features of the
38 cysteine peptidase family, enhanced activity in the presence of a reducing agent,
39 optimum pH between 6.5 – 7.0 and was inhibited by cystatins from *R. microplus*.
40 Moreover, rBbCp substrate specificity evaluation using a peptide phage display library
41 showed a preference for Val > Leu > Phe. Finally, antibodies anti-rBbCp were able to
42 interfere with *B. bovis* growth *in vitro*, which highlights the BbCp as a potential target for
43 drug design.

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45 **1. Introduction**

46 The *Babesia* genus comprises a group of hemoparasites transmitted by ticks [1]
47 and since *Babesia* vectors are distributed worldwide, these piroplasmids are commonly
48 found in mammals' bloodstream including, but not limited to, humans, horses, sheep,
49 dogs, cats and cattle [2, 3]. Bovine babesiosis caused by *B. bovis* induces severe fever,
50 hemolytic anemia, hemoglobinuria, and hypotensive shock leading to high mortality
51 rates in susceptible cattle [4]. Since more than half of the world cattle population is
52 considered to be at risk of babesiosis infections [5], the *Rhipicephalus microplus* - *B.*
53 *bovis* complex represents an important association from the economical perspective.

54 Babesiosis control is currently based on three different strategies, (i) vector
55 control, (ii) cattle immunization and (iii) anti-*Babesia* drugs [6, 7]. Vector control is
56 mainly carried out by acaricides, although tick strains resistant to different compounds
57 has already been described [8, 9]. Cattle immunization has been proposed for both
58 vector and *Babesia* sp. control. Tick immunological control studies resulted in the
59 development of two commercially available vaccines, although the levels of protection
60 vary largely between different regions [10-12]. Cattle immunization against *B. bovis* has
61 been mainly carried out with live vaccines using attenuated parasites [13, 14]; although
62 the use of recombinant vaccines based on *B. bovis* proteins has been proposed and is
63 a subject of active investigation [15, 16]. Currently the most commonly used drug to
64 treat *Babesia* infections is imidocarb [17], although several other drugs are in the
65 development stage [6, 18].

66 In apicomplexan parasites, cysteine peptidases from the C1A subfamily have
67 been identified and shown to be crucial for parasite survival and proliferation [19-22].
68 Therefore, it was proposed that such enzymes could be explored as targets for drug
69 development, in which enzyme specificity plays an important role [23-25]. Notably, *B.*
70 *bovis* treatment with cysteine peptidase inhibitors reduces parasite growth [26], showing
71 the contribution of cysteine peptidases for *Babesia* sp. life cycle. So far, a handful of
72 C1A cysteine peptidases have been characterized [27-29]. One of them, ovipain-2 from
73 *B. ovis*, displays a high degree of conservation with *P. falciparum* falcipain-2; ovipain-2
74 was found in merozoite stages and appears to be secreted into the erythrocyte

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75 cytoplasm, moreover, treatment of *B. ovis in vitro* cultures with anti-ovipain-2 antibodies
76 resulted in a significant decrease of parasite proliferation [30], indicating the
77 participation of ovipain-2 in the process of invasion of or egress from host red blood
78 cells (RBCs). Similarly, bovipain-2, the falcipain-2 ortholog from *B. bovis*, was also
79 found in merozoite stages scattered in the erythrocyte cytoplasm, suggesting its role
80 during parasite egress [31]. Although these studies reinforce the role of cysteine
81 peptidases for *Babesia* survival there is no information regarding their proteolytic
82 specificity. So far only the BbiCPL1 from *B. bigemina* has been biochemically
83 characterized [29], in which a preference for Val > Leu > Phe at the P2 position was
84 found.

85 *B. bovis* genome is available [32] and 17 putative cysteine peptidases are
86 currently annotated, from which four belong to the C1A subfamily [33]. Since C1A
87 cysteine peptidases play an important role in apicomplexan life cycle and represent
88 potential targets for anti-parasitic drug development, we present here the biochemical
89 characterization of a putative C1A cysteine peptidase from *B. bovis* and its possible role
90 during RBC invasion.

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92 **2. Materials and methods**

93 **2.1. Bioinformatic analysis:** The complete mRNA sequence of XP_001612131 was
94 obtained from the *B. bovis* T2Bo strain deposited genome (http://protists.ensembl.org/).
95 Domain analysis was conducted using PFAM (https://pfam.xfam.org/) [34] and detection
96 of putative signal peptides was carried out with SignalP 5.0 [35]. The theoretical
97 molecular weight and isoelectric point were estimated using the Compute pI/MW tool
98 [36] and the topology analysis was carried out with TMHMM V.2.0
99 (http://www.cbs.dtu.dk/services/TMHMM/). An amino acid alignment was performed with
100 Clustal Omega [37] and edited with BioEdit software [38].

101 **2.2. Ticks and *B. bovis* parasites:** Cattle RBCs infected with attenuated *B. bovis* IPV2
102 strain were supplied by Dr. Itabajara da Silva Vaz Junior from Faculdade de Medicina
103 Veterinária from Universidade Federal do Rio Grande do Sul. RBCs were used for total
104 RNA extraction using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's
105 instructions, followed by cDNA synthesis using the Improm-II Reverse Transcription
106 System kit (Promega, WI, USA) following the manufacturer`s recommendations.

