Detection and Characterization of *Theileria sergenti* Proteinases

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ABSTRACT. The lysate of *Theileria sergenti* piroplasms was tested for proteinases using sodium dodecyl sulfate-polyacrylamide gel electrophoresis in which substrate was included in gel matrix. Six proteinases of molecular weight 330, 125, 98, 94, 67 and 58 kilodalton (kDa) were detected. From the results of the Triton X-114 phase partition, 330, 125 and 58 kDa proteinases were partitioned into aqueous phase, which indicated that they were not associated with parasite membranes. All these three enzymes were classified into metalloproteinase family because of their sensitivities to metal-ion chelating compounds, ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline. On the other hand, 98 and 94 kDa proteinases were membrane-associated metalloproteinases which were preferentially inhibited by 1,10-phenanthroline. Another metalloproteinase of 67 kDa which was inhibited by EDTA and 1,10-phenanthroline was not associated with parasite membranes. Proteinases of 98 and 94 kDa degraded heat-denatured hemoglobin.—KEY WORDS: bovine erythrocyte, metalloproteinase, piroplasm, proteinase, *Theileria sergenti*.

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In protozoan parasites, as in other eukaryotic cells, proteinases are involved in numerous physiological events; post-translational modifications of parasite proteins, degradation and utilization of host proteins, and parasite development [2–4, 19, 21, 23, 28]. In pathogenic protozoan species, proteinases are related to their pathogenesis; invasion into host cells [1, 10, 14, 20] and inactivation of host proteins involved in defense mechanism, such as complement factors and macrophage lysosomal components [6, 7, 12].

Nothing is described about proteinases of *Theileria sergenti*, an intraerythrocytic protozoan parasite which causes anemia in cattle [18]. In the present study, we attempted to detect and characterize proteinase(s) of *T. sergenti* piroplasms purified from parasitized bovine erythrocytes.

MATERIALS AND METHODS

Theileria stocks and experimental infection: Shintoku and Chitose stocks of *T. sergenti* were used in this study. They were isolated in Hokkaido and identified as *T. sergenti* by morphological and serological criteria [18] and reactivity with monoclonal antibodies against *T. sergenti* [16]. Two Holstein calves of four and six months of age which had been splenectomized approximately two weeks before infection, were used. Each of them was infected with *T. sergenti* Chitose stock by subcutaneous inoculation of infected blood, or with *T. sergenti* Shintoku stock by feeding them with infected ticks.

Purification of piroplasms: Blood was collected from the cattle when parasitized erythrocytes reached more than 10%, and washed three times with Tris-buffered saline (TBS: 10 mM Tris-HCl/150 mM NaCl, pH 7.4). Purification of piroplasms was carried out as described previously [26].

Proteinase detection and effects of proteinase inhibitors: To detect proteinase activity, gelatin was added to polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulfate (SDS) [17] to a final concentration of 0.2% (w/v) as described by Heussen and Dowdle [15]. Alternatively, hemoglobin denatured at 70°C for 10 min was used as substrate at a final concentration of 0.2% (w/v) [8]. Piroplasm samples were treated with Tris-HCl buffer (0.0625 M, pH 6.8) containing 2% (w/v) SDS and 10% glycerol at room temperature for 10 min. After electrophoresis at 4°C, gels were treated with 2.5% (v/v) Triton X-100 in distilled water twice for 30 min at room temperature to renature proteinases. The gels were incubated at 37°C for 12–18 hrs in HEPES buffer (10 mM, pH 7.4) containing 2mM $CaCl_2$, stained for 1 hr with 0.1% (w/v) Coomassie brilliant blue R-250/20% (v/v) methanol/10% (v/v) acetic acid, and destained with 20% (v/v) methanol/10% (v/v) acetic acid. Enzyme activity was detected as unstained zone in the gel. Molecular weights of proteinases, were determined by using molecular weight standard proteins of 14, 20, 30, 43, 67, and 94 kilodalton

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(kDa: Pharmacia LKB Biotechnology, Sweden) and 70, 140, 210 and 280 kDa (Sigma Chemical Co., U.S.A.). Effects of proteinase inhibitors were examined by incubating the gels for 24 hr at 37°C in the presence of proteinase inhibitors.

