Inhibitory effect of cyclophilin A from the hard tick *Haemaphysalis longicornis* on the growth of *Babesia bovis* and *Babesia bigemina*

Hiroki Maeda,[1] Damdinsuren Boldbaatar,[1] Kodai Kusakisako,[1] Remil Linggatong Galay,[1,2] Kyaw Min Aung,[1] Rika Umemiya-Shirafuji,[3] Masami Mochizuki,[1,2] Kozo Fujisaki,[4] and Tetsuya Tanaka[1,2*]

[1]*Laboratory of Emerging Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan*

[2]*Department of Pathological and Preventive Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, Yoshida, Yamaguchi 753-8515, Japan*

[3]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

[4]*National Agricultural and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan*

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*To whom editorial correspondence should be addressed:

Dr. Tetsuya Tanaka

Laboratory of Emerging Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

Tel./Fax: +81-99-285-3539

E-mail: tetsuya@ms.kagoshima-u.ac.jp

Abstract

Haemaphysalis longicornis is known as one of the most important ticks transmitting *Babesia* parasites in East Asian countries, including *Babesia ovata* and *Babesia gibsoni*, as well as *Theileria* parasites. *H. longicornis* is not the natural vector of *Babesia bovis* and *Babesia bigemina*. Vector ticks and transmitted parasites are thought to have established unique host-parasite interaction for their survival, meaning that vector ticks may have defensive molecules for the growth control of parasites in their bodies. However, the precise adaptation mechanism of tick-*Babesia* parasites is still unknown. Recently, cyclophilin A (CyPA) was reported to be important for the development of *Babesia* parasites in ticks. To reveal a part of their adaptation mechanism, the current study was conducted. An injection of *B. bovis*-infected RBCs into adult female *H. longicornis* ticks was found to upregulate the expression profiles of the gene and protein of CyPA in *H. longicornis* (HlCyPA). In addition, recombinant HlCyPA (rHlCyPA) purified from *Escherichia coli* exhibited significant inhibitory growth effects on *B. bovis* and *B. bigemina* cultivated *in vitro*, without any hemolytic effect on bovine RBCs at all concentrations used. In conclusion, our results suggest that HlCyPA might play an important role in the growth regulation of *Babesia* parasites in *H.*

longicornis ticks, during natural acquisition from an infected host. Furthermore, rHlCyPA may be a potential alternative chemotherapeutic agent against babesiosis.

Key words: Cyclophilin, Tick, recombinant, *Babesia*, RBC, parasite

Introduction

Babesiosis is an important protozoan disease caused by *Babesia* parasites. *Babesia* species are tick-transmitted protozoans that comprise some of the most ubiquitous and widespread parasites of red blood cells (RBCs), affecting a wide range of wild and economically important domestic animals and also humans (Homer et al. 2000; Schnittger et al. 2012; Vannier et al. 2008). From the economic and public health perspectives, sustained and continued research on babesiosis is needed for the development of effective therapeutic medication.

Haemaphysalis longicornis is one of a well-known vector tick of *Babesia* spp. *Babesia ovata* and *Babesia gibsoni*, as well as *Theileria* parasites are transmitted by *H. longicornis*. On the other hand, *Babesia bovis* and *Babesia bigemina* are transmitted by *Rhipicephalus* (*Boophilus*) *microplus* (Schnittger et al. 2012). The precise adaptation

mechanism of tick-*Babesia* parasites is still unknown.

Immunophilin is the generic name of isomerases such as the cyclophilins (CyPs) and FK-binding protein (FKBPs) families. Immunophilin binds specific immunosuppressive drugs; e.g., CyPs and FKBPs bind the cyclic peptide cyclosporine A and the macrolactones FK506 (tacrolimus) and rapamycin (sirolimus), respectively. CyPs have been found in many eukaryotes. They possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. PPIase can catalyze the *cis-trans* isomerization of the peptide bonds preceding proline residues, which involves in a wide range of cellular processes, such as cell division, transcriptional regulation, protein trafficking, and RNA splicing. In addition to enzymatic activities, many immunophilins act as molecular chaperones. Therefore, most members of the CyP family have been shown to function as mediators of intra- and inter-cellular communication (Barik et al. 2006; Bell et al. 2006; Galat 1993, 2004; Krücken et al. 2009).