107 **2.3. Amplification and cloning of BbCp:** Specific oligonucleotides containing the
108 restriction sites for *Xho*I and *Bpu*1102 were designed (Supp. Table 1) based on the
109 genomic sequence XP_001612131. The putative *B. bovis* cysteine peptidase (BbCp)
110 was amplified from the cDNA preparation obtained in 2.1. Reactions were performed in
111 50 µL containing 1 µL of cDNA preparation, 25 pmol of each primer, 100 µM dNTPs, 1.5
112 mM $MgCl_2$ and 5 U Platinum Taq DNA polymerase High Fidelity (Invitrogen, CA, US)
113 and submitted to: 94ºC – 10 min, 25 cycles of 94ºC – 30s, 55ºC – 45 s, 68ºC – 2 min
114 and 68ºC – 10 min. PCR products were observed in a 1% agarose gel and purified
115 using QIAEXII extraction kit (QIAGEN, Hilden, DE). A purified BbCp amplicon and
116 pET14b were digested overnight with *Xho*I (Fermentas, Vilnius, LT) and *Bpu*1102
117 (Fermentas, Vilnius, LT) at 37°C, purified with QIAEXII (QIAGEN, Hilden, DE) and
118 subjected to ligation with T4 ligase (Promega, WI, USA) overnight at 16°C. Finally, the
119 ligation product was used in the transformation of *Escherichia coli* DH5α and positive
120 clones were confirmed by colony PCR using T7 promoter and terminator primers.
121 Clones were sequenced using a Big Dye Terminator cycle sequencing kit (Applied

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122 Biosystems, Warrington, UK) in an ABI Prism 3130 automated sequencer (Applied
123 Biosystems, Warrington, UK).

124 **2.4. Expression, purification and refolding of recombinant BbCp (rBbCp):**
125 Expression of rBbCp was carried out in *E. coli* BL21 pLysS strain. Bacteria were grown
126 until OD_{550} = 0.6 – 0.8, then induced with 1 mM of IPTG for 6 h at 37ºC. The culture
127 medium was centrifuged (10 min, 3,000 x g at 4ºC), the supernatant discarded, the cells
128 suspended in 50 mM Tris-HCl pH 8.0 and lysed with 2 cycles using a *French press*
129 system. After lysis, the sample was centrifuged (20 min, 12,000 x g, 4°C), supernatant
130 collected and the pellet washed with 50 mM Tris-HCl pH 8.0 containing urea (2, 4, 6,
131 and 8.0 M). Protein purification was carried out in two steps; an affinity chromatography
132 with Ni-NTA resin, followed by ionic exchange chromatography with a HiTrap Q resin.
133 After purification, rBbCp was submitted to refolding as described [39].

134 **2.5. Production of anti-rBbCp antibodies:** Purified rBbCp (30 µg) in PBS was mixed
135 1:1 with incomplete Freund's adjuvant (Sigma) to a final volume of 200 µL. The
136 suspension was injected subcutaneously in 3 male mice (BALB/c). Two boosters were
137 applied with 15 day-intervals before the final bleeding. Control animals were inoculated
138 with a PBS:adjuvant preparation. Blood samples were incubated at 37°C for 60 min,
139 centrifuged (20 min, 800 x g at 4°C) and sera collected and stored at -20°C. The
140 protocols used for obtaining murine sera were approved by the Institutional Committee
141 for the use and care of experimental animals (CICUAE, CICVyA, INTA, Argentina)
142 under protocol number 52/2016.

143 **2.6. Characterization of rBbCp proteolytic activity:** Purified rBbCp was pre-
144 incubated with different concentrations of dithiothreitol (DTT, 0 – 6 mM) in 50 mM
145 sodium acetate pH 5.5 in the presence of Z-FR-AMC (4 mM) or Z-RR-AMC (4 mM).
146 Determination of rBbCp optimum pH was carried out in 75 mM Tris, 25 mM glycine, 25
147 mM MES and 25 mM acetic acid with pH varying from 3 – 8 in the presence of 1 mM
148 DTT with Z-FR-AMC (4 mM). For the inhibition assays, rBbCp was activated in 75 mM
149 Tris-HCl, 25 mM glycine, 25 mM MES and 25 mM acetic acid pH 6.5 with DTT (1 mM)
150 for 10 min at 37°C, followed by the pre-incubation with 0.25 µM of E64 (Sigma,

151 Steinheim, DE), APMSF (Sigma, Steinheim, Germany) or EDTA (Fermentas, Vilnius,
152 LT) for 10 min at 37°C, followed by addition of Z-FR-AMC substrate (4 mM). Proteolytic
153 activity was monitored by fluorescence (380 nm excitation/ 460 nm emission) for 15 min
154 at 37°C. The initial rate was determined by linear regression of fluorescence (RFU) per
155 time (min) and the residual activity by the ratio between the proteolytic activity in the
156 presence of the inhibitor and the control reaction (without inhibitor).

