1 **RBC Invasion and invasion-inhibition assays using free merozoites isolated after**

2 **cold treatment of *Babesia bovis in vitro* culture**

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16

17   **Abstract**

18

19   *Babesia bovis* is an apicomplexan hemoprotozoan that can invade bovine red blood

20   cells (RBCs), where it multiplies asexually. RBC invasion assays using free viable

21   merozoites are now routinely used to understand the invasion mechanism of *B. bovis*,

22   and to evaluate the efficacy of chemicals and antibodies that potentially inhibit RBC

23   invasion by the parasite. The application of high-voltage pulses (high-voltage

24   electroporation), a commonly used method to isolate free merozoites from infected

25   RBCs, reduces the viability of the merozoites. Recently, a cold treatment of *B. bovis in*

26   *vitro* culture was found to induce an effective release of merozoites from the infected

27   RBCs. In the present study, we incubated *in vitro* cultures of *B. bovis* in an ice bath to

28   liberate merozoites from infected RBCs and then evaluated the isolated merozoites in

29   RBC invasion and invasion-inhibitions assays. The viability of the purified merozoites

30   (72.4%) was significantly higher than that of merozoites isolated with high-voltage

31   electroporation (48.5%). The viable merozoites prepared with the cold treatment also

32   invaded uninfected bovine RBCs at a higher rate (0.572%) than did merozoites prepared

33   with high-voltage electroporation (0.251%). The invasion-blocking capacities of

34   heparin, a polyclonal rabbit antibody directed against recombinant *B. bovis* rhoptry

35   associated protein 1, and *B. bovis*-infected bovine serum were successfully

36   demonstrated in an RBC invasion assay with the live merozoites prepared with the cold

37   treatment, suggesting that the targets of these inhibitors were intact in the merozoites.

38   These findings indicate that the cold treatment technique is a useful tool for the isolation

39   of free, viable, invasion-competent *B. bovis* merozoites, which can be effectively used

40   for RBC invasion and invasion-inhibition assays in *Babesia* research.

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42   **Keywords:** *Babesia bovis*, Cold treatment, Invasion-inhibition assay, Merozoite

43   isolation, RBC invasion

44

1. Introduction

Babesia bovis, an apicomplexan hemoprotozoan, causes severe clinical bovine babesiosis in cattle worldwide. The cattle disease caused by *B. bovis* is characterized by fever, hemoglobinuria, anemia, icterus, and neurological and respiratory syndromes, with occasional death (Bock et al., 2004; Everitt et al., 1986). *Babesia bovis*, which is biologically transmitted by ticks, has three different reproductive stages in its lifecycle: (i) gametogony, the sexual fusion of gametes within the mid-gut of the tick vector; (ii) sporogony, asexual reproduction in the salivary gland of the tick vector; and (iii) merogony, asexual reproduction in the erythrocytes (RBCs) of the infected animal (Homer et al., 2000).

The life cycle of *B. bovis* in cattle begins with the injection of the sporozoites by infected tick vectors while feeding on cattle blood (Hunfeld et al., 2008). The sporozoites directly invade the host RBCs, where they transform into merozoites and move into the merogony stage (Yokoyama et al., 2006). The rupture of the infected RBCs after the asexual multiplication of the parasite releases the mature merozoites, which then invade uninfected RBCs (Bock et al., 2004). The involvement of several

62   protozoan molecules secreted from the micronemes and rhoptries of *B. bovis* during

63   merozoite invasion of RBCs, including merozoite surface antigens, rhoptry associated

64   protein 1 (RAP-1), apical membrane antigen 1, and thrombospondin-related anonymous

65   protein (Yokoyama et al., 2006), has been documented. Antisera directed against these

66   protozoan antigens are reported to inhibit the asexual growth of the parasite *in vitro*

67   (Gaffer et al., 2004a,b; Mosqueda et al., 2002a,b; Suarez et al., 2000; Yokoyama et al.,

68   2002), but their exact roles in RBC invasion are not fully understood, hindering the