Cyclophilins and their related molecules were identified and characterized previously in many living organisms; however, in ticks, only a few cyclophilin gene sequences were identified, and their functions remain unknown. Recently, we reported that cyclophilin A (CyPA) from the ixodid tick *H. longicornis*, *H. longicornis* CyPA (HlCyPA), has a conserved PPIase domain and is expressed in multiple organs as well

as throughout all developmental stages (Boldbaatar et al. 2008). Recombinant HlCyPA (rHlCyPA) was found to exhibit PPIase activity. After knockdown of the *HlCyPA* gene by RNA interference (RNAi), engorged female ticks had significantly lower body weight and failed to lay eggs. Furthermore, some RNAi-treated ticks died after engorgement. In addition, there was one report on the putative immunophilin gene in *R.* (*B.*) *microplus* ticks that showed high homology with the *HlCyPA* gene, wherein gene silencing significantly increased the infection rate of *Babesia bovis* in the larval progeny (Bastos et al. 2009). These reports strongly suggest that tick immunophilin genes and their products play important roles in tick physiology and as defensive immunological mechanisms against parasites. The current study was conducted to evaluate the response of HlCyPA to *Babesia* infection and its inhibitory growth effects on *Babesia* parasites cultivated *in vitro*. Two bovine *Babesia* parasites, *B. bovis*, *B. bigemina* and non-vector tick, *H. longicornis* were also used in this study to clarify vector-parasite adaptation mechanism. This is the first report on the inhibitory effect of cyclophilin from ticks on a tick-borne pathogen.

Materials and methods

109

110 Ticks and Animals

111

112 The parthenogenetic Okayama strain of *H. longicornis* has been maintained by
113 blood feeding on Japanese white rabbits (Kyudo, Kumamoto, Japan) (Fujisaki 1978) in
114 the Laboratory of Emerging Infectious Diseases, Joint Faculty of Veterinary Medicine,
115 Kagoshima University.

116 Rabbits were kept in accordance with the guidelines approved by the Animal
117 Care and Use Committee of Kagoshima University (Approval number A08010). They
118 were maintained under regulated conditions throughout the experiments.

119

120 Culture of *Babesia* parasites

121

122 Both *Babesia bovis* (the Texan strain) and *Babesia bigemina* (the Argentine
123 strain) were used in this study (Bork et al. 2004). They were maintained on purified
124 bovine RBCs using different culture media for each species (Galay et al. 2012).

125

126 Injection of *B. bovis*-infected RBCs to ticks

127

128 Unfed adult ticks were injected with 0.5 μl of *B. bovis*-infected RBCs or
129 uninfected normal RBCs (Control) through the fourth coxae into the hemocoel, as
130 previously described (Aung et al. 2012). The degree of parasitemia of the *B.*
131 *bovis*-infected RBCs was 5%. After injection, nine ticks were collected every 24 h; one
132 was used for genomic DNA extraction for the detection of *B. bovis*, and three were used
133 for total RNA extraction and complementary DNA (cDNA) synthesis. The remaining
134 five were used for protein extraction. The level of expression of the *HlCyPA* gene was
135 investigated by real-time PCR, and protein expression of HlCyPA was determined by
136 Western blot analysis. At the first step of real-time PCR, *actin*, *tubulin*, *P0*, and *L23*
137 genes were selected for tick reference and evaluated for standardization.

138

139 RNA extraction and cDNA synthesis

140

141 To extract total RNA, ticks were homogenized using Automill (Tokken, Tiba,
142 Japan), to which the TRI® reagent (Sigma, MO, USA) was added. The extracted RNA
143 was purified with the Turbo DNA-*free*™ Kit (Applied Biosystems, Tokyo, Japan).
144 cDNA synthesis was performed with ReverTra Ace-α-® (TOYOBO, Osaka, Japan)

145 following the manufacturer's protocol using 1 μg of total RNA.