Phase partition with Triton X-114: Proteins of *T. sergenti* were subjected to phase partition by Triton X-114 according to the method of Bordier [5]. Purified piroplasms were treated with TBS containing 1% (v/v) Triton X-114 on ice for 30 min. The lysate was centrifuged for 1 hr at 100,000 × g and the pellet was discarded. The supernatant was carefully layered over an equal volume of a 6% (w/v) sucrose cushion in TBS. After incubation at 30°C for 15 min, the sample was centrifuged at 1,000 × g for 15 min at room temperature. The top aqueous phase and bottom detergent phase were collected. Each fraction was precipitated with 4 volumes of acetone and centrifuged at 6,000 × g for 10 min at 4°C. The pellet was solubilized as described above and subjected to proteinase detection.

RESULTS

Detection of piroplasm proteinases: By using SDS-PAGE in which gelatin was added as proteinase substrate, two proteinases were detected in Shintoku stock of *T. sergenti*. The apparent molecular weight of the proteinases was 330 and 125 kDa, respectively (Fig. 1; lane 1). The proteinase activities were irreversibly denatured by the treatment with SDS in the presence of 2-mercaptoethanol (data not shown). In addition to these two proteinases, three major proteinases (98, 94, and 58 kDa) and one minor proteinase (67 kDa) were detected in Chitose stock of *T. sergenti* (Fig. 2; lane 2). No proteinase activities were detected in erythrocyte lysate prepared from uninfected calf (Fig. 1; lane 4 and Fig. 2; lane 1). When heat-denatured hemoglobin was used as proteinase substrate, only 98 and 94 kDa proteinases of Chitose stock were demonstrated (Fig. 3, lane 2).

Phase partition with Triton X-114: The proteinases of 330, 125, and 58 kDa were partitioned exclusively into the aqueous phase, while 98 and 94 kDa proteinases exclusively into the detergent phase (Fig. 1; lanes 2 and 3, and Fig. 2; lanes 3 and 4). Most of the activity of 67 kDa proteinase was detected in the aqueous phase (Fig. 2, lane 4).

Sensitivity of proteinases to inhibitors: Table 1 shows sensitivities of 6 proteinases detected in Shintoku and Chitose stocks to proteinase inhibitors. None of the proteinases were inhibited by

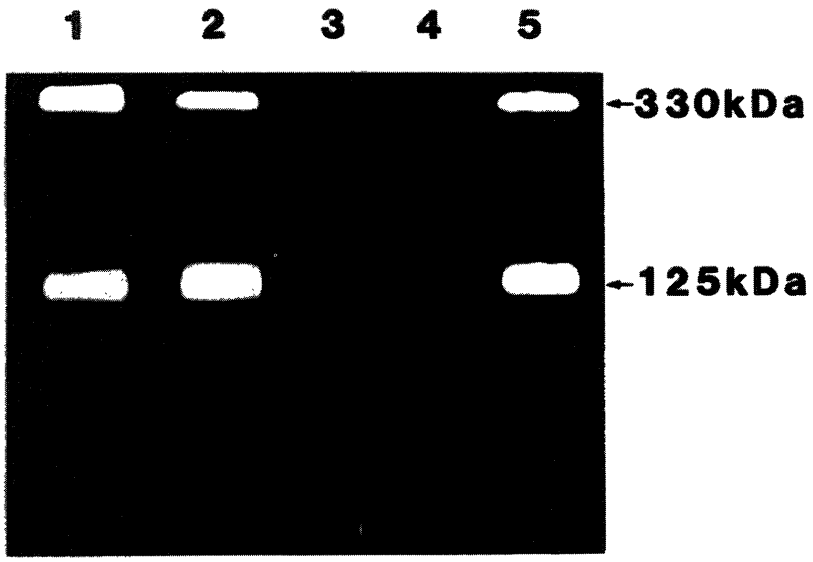

Fig. 1. Detection of *T. sergenti* (Shintoku stock) proteinases by SDS-polyacrylamide gel electrophoresis containing substrate. Gelatin was added to 7.5% polyacrylamide gel at a final concentration of 0.2%. Lane 1: purified piroplasm (Shintoku stock), lane 2: *Theileria*-infected erythrocytes, lane 3: erythrocytes from uninfected calf, lane 4: Triton X-114 phase-partitioned piroplasm sample (detergent phase), lane 5: Triton X-114 phase-partitioned piroplasm sample (aqueous phase).