157 **2.7. Determination of inhibition constants (Ki) of *R. microplus* cystatins for**
158 **rBbCp:** *R. microplus* cystatins 1 [40], 3 [41] and 4 [42] were expressed, purified and
159 titrated with commercial papain previously titrated with E64 - trans-Epoxysuccinyl-L-
160 leucylamido(4-guanidino)butane. The determination of the inhibition constants for rBbCp
161 was carried out in 25 mM MES, 25 mM glycine, 25 mM acetic acid and 75 mM Tris-HCl
162 pH 6.5, at 37ºC. The rBbCp was pre-incubated with 1mM DTT for 10 min at 37ºC,
163 following addition of 1, 3, 5, 7, 10, 15 or 20 μl of Bmcystatin1 (0.71 μM), Rmcystatin3
164 (0.32 μM) or Rmcystatin4 (1.2 μM) and incubation at 37ºC for 10 min. Finally, Z-FR-
165 AMC substrate (4 mM) was added and the fluorescence was monitored as previous
166 described.

167 **2.8. rBbCp substrate profiling using phage display peptide library:** A hexapeptide
168 library [43] was used to study rBbCp specificity. *E. coli* TG1 transformed cells were
169 grown in 2YT medium containing ampicillin (200 μg/mL) and 2% glucose. When A_{550} =
170 0.5 – 0.7 was reached, helper phage M13K07 was added with a multiplicity of infection
171 (MOI) of 50, following manufactures instructions. The culture was centrifuged, the
172 medium replaced with 2YT containing ampicillin (200 μg/mL) and kanamycin (50
173 μg/mL), and the culture was incubated at 37ºC. After 16 h, the culture was centrifuged
174 and the supernatant, containing the fusion phage particle, was collected and selected
175 against rBbCp. Initially, fusion phages were incubated with Ni-NTA magnetic agarose
176 beads (GE) for 6 h at 25ºC, following 6 washes with 50 mM Tris-HCl pH 8.0 with 20 mM
177 imidazole and 100 mM NaCl. Next, beads were incubated in the presence of rBbCp for
178 30 min at 37ºC following addition of 1 μM E64 solution. After peptide cleavage by
179 rBbCp, phages were released from the magnetic beads to the supernatant and
180 collected. These phages were then used in the transfection of *E. coli* TG1 for

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181 amplification of the selected phages. After three round of selections, 96 phagemids
182 were randomly selected, sequenced and the translated peptide represented with
183 WebLogo tool [44].

184 **2.9. Inhibition of *B. bovis in vitro* culture growth:** *B. bovis* S2P strain was maintained
185 *in vitro* in microaerophilous stationary phase (MASP) as described in [45] with a 10%
186 hematocrit. Seroneutralization experiments were carried out essentially as previously
187 described [16, 30]. Shortly, when the percentage of infected RBCs reached 2%,
188 parasites were transferred to a 96 well-plate containing complete culture media
189 supplemented with 40% bovine serum, 5% non-infected RBCs to a final percentage of
190 parasitized RBCs of 0.5% and 20 μL of control serum (non-immunized mice) or anti-
191 BbCp serum in a final volume of 100 μL per well. The amount of mouse serum added
192 was the maximal that would not cause *per se* inhibition of merozoite growth, as
193 determined in previous experiments with normal mouse serum. Every 24 h the medium
194 was replaced and 5 μL of packed erythrocytes were collected from the bottom of each
195 well and smeared into glass slides, which were then Giemsa-stained and analyzed by
196 light microscopy. Percentages of infected RBCs were determined 24 and 48 h post-
197 serum treatment by counting 3000 cells per slide. Three technical replicates were used
198 for each condition.

199 **2.10. Statistical analysis:** The inhibitory constants were determined by fitting the non-
200 linear regression model according to the Morrison's equation [46] using the Grafit 5.0.11
201 software (Erithacus Software Limited, UK). One-way ANOVA followed by Bonferroni
202 multiple comparison test were used to compare the residual activity of rBbCp in the
203 presence of different classes of inhibitors, and to compare the percentages of infected
204 RBCs 48 h post-treatment. Statistical differences were considered significant when $p <
205 0.05$.

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207 **3. Results**

208 **3.1. Analysis of *B. bovis* cysteine peptidase (BbCp) primary structure:** The
209 deposited sequence XP_001612131 comprises a 49.1 kDa protein with a theoretical
210 isoelectric point of 5.39 and no predicted signal peptide. Domain analysis of
211 XP_001612131 revealed the presence of an inhibitory pro-domain I29 (F^{120} – F^{177}) and
212 a catalytic domain from the C1A family (I^{230} – A^{435}). Topology prediction shows a short
213 cytoplasmic stretch (M^{1} to S^{35}), followed by a transmembrane domain (A^{36} to G^{58}) in the
214 N-terminal, while the rest of the protein is predicted as extracellular (K^{59} to A^{435}) (Fig.
215 1A). Moreover, comparison of XP_001612131 primary structure with cysteine
216 peptidases from *B. ovis*, *B. ovata* and *B. bigemina* revealed the presence of a high
217 degree of similarities in the C1A catalytic domain, in which the conserved putative
218 catalytic residues C^{250}, H^{379} and N^{399} can be found (Fig. 1B). The cysteine peptidase
219 DNA sequence amplified from RBCs infected with *B. bovis* Brazilian IPV2 strain, named
220 BbCp, shared 98% of identity with the deposited genomic sequence XP_001612131
221 with substitutions in the inhibitory pro-domain (Sup. Fig. 1).

222 **3.2. Production and refolding of recombinant BbCp (rBbCp):** Recombinant BbCp
223 was obtained in inclusion bodies and became soluble only in the presence of 8.0 M urea
224 (Sup. Fig. 2). After two purification steps (Fig. 2 A and B), purified rBbCp was observed
225 as a major 37 kDa protein band (Fig. 2C – lane 1) and after refolding, rBbCp was
226 observed as a 25 kDa protein and a minor 13 kDa protein band (Fig. 2C – lane 2).