146

147 DNA extraction

148

149 Genomic DNA was also extracted from collected ticks. Homogenized ticks
150 were suspended in an extraction buffer [100 mM Tris-HCl (pH 8.0), 0.5% SDS, 100
151 mM NaCl, 10 mM EDTA], and, after adding proteinase K (10 mg/ml) (KANTO
152 CHEMICAL, Tokyo, Japan), samples were incubated overnight at 55°C. After removal
153 of proteins using Phenol:Chloroform:IsoamylAlcohol (Sigma), ethanol precipitation was
154 performed to collect DNA. DNA samples were purified with an RNaseA solution (4
155 mg/ml) (Promega, WI, USA).

156

157 Expression analysis of the *HlCyPA* gene and detection of the *B. bovis* gene

158

159 The expression analysis of the *HlCyPA* gene was performed by real-time PCR
160 using THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO) with a 7300 real-time PCR
161 system (Applied Biosystems). Gene-specific primers were designed to target the
162 *HlCyPA* gene (Boldbaatar et al. 2008) and the control genes, as shown in Table 1.

163 Standard curves were made from eight-fold serial dilutions of cDNA of adult ticks fed
164 for 3 days. The PCR cycle profile was as follows: 95 °C for 10 min, 40 cycles of a
165 denaturation step at 95°C for 15 sec, and an annealing/extension step at 60°C for 60 sec.
166 The data was analyzed with 7300 system SDS software (Applied Biosystems).

167 Detection of the *B. bovis SSrRNA* gene was performed using PCR as described
168 by Adham et al. (2009) with a slight modification of the thermo cycle profile at 94°C for
169 5 min, 40 cycles of a denaturation step at 94°C for 1 min, an annealing/extension step at
170 72°C for 2 min, and final extension at 72°C for 7 min.

171

172 Protein extraction and Western blot analysis

173

174 Homogenized ticks were suspended in phosphate-buffered saline (PBS),
175 ultrasonicated three times (2 min each; Vibra Cell™; Sonics and Materials, CT, USA)
176 on ice, and finally centrifuged at 500 × g. The supernatant was resolved in 15%
177 SDS-PAGE (Laemmli 1970) under reducing conditions. After SDS-PAGE, the proteins
178 were transferred onto a polyvinylidene difluoride membrane (Immobilon®-P; Millipore,
179 MA, USA). The membrane was blocked overnight with 5% skim milk in PBS and then
180 incubated with a 1:500 dilution of anti-rHlCyPA mouse sera (Boldbaatar et al. 2008) at

37°C for 1 h. Tubulin was used as the control protein (Umemiya-Shirafuji et al. 2012). After washing five times in PBS containing 0.05% Tween20, the membrane was incubated with a 1:50,000 dilution of horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) at 37°C for 1 h. After washing five times in PBS containing 0.05% Tween20, bands were detected using the Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) and viewed using FluorChem®FC2 software (Alpha Innotech, CA, USA).

Expression and purification of recombinant HlCyPA

Recombinant plasmids (Boldbaatar et al. 2008) were used to transform into *Escherichia coli* (BL21), and histidine-tagged rHlCyPA expression was induced by 1 mM Isopropyl-β-D(-)-thiogalactopyranoside (IPTG) (Wako, Osaka, Japan) at 37°C for 6 h. The expressed recombinant protein was purified using a His trap FF column (GE Healthcare) containing 1 ml of chelating sepharose with nickel ions using the Bio Logic Duo Flow Base System (BIO-RAD, Tokyo, Japan). The purified recombinant protein was dialyzed against PBS. The concentration of rHlCyPA was determined using the Micro BCA™ protein assay kit (Thermo Fisher Scientific, MA, USA) and rHlCyPA