69   development of an effective vaccine against this parasite. Other molecules that induce

70   neutralizing antibodies against *B. bovis* are still being investigated (Silva et al., 2010;

71   Suarez et al., 2011; Terkawi et al., 2013). *In vitro* invasion assays are essentially used to

72   clarify the mechanism of RBC invasion, which involves parasite molecules (Sun et al.,

73   2011). Similarly, invasion-inhibition assays (*in vitro* neutralization assays) are useful

74   for investigating neutralizing antibodies raised against parasite antigens that are

75   potential candidate vaccines (Gaffer et al., 2004a,b) or for analyzing the utility of

76   invasion-blocking chemicals as possible chemotherapeutic agents against *B. bovis* (Bork

77   et al., 2004; Nakamura et al., 2007; Okubo et al., 2007). High-quality, free, viable,

78   invasion-competent *B. bovis* merozoites are essential for both invasion and

79   invasion-inhibition assays, if reliable results are to be obtained.

80   At present, two main methods are used to prepare free merozoites from *in vitro*

81   cultures of *B. bovis*. With the first method, the merozoites are isolated from the

82   supernatants of *in vitro* cultures (Hines et al., 1992; Rodriguez et al., 1986; Sun et al.,

83   2011), whereas in the second method, the merozoites are released from RBCs

84   physically ruptured by the application of high-voltage pulses (high-voltage

85   electroporation) and collected (Franssen et al., 2003). Large volumes of *in vitro* cultures

86   are required for the first method, to obtain sufficient free merozoites for RBC invasion

87   assays, because the numbers of free merozoites in culture supernatants are very low

88   (Suarez and McElwain, 2008; Sun et al., 2011). In contrast, large quantities of free

89   merozoites can be obtained with high-voltage electroporation, but the viability of the

90   merozoites is greatly affected by the harsh isolation method (Rodriguez et al., 2014).

91   Therefore, the establishment of a new method is required to obtain large quantities of

92   free, viable, invasion-competent merozoites from *in vitro* cultures of *B. bovis*.

93   A previous study reported that when *in vitro* cultures of *Babesia* parasites (*B.*

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94   *divergens* and *B. major*) were stored at 4 °C, the paired form of the intraerythrocytic

95   parasites was released into the culture medium as free merozoites (Konrad et al., 1985).

96   Consistent with this observation, Rodriguez et al. (2014) reported the use of cold

97   treatment to isolate free merozoites of *B. bovis*. However, the merozoites liberated by

98   that group were not evaluated for their potential suitability for invasion and

99   invasion-inhibition assays. Therefore, in the present study, *in vitro* cultures of *B. bovis*

100   were subjected to cold treatment in an ice bath. The free merozoites released from the

101   infected RBCs were purified and analyzed for their potential utility in RBC invasion

102   and invasion-inhibition assays.

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104 **2. Materials and methods**

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106 *2.1.* In vitro *culture of* B. bovis

107   *Babesia bovis* (Texas strain) was cultured in purified bovine RBCs in

108 serum-free GIT medium (Wako, Osaka, Japan), as described previously (Bork et al.,

109 2005). Parasite cultures with about 30% parasitemia were used to isolate *B. bovis*

110 merozoites.

111

112 *2.2. Optimization of the duration of cold treatment and purification of free merozoites*

113   An aliquot (1 ml) of an *in vitro* culture of *B. bovis*, containing 100 µl of RBCs

114 (10% hematocrit), was subjected to cold treatment for 1, 2, 3, or 4 h in an ice bath. After

115 treatment, each culture was resuspended in 10 ml of GIT medium, and slowly overlaid

116 onto 2 ml of 30% (1.043 g/ml density) Percoll/phosphate-buffered saline (PBS) solution

117 (GE Healthcare, Buckinghamshire, UK) at the bottom of a 50 ml centrifuge tube