227 **3.3. Characterization of rBbCp proteolytic activity:** Purified rBbCp proteolytic activity
228 was 50-fold enhanced in the presence of the reducing agent DTT (1 mM), although no
229 further activity increases were observed at higher DTT concentrations (Fig. 3A).
230 Optimum pH was between 6.5 – 7.0 (Fig. 3B). As expected, rBbCp proteolytic activity
231 was completely inhibited in the presence of E64, while no inhibition was observed in the
232 presence of AMPSF or EDTA (Fig. 3C). Moreover, rBbCp was also inhibited by *R.*
233 *microplus* cystatins 1 (Ki = 2.89 nM) and 3 (Ki = 0.13 nM) but not by the midgut
234 Rmcystatin-4 (Table 1).

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235 **3.4. Recombinant BbCp specificity study:** After three rounds of selection no further
236 phage enrichment was observed (Sup. Table 2). Interesting, from the 96 selected
237 sequenced phages no consensus peptides was found (Fig. 4A), indicating that rBbCp
238 does not have a strict specificity, although a high prevalence of peptides (81 from 96)
239 containing Val (49%), Leu (42%) and Phe (11%) residues were found. Together they
240 corresponded to 84% of the total selected peptides (Fig. 4B). Moreover, the kinetic
241 parameters Kcat, Km and Kcat/Km were estimated for the substrates Z-FR-AMC and Z-
242 RR-AMC (Table 2). No proteolytic activity was observed for Z-RR-AMC while a high
243 catalytic efficiency was observed for Z-FR-AMC (Kcat/Km = 1.11×10^3 M^{-1} s^{-1}).

244 **3.5. Effect of anti-rBbCp serum in *B.bovis in vitro* growth:** Cultures exposed for 48
245 h to anti-rBbCp presented a reduction of 32% of infected RBCs when compared to
246 control groups, non-treated cultures or cultures treated with control-serum (Fig. 5), while
247 no statistical difference was observed between the percentages of infected RBCs in the
248 two latter control groups.

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250 **4. Discussion**

251 Cattle babesiosis, caused by *B. bovis*, is a major concern for livestock production
252 worldwide [2, 5], yet knowledge regarding the molecular mechanisms of parasite
253 survival is still sparse. Since cysteine peptidases play an important role for
254 apicomplexan survival and proliferation [47] it was proposed that such molecules could
255 be used as targets for parasite control, in which enzyme specificity is determinant [49].
256 Therefore, in this study, we carried out a kinetic characterization of a novel cysteine
257 peptidase from *B. bovis*, named BbCp. Primary structure of BbCp revealed high
258 similarities with other cysteine peptidases from apicomplexan parasites (Fig. 1),
259 including a conserved inhibitory pro-domain I29 and a catalytic domain belonging to the
260 C1A family [48].

261 After refolding, purified rBbCp appears as a major 25 kDa protein band (Fig. 3C –
262 lane 2) and a minor 13 kDa band, corresponding to the expected molecular weight of
263 the catalytic and inhibitory domains, indicating the presence of auto-activation
264 mechanism already demonstrated for other cysteine peptidases [49, 50]. Proteolytic
265 activity of rBbCp was enhanced in the presence of a reducing agent (Fig. 3A) and it
266 presented optimum pH between 6.5 – 7.0. Other apicomplexan cysteine peptidases
267 also presented high proteolytic activity at neutral pH [29, 51], indicating a possible
268 adaptation to the host environment in order to facilitate protein degradation and
269 therefore egress from the RBCs.

270 Peptidases belonging to the C1A family have their specificity determined by the
271 interactions between the substrate side chains and the S2 subsite [52], although the
272 residues predicted to compose the S2 pocket are not conserved among parasites [53].
273 Recombinant BbCp substrate profiling using a peptide phage display library revealed a
274 preference for Val > Leu > Phe (Fig. 4), a similar pattern to that observed for *B.*
275 *bigemina* cysteine peptidase babesipain-1 [29]. *P. facilparum* falcipains 2 and 3, on the
276 other hand, have a preference for Leu > Phe at P2 position [54, 55]. The difference
277 between babesipain-1 and falcipain P2 specificity was thought to be due the presence
278 of a Phe residue at the bottom of the S2 pocket (residue 447 from babesipain-1),
279 instead of a negative charged Asp/Glu from falcipain-2 and 3, respectively (Sup. Fig. 3),

280 which would offer a narrower space and preferentially accommodate small residues
281 [29]. However, it was recently demonstrated that the charged residue is responsible for
282 stabilizing a positively charged P2 residue of the substrate and does not interfere with
283 the binding of aromatic or branched side chains from hydrophobic residues [56, 57].
284 Corroborating those findings, rBbCp, which possesses a Met residue in the S2 pocket
285 (Sup. Fig. 4), presented no major proteolytic activity towards Z-RR-AMC and high
286 catalytic efficiency for Z-FR-AMC (Table 2). Moreover, the residue I94 of the S2 pocket
287 of falcipain-3 is responsible for the Leu preference at P2 position [56], therefore the
288 same preference found between BbCp and babesipain-1 (Val > Leu) could be explained
289 by the conservation of the Ser residue at this position (Sup. Fig. 4). Since BbCp,
290 babesipain-1 and ovipain-2 present the conserved Ser residue at the S2 pocket (Sup.
291 Fig. 4) and possibly the same specificity for the P2 substrate, it can be hypothesized
292 that an anti-*Babesia* drug that targets these cysteine peptidases could be used in the
293 control of multiple species of the parasite. It is important to state that the amino acid
294 residues that compose the S2 pocket of falcipains 2 and 3 were identified by structural
295 studies [58]. Thus, the discussion presented here based on the comparison of primary
296 sequence of falcipains, BbCp and babesipain-1 needs to be validated with structural
297 studies and with different substrates in the future.