199 was stored at -30°C until use. The PPIase activity was also confirmed as described by
200 Boldbaatar et al. (2008).
201
202 Hemolysis assay
203
204 The hemolytic activity of rHlCyPA was determined according to the method
205 described by Stark et al. (2002). Briefly, bovine RBCs were washed with PBS. Then,
206 from 0.01 to 3.3 μM concentrations, rHlCyPA was mixed with bovine RBCs in a
207 96-well plate (Nunc, Roskilde, Denmark). The plate was incubated at 37 °C for 1 h and
208 centrifuged at 1000 × g for 5 min. The supernatant was collected, and the degree of
209 hemolysis was assessed by measuring the absorbance at 550 nm in a microplate reader
210 Model 680 (BIO-RAD). PBS and Triton-X were used as agents for preparing the 0 and
211 100% hemolyses.
212
213 Effect of recombinant HlCyPA on *Babesia* parasites *in vitro*
214
215 The culture media of *Babesia* parasites were changed daily, and rHlCyPA was
216 added each day at different concentrations of 3.3, 33, 330 nM, and 3.3 μM. An equal

volume of PBS was used for the control group. Blood smears with Giemsa staining were made daily to calculate the parasitemia and observe morphology of *Babesia* parasites. Three replicated wells were tested on the each group. Parasitemia was calculated as the percentage of infected RBCs to 1,000 RBCs counted.

Statistical analysis

All experiments were conducted with two or three separate trials. Data were statistically analyzed using the Student's *t*-test; results are presented as the mean ± SE, and $P < 0.05$ was considered statistically significant.

Results

Expression profiles of the *HlCyPA* gene and protein in *H. longicornis* females injected with *B. bovis*-infected RBCs

In the group of adult female *H. longicornis* ticks injected with *B. bovis*-infected RBCs, the *HlCyPA* gene expression increased faster than the control group (*H.*

235 *longicornis* female ticks injected with normal bovine RBCs), and a significant difference
236 (*$P < 0.05$, Control group vs. *B. bovis*-infected RBC-injected group) was observed 1-3
237 days after the injection (Fig. 1a). In addition, the expression levels of HlCyPA protein
238 showed a similar pattern compared to gene expression (Fig. 1b). The protein expression
239 levels were quantified by using the densitometry analysis. During the first two days after
240 the injection, protein expression of *B. bovis*-infected RBC-injected group tended to
241 increase faster than in the control group. In addition, *B. bovis* DNA was detected by
242 PCR to confirm the success of the injection (Supplementary Fig. 1).
243
244 Hemolytic activity of recombinant HlCyPA against bovine RBCs
245
246 No hemolysis was observed in bovine RBCs incubated with any concentration
247 of rHlCyPA from 0.01 to 3.3 μM. The hemolytic activity of rHlCyPA was compared
248 with Triton-X and expressed as % hemolysis. The percentage of hemolysis was lower
249 than 5% and almost negligible. at all concentrations of rHlCyPA determined
250 (Supplementary Fig. 2).
251 Effect of recombinant HlCyPA on the growth of *B. bovis* and *B. bigemina in vitro*
252

253 There were no significant differences on the growth of *B. bovis* (Fig. 2a) and *B.*
254 *bigemina* (Fig. 2b) in the presence of rHlCyPA, at concentrations from 330 nM and
255 lower. However, the growth of both species was completely inhibited in the culture with
256 3.3 μM rHlCyPA at 3 and 4 days (Fig. 2). In addition, in the presense of 3.3 μM
257 rHlCyPA, *Babesia* parasites were sparsely observed under light microscopy, and most of
258 them had an abnormal ring-form-like morphology (Fig. 3).
259
260 **Discussion**
261
262 *Babesia* is one of best-known parasites transmitted by ticks and has been
263 considered to be seriously injurious to tick biology (Florin-Christensen and Schnittger
264 2009). Therefore, it was speculated that ticks may have developed defensive molecular
265 mechanisms to reduce and/or attenuate the harmful and injurious effects of *Babesia*
266 parasites. On the other hand, *Babesia* parasites are believed to be capable of avoiding
267 the defensive mechanisms of ticks (Florin-Christensen and Schnittger 2009; Sonenshine
268 and Hynes 2008). The existing and sustainable host-parasite relationship between ticks
269 and *Babesia* parasites is assumed to be maintained on the basis of superb molecular
270 mechanisms for conflict of interest or potential conflict of interest (Chauvin et al. 2009;