118 (Corning, Corning, NY, USA). The tubes were then centrifuged (Himac CF-9RX,

119 Hitachi Koki, Tokyo, Japan) at $280 \times g$ for 5 min at 4 °C, and then at $330 \times g$ for 20 min.

120   An aliquot (10 ml) of the supernatant was transferred to a new tube and centrifuged at

121     $1,500 \times g$ for 5 min at 4 °C. The resulting pellet, containing free merozoites, was

122   washed twice with GIT medium and then suspended in 1 ml of GIT medium. The

123   concentration of purified merozoites was determined with a disposable hemocytometer

124   (AR Brown, Tokyo, Japan). The viability of the merozoites was assessed with

125   6-carboxyfluorescein diacetate (6-CFDA; Invitrogen Corp., Carlsbad, CA, USA), which

126   stains only live merozoites, and propidium iodide (PI; Dojindo, Kumamoto, Japan),

127   which stains both live and dead merozoites, as described previously (McElwain et al.,

128   1987; Zotta et al., 2012). Briefly, 100 µl of merozoite-containing GIT was mixed with

129   6-CFDA and PI at final concentrations of 50 µM and 20 µM, respectively, and

130   incubated at room temperature for 15 min. After the samples were washed with PBS,

131   the numbers of total (both live and dead merozoites) and the numbers of live merozoites

132   were counted with a disposable hemocytometer under a fluorescence microscope

133   (Keyence, Osaka, Japan) to calculate the viability of the merozoites.

134

135   *2.3. Dynamics of intraerythrocytic parasite stages during cold treatment*

136   An aliquot (1 ml) of *B. bovis in vitro* culture, containing 100 µl of RBCs, was

137   incubated in an ice bath or at 37°C for 1, 2, 3, or 4 hours. Erythrocyte smears prepared

138   at the each time point of incubation were stained with Giemsa and observed under a

139   light microscope to determine the percentages of paired and single parasite stages

140   within the erythrocytes. The experiment was repeated thrice.

141

142   *2.4. Comparison of RBC invasion efficiencies of free merozoites obtained with cold*

143   *treatment or high-voltage electroporation*

144   The efficiencies of the free merozoites of *B. bovis* obtained with cold

145   treatment or electroporation in invading fresh RBCs *in vitro* were analyzed and

146   compared. *Babesia bovis* cultures (100 μl) with 30% parasitemia were incubated on ice

147   for the optimal time. The liberated free merozoites were purified and their viability

148   analyzed, as described in section 2.2. *Babesia bovis*-infected RBCs sourced from the

149   same *in vitro* culture were also used to isolate the merozoites by the application of

150   high-voltage pulses, as described previously, with minor modifications (Franssen et al.,

151   2003; Sun et al., 2011). Briefly, 100 µl of *B. bovis*-infected RBCs with 30% parasitemia

152   were mixed with 300 μl of GIT medium. The mixture was added to a 0.2 cm cuvette

153   (Bio-Rad, Hercules, CA, USA) and subjected to five intermittent (10 s, 0 °C),

154   high-voltage pulses (1.5 kV, 400 Ω, 25 μF) with a Bio-Rad Gene Pulser II to rupture the

155   RBCs. The mixture was then washed three times with GIT medium to eliminate the

156   debris from the burst RBCs. The viability of the free merozoites was determined as

157   described in section 2.2.

158   To assess and compare the RBC invasion efficiencies of the isolated

159   merozoites, 50 μl of an uninfected bovine erythrocyte mass, containing 2.21×10^6

160   RBCs, was added to 1 ml of GIT medium containing 9.28×10^6 free viable merozoites

161   (at a multiplicity of infection [MOI] of 4.2) isolated with the cold treatment or

162   electroporation, in triplicate in 24-well plates (Thermo Scientific, K. K., Waltham, MA,

163   USA), and incubated in an atmosphere of 5% CO_2 and 5% O_2 at 37 °C. Thin blood

164   smears were prepared after incubation for 5, 20, or 60 min, and the percentage

165   parasitemia was determined with light microscopy, based on the numbers of parasitized

166   RBCs in 5,000 total RBCs (Bork et al., 2005).

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168   *2.5. RBC invasion-inhibition assays*

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170   *2.5.1. Invasion-inhibition assay using heparin*

171   Heparin, which is reported to inhibit the invasion of *B. bovis* (Bork et al.,

172   2004), was used to assess whether the merozoites obtained with the cold treatment were

173   suitable for invasion-inhibition assays to investigate invasion-blocking chemicals.