298 Cysteine peptidases of the apicomplexan parasites *Toxoplasma gondii* and
299 *Plasmodium falciparum* have been demonstrated to participate in parasite invasion and
300 egress from host cells [59]. In the work at hand, treatment of *B. bovis* cultures with anti-
301 rBbCp antibodies resulted in a reduction of 32% of infected RBCs after 48 h when
302 compared to control groups (Fig. 5). Similarly, treatment of *B. ovis* cultures with anti-
303 ovipain-2 antibodies also resulted in the reduction of infected RBCs [30]. These studies
304 suggest that these cysteine peptidases could participate in the interaction between
305 parasite and host cells during the asexual stages of their life cycles. Since the immature
306 proteins have a predicted transmembrane domain close to the N-terminus, while most
307 of the proteins are predicted as extracellular, it is likely that they are first surface-
308 exposed, before undergoing cleavage yielding the shorter catalytic forms. Thus, these
309 surface cysteine protease forms would be susceptible to antibody neutralization [30 and

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310 this study]. Loss-of-function studies need to be performed to confirm the relevance of
311 cysteine proteases for parasite survival. Recent advances in *B. bovis* genetic
312 manipulation techniques will allow this approach in the future [60, 61].

313 Although the relevance of parasitic cysteine peptidases for parasite survival in
314 the vertebrate host has been established for some apicomplexan parasites, less is
315 known about their role in the invertebrate host. Transcriptomic and proteomic studies
316 with *B. bigemina*-infected salivary glands of *R. annulatus* revealed that transcripts with
317 high similarities with the cystatin family (cysteine peptidase inhibitors) were modulated
318 during infection [62]. In *H. longicornis,* the cystatin Hlcys-2 was found up-regulated in *B.*
319 *gibsoni*-infected larvae, and the recombinant inhibitor was able to interfere with *B. bovis*
320 growth *in vitro* [63]. Together, these studies highlight the importance of vector cystatins
321 and parasitic cysteine peptidases interplay during the parasite life cycle. Interestingly,
322 Rmcystatin-4, a type-2 cystatin found almost exclusively in the tick midgut [42], did not
323 display inhibitory activity towards rBbCp (Table 1). However, both Rmcystatin-3, a type-
324 2 cystatin found in tick hemocytes and related to tick immunity [41], and Bmcystatin-1, a
325 type-1 cystatin from fat bodies and ovary [40], inhibited rBbCp with high affinity (Ki =
326 0.13 nM and Ki = 2.89 nM, respectively). Based on our results, we hypothesize that no
327 inhibition by midgut cystatins (i.e. Rmcystatin-4) could favor parasite survival and
328 midgut epithelial crossing. However, cystatins from other tissues such as Bmcystatin-1
329 and Rmcystatin-3 could be relevant as one of the tick mechanisms to control excessive
330 parasite proliferation that could lead to deleterious effects in tick tissues and even death
331 [64]. However, further functional studies have to be carried out in order to elucidate the
332 complex interplay between *Babesia* cysteine peptidases and tick cystatins.

333

334 **5. Conclusion**

335 In this study, we describe the biochemical characterization of a putative cysteine
336 peptidase from *B. bovis* named BbCp. The active recombinant BbCp was obtained in
337 bacteria after the refolding process and presented increased proteolytic activity in the
338 presence of DTT and an optimum pH between 6.5 – 7.0. The rBpCp was inhibited by *R.*

339 *microplus* Bmcystatin-1 and Rmcystatin-3 but not by Rmcystatin-4. Moreover, substrate
340 profiling of rBbCp revealed preference for Val > Leu > Phe residues. Finally, anti-rBbCp
341 antibodies were able to reduce *B. bovis in vitro* growth, suggesting a possible role of
342 BbCp in the protozoa survival. Our results indicate that BbCp is an attractive target for
343 the development of control measures against *B. bovis* infections.

344

345 **Conflict of interest**

346 The authors declare that there is no conflict of interest.

347

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362 **Author's contribution**

363 Conceived and designed the experiments: Lu, S., Florin-Christensen, M. And Tanaka,
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368 Drafting the article: Lu, S. and Tanaka, A.S.

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369 Critical revision of the article: Tanaka, A.S. and Florin-Christensen, M.