271 Florin-Christensen and Schnittger 2009).

272 The cattle tick, *R.* (*B.*) *microplus*, is a known natural vector of *B. bovis* (Bock et
273 al. 2004; Schnittger et al. 2012), and their immunophilin gene hinders *B. bovis* infection,
274 which suggests that the gene plays an important role in the control of the transmission of
275 protozoa (Bastos et al. 2009). *H. longicornis* is also an important tick vector of *Babesia*
276 spp. (Schnittger et al. 2012); however, it is not a natural vector for *B. bovis* and *B.*
277 *bigemina* (Bock et al. 2004). These reports suggest that *H. longicornis* might not have
278 established a control strategy for these *Babesia* species or may have developed some
279 defense mechanisms for them. Interestingly, in *H. longicornis*, an immunophilin gene
280 has been identified and characterized, *HlCyPA* possessing 90% identity with the
281 immunophilin gene of *R.* (*B.*) *microplus*. Silencing of *HlCyPA* through RNAi has led to
282 a significant reduction in the body weight of engorged ticks and their failure to lay eggs
283 (Boldbaatar et al. 2008). This result indicates that HlCyPA represents a major
284 cyclophilin protein in *H. longicornis* involved in blood ingestion, tick viability, and
285 oocyte development. Therefore, HlCyPA might also be an important protein involved in
286 a tick's innate immunity.

287 In this context, this study was conducted to investigate the possible role of
288 HlCyPA against two *Babesia* parasites. To understand the interaction of HlCyPA and

289 *Babesia* parasites, *B. bovis*-infected RBCs were injected into *H. longicornis*. As shown
290 in Fig. 1, the injection of *B. bovis*-infected RBCs may have caused the upregulation of
291 *HlCyPA* gene and its product. These results suggest that HlCyPA might be related in the
292 tick immune response against *Babesia* parasites. In a previous study, we showed that the
293 *HlCyPA* gene was expressed in many organs, and the expression level was the highest in
294 the midgut and salivary glands (Boldbaatar et al. 2008). Both of them are important
295 organs involved in the multiplication and transmission of *Babesia* parasites in vector
296 ticks (Chauvin et al. 2009; Florin-Christensen and Schnittger 2009). These results
297 suggest that HlCyPA may be related to the tick's immune response to *Babesia* parasites.
298 In *H. longicornis*, a cysteine protease, longipain, is known to be highly expressed in the
299 midgut as well as HlCyPA and act as a defense molecule against invading *Babesia*
300 parasites (Tsuji et al. 2008). The defensin-like peptide, longicin, was also found to
301 possess activities against different pathogens, e.g., antimicrobial activity, fungicidal
302 activity, and parasiticidal activity, including babesiacidal activity (Tsuji et al. 2007).
303 Additional studies demonstrated that the synthetic partial peptide, P4 of longicin,
304 showed similar activities, including parasiticidal action, against *Toxoplasma gondii*
305 (Rahman et al. 2010; Tanaka et al. 2012). These results suggest that HlCyPA may act
306 synergistically with longipain and/or longicin to eliminate parasites, bacteria, and

307 viruses.

308 To further evaluate the effect of HlCyPA on *Babesia* parasites, rHlCyPA was
309 prepared (Boldbaatar et al. 2008). In this study, a dose-dependent inhibitory effect of
310 rHlCyPA on the growth of *B. bovis* and *B. bigemina* was observed (Fig. 2), and
311 rHlCyPA affected their morphology (Fig. 3). Even though lower concentrations of
312 rHlCyPA seemed to inhibit *B. bigemina* more efficiently than *B. bovis*, this may be due
313 to the more rapid increase of *B. bovis* than of *B. bigemina*. Coagulation disorders,
314 cytoadherence, and the hypotensive state seen in acute *B. bovis* infections are not
315 features of *B. bigemina* infections (Bock et al. 2004). These differences in pathogenicity
316 probably reflect a distinction in the metabolic or infection mechanisms between *B. bovis*
317 and *B. bigemina*. Therefore, these differences may affect the susceptibility of *B. bovis*
318 and *B. bigemina* to rHlCyPA. On the other hand, no toxic effects of rHlCyPA against
319 host RBCs were observed (Supplementary Fig. 2). This result revealed that the
320 babesiacidal effect of rHlCyPA is not due to the hemolysis of host RBCs. These results
321 support the idea that HlCyPA plays an important role in controlling the multiplication of
322 *Babesia* parasites as a defensive molecule in vector ticks.