174   Uninfected bovine RBCs (50 μl) were added to GIT medium containing 4, 20, or 100

175   units (U) of heparin sodium (Wako) and purified free merozoites at an MOI of 4.2, in

176   triplicate in a 24-well plate, as described in section 2.3. The cultures were incubated,

177   and the percentage parasitemia was determined after incubation for 60 min.

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179   *2.5.2. Invasion-inhibition assays using* B. bovis-*specific antibodies*

180   Two kinds of antibodies, an antiserum raised in a rabbit against recombinant

181   *B. bovis* RAP-1 (rRAP-1) and serum from a cow experimentally infected with *B. bovis*,

182   were used in this study to assess whether the merozoites isolated with the cold treatment

183   can be used effectively in invasion-inhibition assays to investigate *B. bovis*-specific

184   neutralizing antibodies. Recombinant RAP-1 was expressed and purified as a

185   glutathione *S*-transferase (GST) fusion protein (GST–rRAP-1), as described previously

186   (Yokoyama et al., 2002). Japanese white rabbits (CLEA, Tokyo, Japan) were

187   immunized with the GST–rRAP-1 or GST antigen (control) to produce polyclonal

188   antibodies (rabbit anti-GST–rRAP-1 or rabbit anti-GST, respectively), with a previously

189   described method (Terkawi et al., 2013). The serum from a cow experimentally infected

190   with *B. bovis* was described in a previous report (Terkawi et al., 2011). Immunization

191   experiments in animals were conducted in accordance with the standards for the care

192   and management of experimental animals stipulated by the Obihiro University of

193   Agriculture and Veterinary Medicine, Hokkaido, Japan.

194   In the RBC invasion-inhibition assays performed in triplicate in a 96-well

195   plate, uninfected bovine RBCs were added to GIT medium containing rabbit

196   anti-GST–rRAP-1 immune serum (diluted 1:100, 1:500, or 1:1000) or experimentally

197   infected *B. bovis* bovine serum (diluted 1:100, 1:500, or 1:2500) with purified free

198   merozoites at an MOI of 4.2. Medium without serum, medium with rabbit anti-GST,

199   and medium with uninfected bovine serum were used as the negative controls. The

200   percentage parasitemia in each culture was determined after incubation for 60 min, and

201   then compared with those of the corresponding controls.

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203   *2.6. Statistical analyses*

204   All data were analyzed with an independent-samples Student's *t* test. *P* values <

205   0.05 were considered statistically significant.

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207

208 **3. Results**

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210 *3.1. Isolation of free viable merozoites with cold treatment*

211 *In vitro* cultures of *B. bovis* were incubated for 1–4 hours in an ice bath to

212 induce the release of free merozoites from the infected RBCs. The merozoites liberated

213 from the RBCs were purified with Percoll-gradient centrifugation, and the numbers of

214 viable merozoites were counted. The results confirmed that the cold treatment released

215 free viable merozoites from the infected RBCs, and that the maximum number of viable

216 merozoites was obtained after ice-bath incubation for 2 h (Fig. S1). When the cultures

217 were incubated for more than 2 h, the viable merozoite counts declined with time.

218 Therefore, a period of 2 h was considered optimal for the ice-bath treatment, and was

219 used in subsequent experiments involving cold-treatment-based merozoite isolation.

220 The viability of the merozoites isolated with the cold treatment or

221 high-voltage electroporation was compared. The viability of the merozoites isolated

222 with the cold treatment (72.4%) was much higher than the viability of those isolated

223 with high-voltage electroporation (48.5%; $P < 0.05$).