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569 **Figure captions:**

570 **Figure 1: (A)** Schematic representation of *B. bovis* XP_001612131.1 conserved
571 domains with the putative transmembrane region from A^{36} to G^{58} (TM). The
572 propeptidase presents an inhibitory pro-domain (I29) from F^{120} to F^{177} and a C1A
573 catalytic domain (I^{230} – A^{435}) with the putative catalytic residues C^{250}, H^{379} and N^{399}. **(B)**
574 Amino acid alignment of BbCp (*B. bovis*) with other apicomplexan cysteine peptidases;
575 *B. ovis* (ALJ75577.1), *T. equi* (XP_004828689.1), *B. ovata* (XP_028867161.1) and *B.*
576 *bigemina* (XP_012766088.1). Identical residues are black-boxed and similar residues
577 are gray-boxed. The inhibitory pro-domain I29 (dashed) and the catalytic C1 domains
578 are indicated with lines. The putative catalytic residues are indicated by arrows.

579 **Figure 2:** Recombinant BbCp purification and refolding: **(A)** Affinity-chromatography
580 using a Ni-NTA resin. Column was equilibrated with 25 mM Tris-HCl buffer pH 8.0
581 containing 0.3 M NaCl and 8.0 M urea. Proteins were eluted in two steps, with
582 equilibration buffer containing 40 mM imidazole (P1) or 200 mM imidazol (P2). P2 was
583 dialised in 25 mM Tris-HCl pH 8.0 containing 8.0 M urea and applied in an **(B)** Ionic
584 exchange chromatography using a HiTrap Q resin.The column was equilibrated with 25
585 mM Tris-HCl buffer pH 8.0 containing 8.0 M urea. Protein elution was carried out with a
586 crescent linear gradient with 25 mM Tris-HCl buffer pH 8.0 containing 8.0 M urea and
587 1.0 M NaCl. Arrows indicate fractions in which rBbcp was observed by SDS-PAGE. **(C)**
588 Purified rBbCp was analysed in SDS-PAGE 12% before (1) and after (2) refolding
589 process.

590 **Figure 3:** Characterization of rBbCp proteolytic activity. **(A)** Recombinant BbCp was
591 assayed in 50 mM sodium acetate buffer pH 5.5 in the presence of different
592 concentration of DTT (0 – 6 mM). **(B)** rBbCp optimum pH was determined with 75 mM
593 Tris, 25 mM glycine, 25 mM MES and 25 mM acetic acid ($3.0 < pH < 8.0$) with 1 mM
594 DTT and Z-FR-AMC (0.1 mM). **(C)** Inhibition assay of rBbCp using E64 (0. 25 µM),
595 AMPSF (0.25 µM) and EDTA (0.25 µM).

596 **Figure 4:** rBbCp substrate specificity profile by peptide phage display library. **(A)**
597 Graphical representation of the amino acid recurrence at the six mutate position from 96

598 selected sequenced clones from rBbCp using WebLogo tool [44]. **(B)** Venn-diagram
599 showing the prevalence of Val, Leu and Phe residues found in the selected peptide
600 sequences.

601 **Figure 5:** Effect of anti-rBbCp antibodies in *B. bovis* growth *in vitro*. Growth curve of *B.*
602 *bovis* S2P in normal growth medium (no sera) or in medium containing murine control
603 serum or anti-BbCp serum. * Different from the control sera group with $p = 0.047$.

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Table 1: Inhibitory constant (Ki) of different cystatins from *R. microplus* for BbCp.

Inhibitor	K_i (nM)	Reference
Bmcystatin-1	2.89	[37]
Rmcystatin-3	0.13	[38]
Rmcystatin-4	N.I	[39]

N.I – No inhibition was observed at 1.2 μM of inhibitor.

Table 2: Kinetic parameters for Z-FR-AMC and Z-RR-AMC hydrolysis by recombinant BbCp.

Substrate	Kcat (s^{-1})	Km (μM)	Kcat/Km (M^{-1} s^{-1})
Z-FR-AMC	0.012	11	1.11×10^3
Z-RR-AMC	ND	ND	ND

ND – No detection of substrate cleavage by rBbCp (3 times more concentrated).

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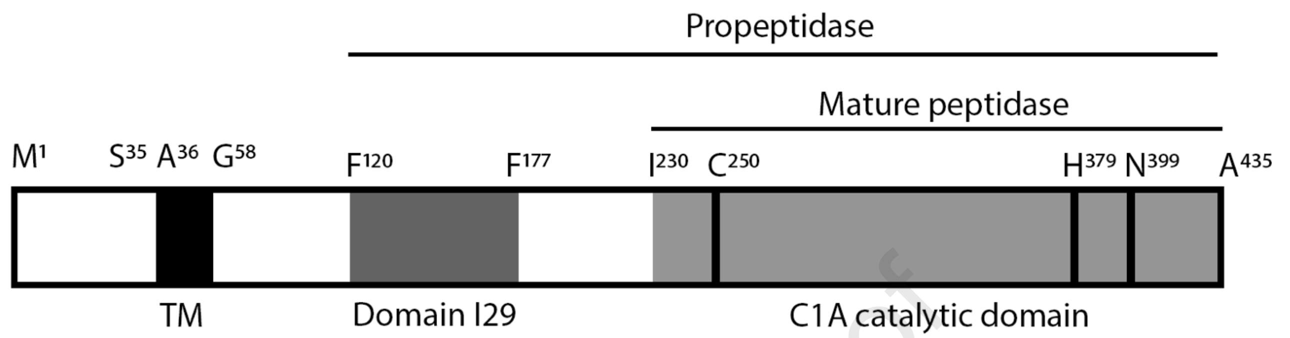
Figure 1