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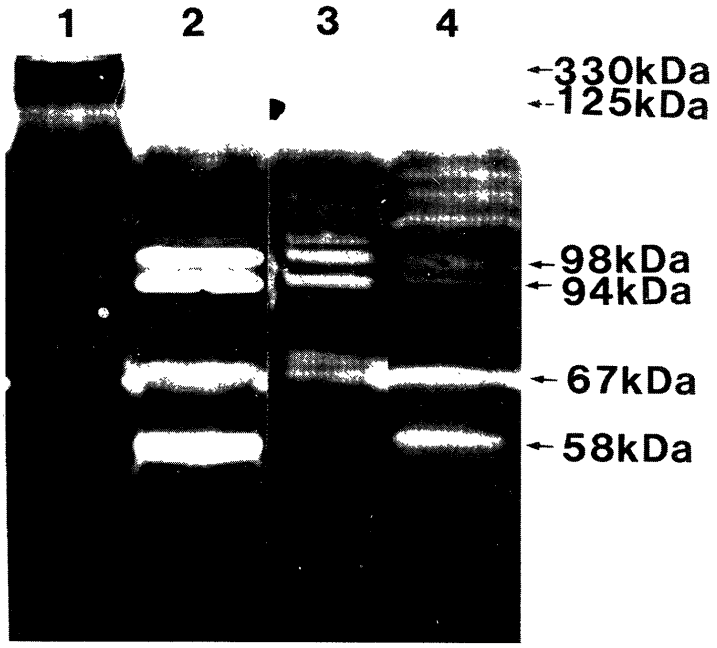

Fig. 2. Detection of *T. sergenti* (Chitose stock) proteinases by SDS-polyacrylamide gel electrophoresis containing substrate. Gelatin was added to 10% polyacrylamide gel to give a final concentration of 0.2%. Lane 1: erythrocytes from uninfected calf, lane 2: purified piroplasm, lane 3: Triton X-114 phase-partitioned piroplasm sample (detergent phase), lane 4: Triton X-114 phase-partitioned piroplasm sample (aqueous phase).

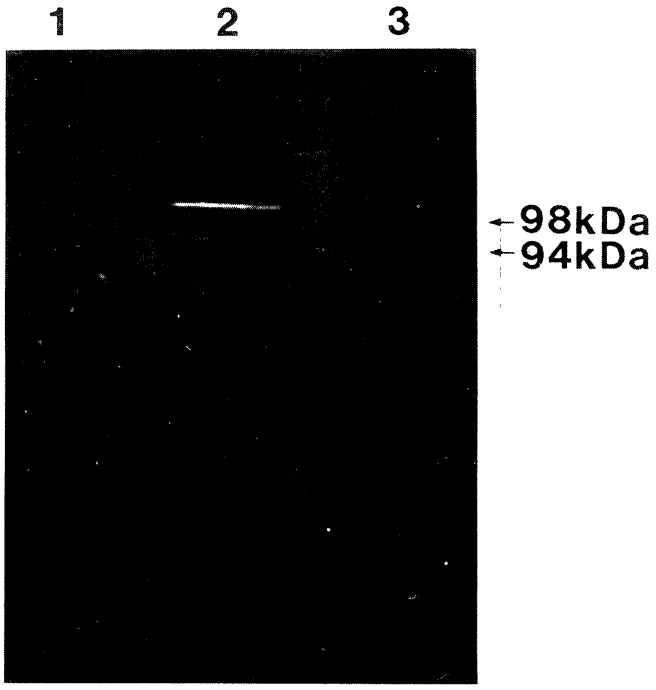

Fig. 3. Detection of *T. sergenti* (Chitose and Shintoku stocks) proteinases by SDS-polyacrylamide gel electrophoresis containing substrate. Heat-denatured hemoglobin was added to 10% polyacrylamide gel to give a final concentration of 0.2%. Lane 1: erythrocytes from uninfected calf, lane 2: purified piroplasm (Chitose stock), lane 3: purified piroplasm (Shintoku stock).