224   When the *in vitro* culture was incubated in an ice bath for 2 or more hours,

225 the percentage of paired merozoites within erythrocytes significantly decreased as

226 compared to that determined at 0-hour incubation (Table 1). Therefore, the percentage

227 of single forms relatively increased during cold treatment. In contrast, such differences

228 were not observed in culture that had been incubated at 37°C.

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230 *3.2. RBC invasion assays using viable merozoites prepared with cold treatment or*

231 *high-voltage electroporation*

232   The free *B. bovis* merozoites prepared with cold treatment or high-voltage

233 electroporation were incubated with uninfected bovine RBCs in GIT medium, and

234 parasitemia was monitored at different time points (5, 20, and 60 min) to determine the

235 invasion efficiencies of the merozoites. The parasitemia in the cultures initiated with

236 free merozoites obtained with both methods gradually increased with increasing

237 incubation time, but the merozoites released by the cold treatment caused significantly

238 greater parasitemia at all the time points examined than did those liberated with

239 high-voltage electroporation ($P < 0.05$; Fig. 1). Consistent with these observations, the

240   invasion efficiency of the viable merozoites isolated with the cold treatment (0.572%)

241   was higher than that of the merozoites isolated with high-voltage electroporation

242   (0.251%), when calculated from the MOI (4.2) and the 60 min parasitemia values.

243

244   *3.3 Invasion-inhibition assays using invasion-blocking agents*

245   To confirm the potential utility of the free merozoites liberated with the cold

246   treatment in the *in vitro* assays used to screen chemical and biological agents that may

247   inhibit RBC invasion by *B*. *bovis* merozoites, invasion-inhibition assays were performed

248   using heparin or *B*. *bovis*-specific antibodies. RBC invasion by the free viable

249   merozoites prepared with the cold treatment was significantly inhibited by 4–100 U of

250   heparin but not in the control cultures, which contained no heparin ($P < 0.05$; Fig. 2A).

251   When rabbit anti-GST–rRAP-1 antiserum and the *B*. *bovis*-infected bovine serum were

252   analyzed in the invasion-inhibition assays with free merozoites isolated with the cold

253   treatment, RBC invasion by the merozoites was significantly inhibited by both sera at

254   low dilutions (1:100) compared with the controls (Figs. 2B and 2C, respectively),

255   suggesting that the free merozoites prepared with the cold treatment can be effectively

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256   used to screen chemical and biological agents that inhibit RBC invasion by *B. bovis*.

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4. Discussion

The currently available methods used to isolate free, viable merozoites, which are essential for RBC invasion and invasion-inhibition assays, are constrained by either the requirement for a large volume of *in vitro* culture or the low viability of the merozoites obtained (Rodriguez et al., 2014; Suarez and McElwain, 2008; Sun *et al.,* 2011). On the other hand, the cold treatment method reported by Rodriguez et al. (2014) could be an alternative technique to obtain free, viable *B. bovis* merozoites. However, the merozoites liberated by cold treatment were not evaluated for their utility in RBC invasion and invasion-inhibition assays. Therefore, in the present study, we analyzed the suitability of the free merozoites prepared with the cold treatment for RBC invasion and invasion-inhibition assays.

When *in vitro* cultures of *B. bovis* were incubated in an ice bath for 1–4 h, the number of free viable merozoites isolated increased steadily for 2 h, after which a significant reduction was observed, suggesting that the viability of the free merozoites released into the culture medium was affected by exposure to cold for more than 2 h. Therefore, 2 h was considered the optimal duration of the cold treatment (Rodriguez et

276   al., 2014). We then compared the viability of free merozoites isolated with the cold

277   treatment or high-voltage electroporation. In agreement with Rodriguez et al. (2014),

278   the viability of the cold-isolated merozoites was significantly higher than that of

279   merozoites isolated with high-voltage pulses. The physical damage caused by the

280   high-voltage pulses could explain the lower viability of these liberated merozoites

281   (Rodriguez et al., 2014).