323 Many agents possessing babesiacidal activity have been reported. In this study,
324 rHlCyPA showed an inhibitory growth effect on *Babesia* parasites *in vitro* (Fig. 2) at a

lower or similar concentration to triclosan (Bork et al. 2003), heparin (Bork et al. 2004), nerolidol (AbouLaila et al. 2010), artesunate (Goo et al. 2010; Nagai et al. 2003), or fusidic acid (Salama et al. 2012). This result suggests that rHlCyPA could be a potential anti-babesial agent as powerful as these drugs.

Meanwhile, recent reports suggested that cyclophilin may also play an important role in the host-response to viruses (Luban et al. 2007; Nagy et al. 2011; Zhou et al. 2012). Particularly, CyPA in chicken and human cells restricts influenza A virus replication through interaction and degradation with the viral M1 protein. Moreover, this inhibitory effect of CyPA on the influenza virus infection process is not dependent on its isomerase activity (Liu et al. 2009; Liu et al. 2012; Xu et al. 2010). These results suggest that the CyPA is also involved in some viral diseases and several effects of CyPA do not require PPIase activity. There are many tick-borne viruses ("tiboviruses") have been detected (Hub á lek and Rudolf 2012). Tick CyPA might be involved tick-virus interaction.

In this study, the biological action of HlCyPA was not fully elucidated; however, it is predicted that HlCyPA may also have other functions in ticks and in the transmission of tick-borne diseases. Thus, further studies will be required to determine its potential as an alternative chemotherapeutic agent. Moreover, the effect of HlCyPA

in vivo should be evaluated. Further understanding on the underlying mechanism of the babesiacidal effect of HlCyPA, as well as its other functions in the hard tick, may contribute to control of both babesiosis and its vector.

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Figure legends

Fig. 1 (a) Gene expression of *HlCyPA* 1-7 days after injection of *B. bovis*-infected RBCs. *B. bovis*, *B. bovis*-infected RBC-injected group; RBC, RBC-injected group for control. $^{*}P < 0.05$, significantly different, RBC vs. *B. bovis*. (b) Protein expression of HlCyPA 1-7 days after injection of *B. bovis*-infected RBCs. *B. bovis*, *B. bovis*-infected RBC-injected group; RBC, RBC-injected group for control; Numbers indicate days after injection. The line graph shows the relative expression of CyPA to tubulin determined using densitometry.

469

470 **Fig. 2** Effect of recombinant HlCyPA on the growth of *B. bovis and B. bigemina. In*
471 *vitro* culture of *B. bovis* (a) and *B. bigemina* (b) with different concentrations of
472 rHlCyPA. Parasitemia was monitored for 4 days. $^{*}P < 0.05$, significantly different,
473 Control vs. rHlCyPA-treated group.

474

475 **Fig. 3** Light micrograph of Giemsa-stained blood smear showing parasite morphology
476 from Control and 3.3 μM rHlCyPA-treated groups. a, *B. bovis*; b, *B. bigemina*. Arrows
477 indicate ring-form-like parasites. Bar: 5 μm

478

479 **Supplementary Fig. 1** Detection of *B. bovis* DNA from *H. longicornis* injected with *B.*
480 *bovis*-infected RBCs. *B. bovis*, *B. bovis*-infected RBC-injected group; RBC,
481 RBC-injected group for control. Numbers indicate days after injection.

482

483 **Supplementary Fig. 2** Hemolysis assay of recombinant HlCyPA. Each percentage
484 represents the ratio vs. Triton-X as 100% hemolysis. PBS was also used for 0%
485 hemolysis.