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Table 1. Inhibition of *T. sergenti* proteinases by proteinase inhibitors

Inhibitor	Concentration	Inhibition of proteinase (kDa)					
		330	125	98	94	67	58
Aprotinin	0.3 μM	–[a)]	–	–	–	–	–
3, 4-Dichloroiso-coumarine	200 μM	–	–	–	–	–	–
Leupeptin	1 μM	–	–	–	–	–	–
PMSF[b)]	1,000 μM	–	–	–	–	–	–
TLCK[c)]	100 μM	–	–	–	–	–	–
Bestatin	130 μM	–	–	–	–	–	–
E-64[d)]	2.8 μM	–	–	–	–	–	–
Pepstatin	1 μM	–	–	–	–	–	–
Phosphoramidon	569 μM	–	–	–	–	–	–
EDTA[e)]	5 mM	+	+	–	–	–	+
1, 10-phenanthroline	10 mM	+	+	+	+	+	+

a) —; Not inhibited, +; Inhibited.
b) Phenylmethylsulfonyl fluoride.
c) N-α-tosyl-L-lysine-chloromethylketone.
d) L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino) butane.
e) Ethylenediaminetetraacetic acid.

serine-, acid- or thiol-proteinase inhibitors. Ethylenediaminetetraacetic acid (EDTA), a metalloproteinase inhibitor, inhibited the activities of 330, 125 and 58 proteinases, but not those of the proteinases of 98, 94 and 67 kDa. Another metalloproteinase inhibitor, 1, 10-phenanthroline inhibited activities of all 6 proteinases.

DISCUSSION

In this study, we demonstrated proteinase activities of *T. sergenti* piroplasms by electrophorectic analysis in SDS-polyacrylamide gel containing proteinase substrate. Of six piroplasm proteinases, 330, 125 and 58 kDa proteinases were not associated with membranes as these were detected in aqueous phase after Triton X-114 phase partitioning. All these three enzymes were classified into metalloproteinase family because of their sensitivities to metal-ion chelating compounds, EDTA and 1, 10-phenanthroline. On the other hand, 98 and 94 kDa proteinases were membrane-associated metalloproteinases which were preferentially inhibited by 1, 10-phenanthroline. Another proteinase of 67 kDa was a metalloproteinase which was not associated with membranes.

The metalloproteinases of *T. sergenti* were not inhibited by phosphoramidon, a potent inhibitor of other metalloproteinases including thermolysin and neutral endopeptidase. Metalloproteinases in the astacin family are insensitive to this inhibitor [11]. However, in order to determine whether *T. sergenti* proteinases belong to the astacin family of metalloproteinase, primary sequences of amino acid are required.

Erythrocytes are known to contain several proteinases in their membrane and cytosol [23, 27]. However, the proteinases demonstrated here are considered to be of parasite-origin because purified piroplasm fraction is free from erythrocyte membrane components as revealed by electronmicroscopic observation and SDS-PAGE analysis [24, 25]. Erythrocyte proteins including hemoglobin are possibly incorporated into piroplasms as indicated by two-dimensional gel electrophoresis analysis [26]. However, a possibility that the proteinases detected in this study are those present in erythrocyte cytosol and incorporated into parasites or absorbed on the surface of the parasites may be excluded because uninfected bovine erythrocytes do not show any proteinase activity detectable by the proteinase assay method used in this study.