282   We also analyzed the ratio of different morphological forms of the parasite in

283   the RBCs before and after the cold treatment, and found that the paired merozoites

284   decreased significantly after the cold treatment, while the single forms relatively

285   increased. This suggests that the cold treatment induces the preferential release of the

286   paired merozoites, which are ready to egress and invade new RBCs (Konrad et al.,

287   1985). In contrast, although large numbers of merozoites can be obtained with

288   high-voltage electroporation, different morphological forms, including immature stages

289   and dead parasites, are invariably released with this technique, resulting in a low

290   viability of isolated merozoites. This could also explain why the merozoites isolated

291   with the cold treatment invaded the RBCs more efficiently than those isolated with

292   high-voltage electroporation, even though the viable merozoites prepared with both

293   methods were added to the culture at the same MOIs. Furthermore, even the paired

294   merozoites liberated by high-voltage pulses may not invade the RBCs effectively,

295   because their membrane properties may have been altered by the harsh treatment.

296   Having confirmed the viability and RBC invasion efficacy of the free

297   merozoites prepared with the cold treatment, we tested whether these merozoites can be

298   effectively used for RBC invasion-inhibition assays, which are used widely to

299   investigate invasion-blocking chemicals and to analyze the antibodies raised against

300   candidate vaccine antigens. Lower parasitemia was observed in cultures containing

301   heparin or antibodies directed against *B. bovis* than in the corresponding controls,

302   suggesting that the targets of these inhibitors are intact in the merozoites prepared with

303   the cold treatment and that they can therefore be effectively used in invasion-inhibition

304   assays.

305   In summary, the cold treatment technique, which unlike high-voltage

306   electroporation requires no sophisticated equipment, is an effective tool with which to

307   prepare large quantities of free, viable, invasion-competent merozoites from *in vitro*

308   cultures of *B. bovis*. The merozoites obtained with this method are well suited to the

309   RBC invasion and invasion-inhibition assays. Therefore, the cold treatment will

310   facilitate research into the mechanism of RBC invasion by *B. bovis*, novel candidate

311   vaccines, and invasion-blocking chemicals, allowing the development of improved

312   control and preventive strategies for *B. bovis*.

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Figure Legends

441

442 **Fig. 1. Comparison of RBC invasion efficiencies of free viable merozoites prepared**

443 **with the cold treatment or high-voltage electroporation.** Free viable merozoites

444 prepared with the cold treatment or high-voltage electroporation were added in triplicate

445 to culture plates containing uninfected bovine RBCs at MOI = 4.2. The percentage

446 parasitemia of the cultures was measured after incubation for 5, 20, and 60 min. The

447 asterisks indicate that the parasitemia in cultures initiated with merozoites prepared with

448 the cold treatment was significantly higher ($P < 0.05$) at all three time points than that

449 of cultures initiated with merozoites obtained with high-voltage electroporation,

450 indicating that the merozoites prepared with the former method invaded the RBCs more

451 efficiently than those obtained with the latter technique.

452

453 **Fig. 2. RBC invasion-inhibition assays using free viable merozoites obtained with**

454 **the cold treatment.** The suitability of free viable merozoites prepared with the cold

455 treatment for RBC invasion-inhibition assays was assessed using heparin (**A**), rabbit

456 anti-GST–rRAP-1 antiserum (**B**), and *B. bovis*-infected bovine serum (**C**). Uninfected

457   bovine RBCs were incubated (at MOI = 4.2) in duplicate with free merozoites prepared

458   with the cold treatment in the presence of 4, 20, or 100 U of heparin (**A**), rabbit

459   anti-GST–rRAP-1 (diluted 1:100, 1:500, or 1:1000) or rabbit anti-GST antiserum

460   (diluted 1:100) (**B**), or *B. bovis*-infected bovine serum (diluted 1:100, 1:500, or 1:2500)

461   or uninfected bovine serum (diluted 1:100) (**C**). Culture medium containing no inhibitor

462   was also used as a control. Parasitemia was determined after incubation for 60 min.