A



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B

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BbCp        ---------- MDVYEPIGDD LPERENRIKL QDLYQRFCAF LRRGSAFVHL CTAAAVELFA FGIFAVIGKS
B.ovis      MASSPLLQDD LDVYSQVEGD WQHRDSRPKP TPWYYRLYTF LRNGSTGVHF AIALVCFLIG FGIFASLGMG
T.equi      MEEIEVVKHD PVERTDTEAT L-CDDIKIQK NARRATILAF CKRYSALIIA SVSSVLFITT FVAIALSSGS
B.ovata     MTSETAVQDD LASRAETSDD LLKNGFGGKP DPWHSRLRAF FRRRGKCFFA LLAALVELLC FVIVAVIGND
B.bigemina  MAGKVEVLDD VVSRAETAED LLKNGSGGKP EPWHSRLRAF FRRRGKCYYA LLAALVEALC FTAVAVIGGD

BbCp        SGPSDWEIAI RNELLHRNFE NHATVLGEYS EDFAAIIVEA LKRDQVLIGA STAVELYIQF NDFNRDFKRH
B.ovis      SGPSEAEIAI RNELIQRNFE NYATVEGGVQ EDFAAIIVAA LGKEATALGA TSAVEFYIQF SDFSREHARK
T.equi      ATITKNKAIL VEAFKNIPFV NHVTIEGCEE EDYPLLIAEV MHYTAIQFDA DEEAATYVEF NSFNEKYNKK
B.ovata     TLYSSQERAV RKAIVRLKFE NYNTVEGVTD EDFAYIVCRA LGASTTLLNP EKDAEIYVQY NDFNRQYNRS
B.bigemina  SQYAAQERAV REAIVRLKFE NHGTVEGVTD EDFAYIVCRA LGASTRTLNP ERDAEIYVQY NDFNRQYDRR

BbCp        DNSISEKIER FATEYRNVTR IREFNMNVHK TYTMKINQFA DMTPEQFMSL QGLRASKIRV SKGIPDSQVA
B.ovis      DASVYDKTQR FLMEYKNVSM IRLFNDKKRR GYRMEMNQFA DMSPDEFMSM HGVRVTLPKV GVRQRGT-LK
T.equi      HTSWKHKKQC FINFRGNVSS INEHNAREDK TFTKEMNHMG DLSAKEFLDR YTKKVEIQFP EV--------
B.ovata     HASVMEKVRR FGVFHANVRR IDAFNSMKDR TFEMGVNKFA DMTTDEFMAL QGARVSTPKF DRSAVALVL-
B.bigemina  HTSVMEKARR FGVFHANVHT IDAFNAMENR TFEMGVNRFA DMTAEEFMAL QGSRVDTPQY ERGGVAMAL-

BbCp        AVGNQKGPNL KSE-----VR QTGNRFADIS PEDFIDLRKD NYMTFVKDQG NCGSCWAFSL IGVAEPFFKH
B.ovis      VSGVSGGSTS LLEVSSAPAD VSPDEGSALG PEEDIDLRRD GYMTFVKDQG KCGSCWAFAT VGVLEPFFKH
T.equi      ---------- ---------- -PANHAWNNV RPMALDLRKR DFMTFVKDQG EEGCSWAYSA TAIAESYHKY
B.ovata     ---------- ---------- ------DGTI ETVDIDLRRD GRMTAVKDQG TCGSCWAFAA LAVVEAYFKE
B.bigemina  ---------- ---------- ------DGTV DIADIDLRRD GIMTFVKDQG ACGSCWAFAT IAVVESYFKK

BbCp        KRDIDVVLSE QNIVDCVKEC HGCDYGNSYF AYEYIRDHGV YRIASYPYTA KSGECVEPLN EPRLTISRFG
B.ovis      KRNTDVILSE QNIVDCVAEC HGCQYGNSYF AYEYARDKGL YRNASYPYSA TCGICTIPEG EPRFTLAKFG
T.equi      NRNIDISLSE KQLIDGVKES GSTPVNNPFL GYKYIKDLGL VESSTYETD- -----VEITD APRYTIGSYS
B.ovata     QRSLILDLSE QQLVDCVEEC HGCNGGDSYQ AYQYITSRGV YTRAAYPYNA QEGQCMSPPG EPRYRLQEFR
B.bigemina  YRSLDLDLSE QQLVDCVKEC HGCQYGDSYH AYEYVTANGC YTRASYPYVA EQGQCMTPAG HPRYRLYEFG

BbCp        LSENPDLPQL LKQYGPLTVY VAVNVDWQFY SSGILDSCAD EINHAVVLAG VGQDDDGPFW LIKNSWGTSW
B.ovis      YSENPDIVQL LKQYGPLTVY VAVSTEWQFY GSGILDHCGE EINHAVVLAG VGKDEHGPYW LIKNSWGTTW
T.equi      YTFNPDIVSL LFNSGPLTIA VSVSEDWQFY KSGTINKCGA EINHEVILVG VGFDRESNYW IIKNSFGAEW
B.ovata     IAESPDIVHL LKMHGPLTVY AAVTPMWQFY KSGIINYCGD TVNHAVTLAG AGQIDQEAFW LIKNSWGTSW
B.bigemina  FTESPDIVQL LKAHGPLTVY VAVTPMWQFY KSGVINYCGE TVNHAVVLAG AGQADKEAFW LIKNSWGTSW