Functions of these proteinases remain uninvestigated. As two proteinases of 98 and 94 kDa showed activity against heat-denatured hemoglobin, they may function in utilizing host cell proteins as their nutrients. Hemoglobin-degrading proteinases of intraerythrocytic protozoan parasites are reported in *Plasmodium falciparum* [2, 28] and *Babesia bovis* [8]. However, whether these hemoglobin-degrading proteinases are indispensable for intraerythrocytic growth of *T. sergenti* piroplasms is not clear because one of the stocks used in this study lacked these proteinases.

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A proteinase (gp 63) which is localized on the surface of *Leishmania* [12] may be involved in the pathogenesis; attachment to and invasion into mammalian cells, degradation of serum proteins, particularly immunoglobulins and complement components accumulated on the parasite surface, and resistance against intracellular killing by macrophage phagolysomal components [6, 7]. Plasmodial proteinases are supposed to play important roles in rupture of infected erythrocyte by degrading membrane-skeleton proteins [9], and in reinvasion into erythrocytes as this step is inhibited by proteinase inhibitors [1, 10, 14, 20]. We are now determining whether the proteinases detected in *T. sergenti* piroplasms are involved in degradation of erythrocyte membrane-skeleton proteins such as spectrins and band 4.1 and 4.2 proteins, and in attachment and reinvasion to host cells.

In 6 proteinases of *T. sergenti*, 98, 94, 67 and 58 kDa proteinases which were detected only in Chitose stock are considered to be stock-specific. Alternatively, expression of these proteinases is associated with certain development stage of parasite *in vivo*. In malaria parasites and trypanosomes, stage specific proteinases are reported [3, 13, 22] and they are supposed to play crucial roles in parasite development. However, it is difficult to determine stage-specific expression of proteinases because the methods for synchronizing *T. sergenti* parasite *in vitro* have not been established.

Finally, proteinases are considered to be possible target molecules for chemotherapy. Proteinase inhibitors which specifically inhibit parasite proteinases without action on host cell function are expected to be effective in the treatment of protozoan parasitosis including malaria and trypanosomiasis [19, 21]. We are now purifying the proteinases of *T. sergenti* in order to characterize them more precisely.