463   Data are expressed as the percentage of the mean parasite growth rate relative to that in

464   the control culture without inhibitors. The asterisks indicate that the relative growth

465   rates were significantly reduced ($P < 0.05$) by heparin (4–100 U), rabbit

466   anti-GST–rRAP-1 antiserum (1:100), and *B. bovis*-infected serum (1:100) compared

467   with the growth rates of the corresponding controls, indicating that RBC invasion by the

468   merozoites was inhibited by heparin and by the specific antibodies directed against *B.*

469   *bovis*.

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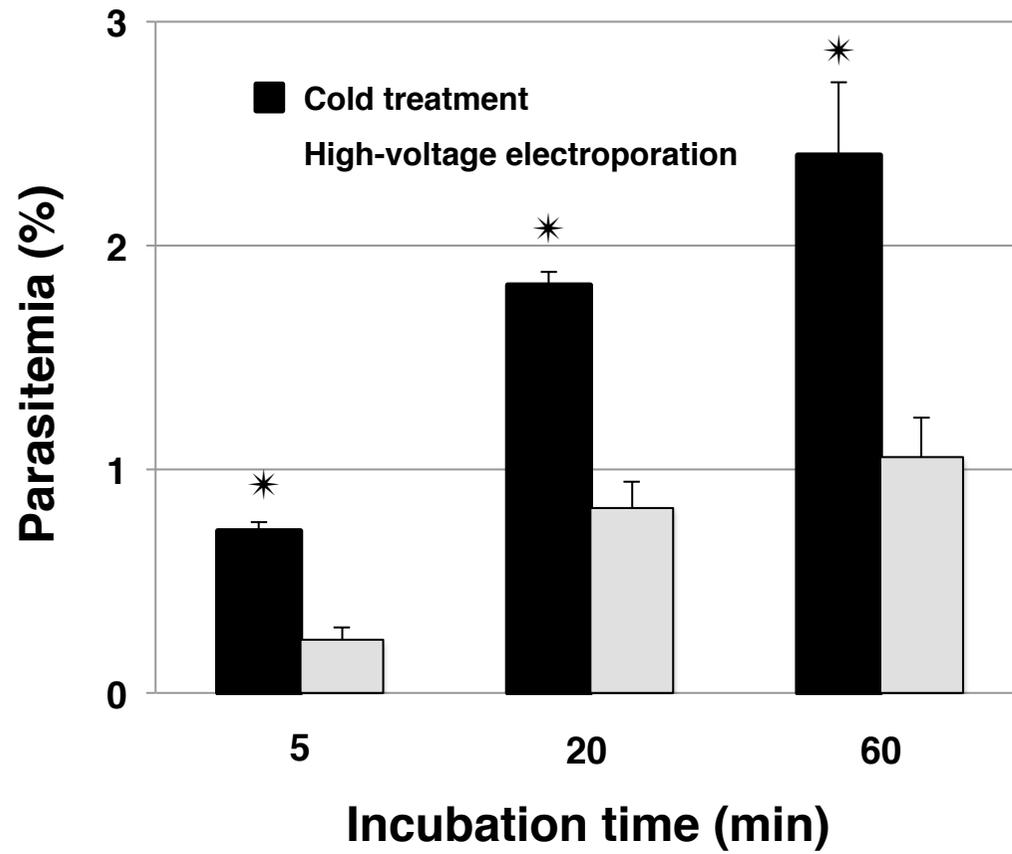
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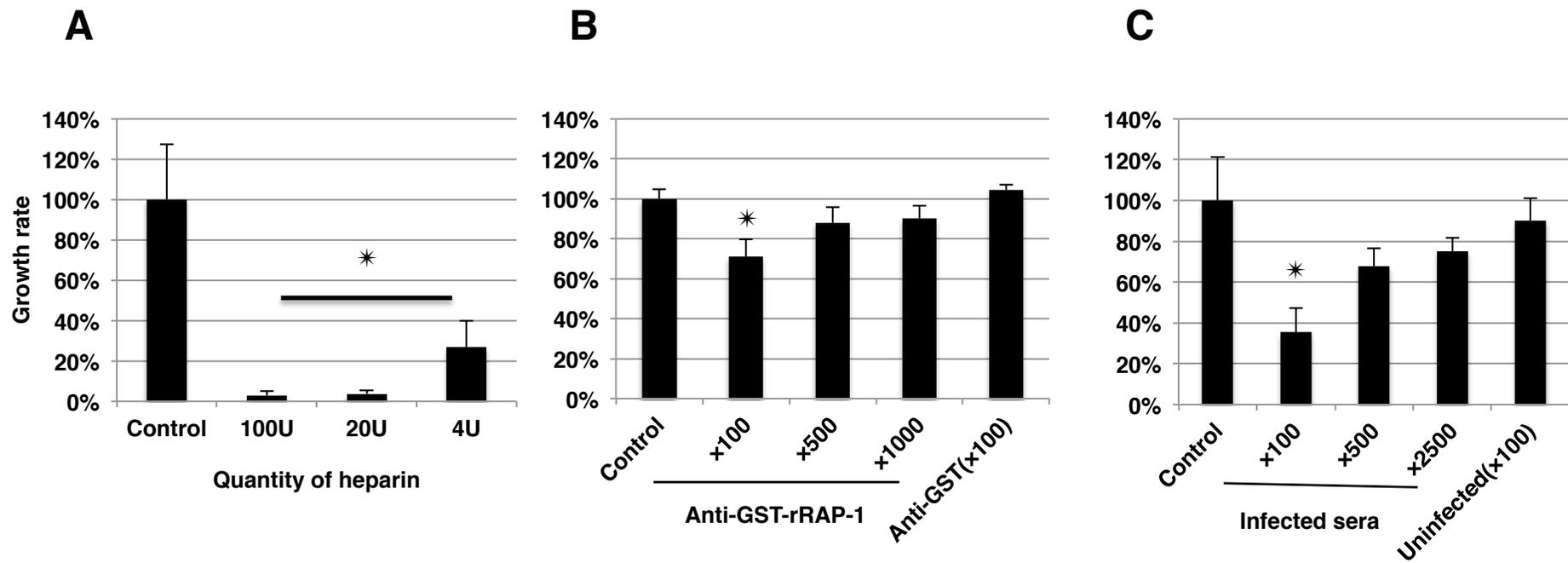
Fig. 2