BbCp        GEEGYVRIAR GSSAFDNECG IAHMALYASA
B.ovis      GEQGYVKIAR GSSAFDSECG LSHMALYASA
T.equi      GDDGYIKLLR GNPEEADDCG VASEAMYSV-
B.ovata     GEEGYVRVAR GSSSLKDECG MSHVALEAVS
B.bigemina  GEEGYVRIAR GSSTLKDECG LSNVAMEAVH
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Figure 2

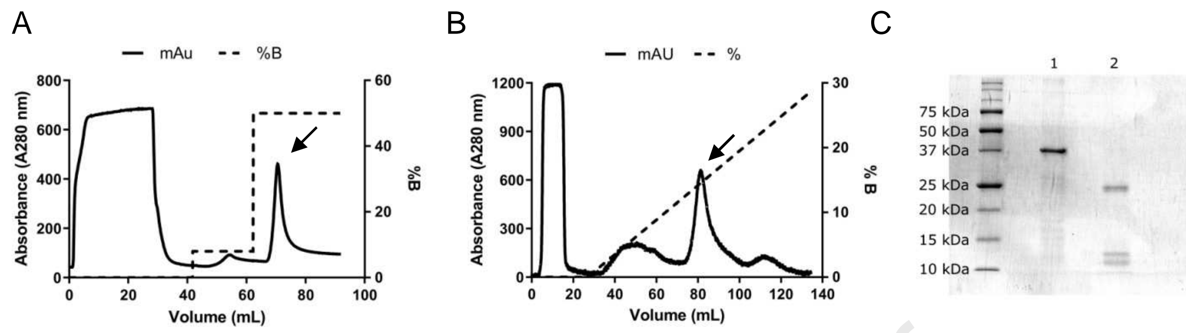

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Figure 3

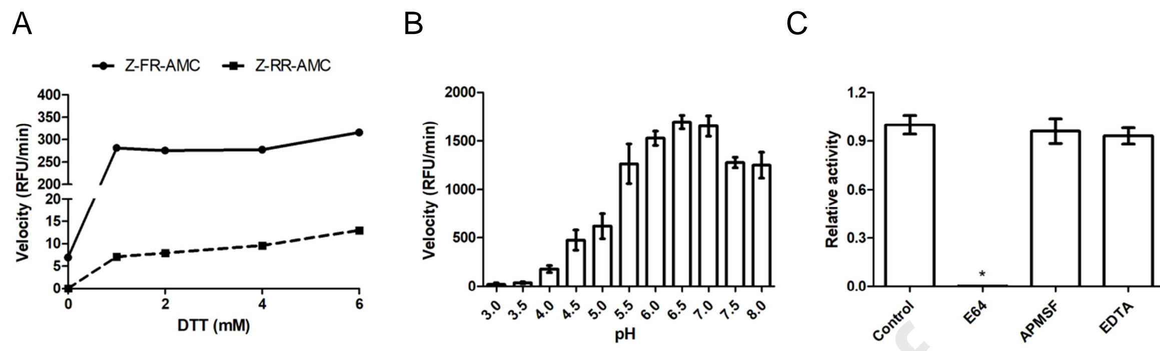

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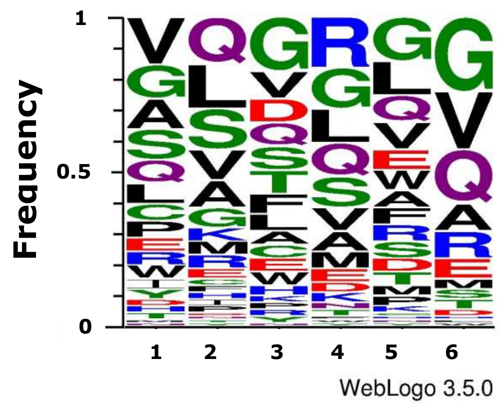
Figure 4

A



B

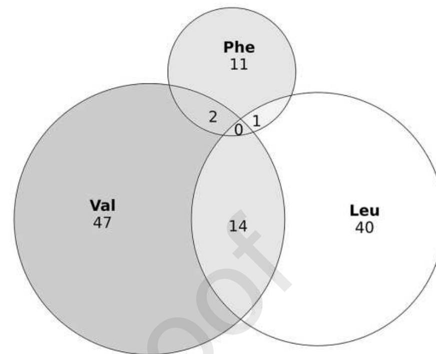


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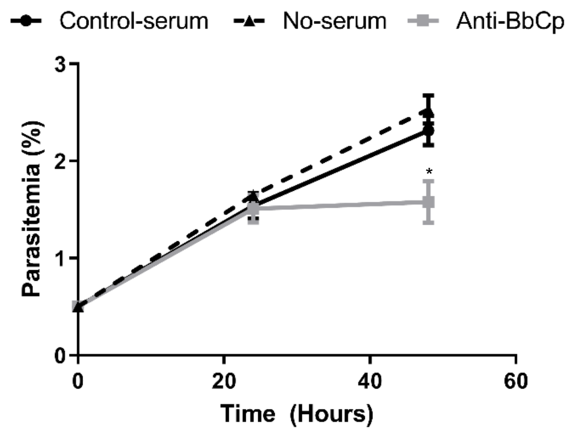
Figure 5



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Conflict of interest

The authors declare there is no conflict of interest.

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