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REFERENCES

1. Banyal, H. S., Misra, G. C., Gupta, C. M., and Dutta, G. P. 1981. Involvement of malarial proteases in the interaction between the parasite and host erythrocyte in *Plasmodium knowlesi* infections. *J. Parasitol.* 67: 623–626.
2. Bailly, E., Savel, J., Mahouy, G., and Jaureguiberry, G. 1991. *Plasmodium falciparum*: Isolation and characterization of a 55-kDa protease with a cathepsin D-like activity from *P. falciparum*. *Exp. Parasitol.* 72: 278–284.
3. Bonaldo, M. C., D'Escoffier, L. N., Salles, J. M., and Goldenberg, S. 1991. Characterization and expression of proteases during *Trypanosoma cruzi* metacyclogenesis. *Exp. Parasitol.* 73: 44–51.
4. Braun-Breton, C., Rosenberry, T. L., and Pereida da Silva L. 1988. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. *Nature (Lond.)* 332: 457–459.
5. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256: 1604–1607.
6. Bordier, C. 1987. The promastigote surface protease of *Leishmania*. *Parasitol. Today* 5: 151–153.
7. Chaudhuri, G., Chaudhuri, M., Pan, A., and Chang, K. 1989. Surface acid proteinase (gp63) of *Leishmania mexicana*. *J. Biol. Chem.* 264: 7483–7489.
8. Commins, M. A., Goodger, B. V., and Wright, I. G. 1985. Proteinases in the lysate of bovine erythrocytes infected with *Babesia bovis*: Initial vaccination studies. *Int. J. Parasitol.* 15: 491–495.
9. Degeurcy, A., Hommel, M., and Schrevel, J. 1990. Purification and characterization of 37-kilodalton proteases from *Plasmodium falciparum* and *Plasmodium berghei* which cleave erythrocyte cytoskeletal components. *Mol. Biochem. Parsitol.* 38: 233–244.
10. Dluzewski, A. R., Rangachari, K., Wilson, R. J. M., and Gratzer, W. B. 1986. *Plasmodium falciparum*: Protease inhibitors and inhibition of erythrocyte invasion. *Exp. Parasitol.* 62: 416–422.
11. Dumermuth, E., Sterchi, E. E., Jiang, W., Wolz, R. L., Bond, J. S., Falnnery, A. V., and Beynon, R. J. 1991: The astacin family of metalloendopeptidases. *J. Biol. Chem.* 266: 21318–21385.
12. Etges, R., Bouvier, J., and Bordier, C. 1986. The major surface protein of *Leishmania* promastigotes is a protease. *J. Biol. Chem.* 261: 9098–9101.
13. Greig, S. and Ashall, F. 1990. Electrophoretic detection of *Trypanosoma cruzi* peptidases. *Mol. Biochem. Parasitol.* 39: 31–38.
14. Hadley, T., Aikawa, M. ,and Miller, L. H. 1983. *Plasmodium knowlesi*: Studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. *Exp. Parasitol.* 55: 306–311.
15. Heussenn, C. and Dowdle, E. B. 1980. Electrophoresis analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.* 102: 196–202.
16. Kobayashi, N., Onuma, M., Kirisawa, R., Ohgitani, T., Takahashi, K., Sasaki, N., and Kawakami, Y. 1987. Monoclonal antibodies against intraerythrocytic merozoites (piroplasms) of *Theileria sergenti*. *Jpn. J. Vet. Res.* 49: 697–702.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227: 680–685.
18. Minami, T., Fujinaga, T., Furuya, K., and Ishihara, T. 1980. Clinico-hematologic and serological comparison of Japanese and Russian strains of *Theileria sergenti*. *Natl. Inst. Anim. Health Q. (Jpn.)* 20: 44–52.
19. North, M. J., Mottram, J. C., and Coombs G. H. 1990. Cysteine proteinases of parasitic protozoa. *Parasitol. Today* 6: 270–275.
20. Olaya, P. and Wasserman, M. 1991. Effect of calpain

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inhibitors on the invasion of human erythrocytes by the parasite *Plasmodium falciparum*. *Biochem. Biophys. Acta* 1096: 217–221.
21. Rockett, K. A., Playfair, J. H. L., Ashall, F., Targett, G. A. T., Anglinker, H., and Shaw, E. 1990. Inhibition of intraerythrocytic development of *Plasmodium falciparum* by proteinase inhibitors. *FEBS Lett.* 259: 257–259.
22. Rosenthal, P. J., Kim, K., McKerrow, J. H., and Leech, J. H. 1987. Identification of three stage-specific proteinases of *Plasmodium falciparum*. *J. Exp. Med.* 166: 816–821.
23. Schrével, J., Deguercy, A., Mayer, R., and Monisgny, M. 1990. Proteases of malaria-infected red blood cells. *Blood Cells* 16: 563–584.
24. Sugimoto, C., Kawazu, S., Kamio, T., and Fujisaki, K. 1991. Protein analysis of *Theileria sergenti/buffeli/orientalis* piroplasms by two-dimensional polyacrylamide gel electrophoresis. *Parasitology* 102: 341–346.
25. Sugimoto, C., Sato, M., Kawazu, S., Kamio, T., and Fujisaki, K. 1991. Purification of merozoites of *Theileria sergenti* from infected bovine erythrocytes. *Parasitol. Res.* 77: 129–131.
26. Sugimoto, C., Kawazu, S., Sato, M., Kamio, T., and Fujisaki, K. 1992. Preliminary biochemical characterization of 'veil' structure purified from *T. sergenti*-, *T. buffeli*- and *T. orientalis*-infected bovine erythrocytes. *Parasitology* 104: 207–213.
27. Vander Jagt, D. L. 1990. Commentary; Proteases in malaria-infected red blood cells. *Blood Cells* 16: 585–590.
28. Vander Jagt, D. L., Hunsaker, L. A., and Campos, N. M. 1986. Characterization of a hemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 18: 389–400.

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