Table 1. The changes in the percentage of paired and single stages of *B. bovis* within the erythrocytes during the incubation of *in vitro* cultures in ice bath

Hours post-incubation	Intraerythrocytic stages (%)[a]		SD[b]	*P* value[c]
	Paired	Single		
Ice-bath incubation				
0	46.41	53.59	2.01	
1	43.14	56.86	0.93	0.104
2	39.66	60.34	1.12	0.014[d]
3	40.84	59.16	1.86	0.045[d]
4	39.15	60.85	0.44	0.007[d]
37°C incubation[e]				
0	45.75	54.25	2.87	
1	45.99	54.01	3.28	0.940
2	45.07	54.93	2.58	0.815
3	44.42	55.58	1.11	0.576
4	45.37	54.63	1.16	0.873

[a] Giemsa-stained erythrocyte smears prepared at each time point of incubation were observed under a light microscope to determine the percentage of paired and single parasite stages.
[b] SD, Standard deviation
[c] P values were calculated as compare to 0-hour incubation.
[d] The percentage of paired merozoites within the erythrocytes significantly decreased ($P < 0.05$) in *in vitro* cultures incubated in ice-bath for 2 or more hours.
[e] The percentages of paired as well as single parasite stages did not change with time when the *in vitro* cultures were incubated at 37°C for 4 hours.
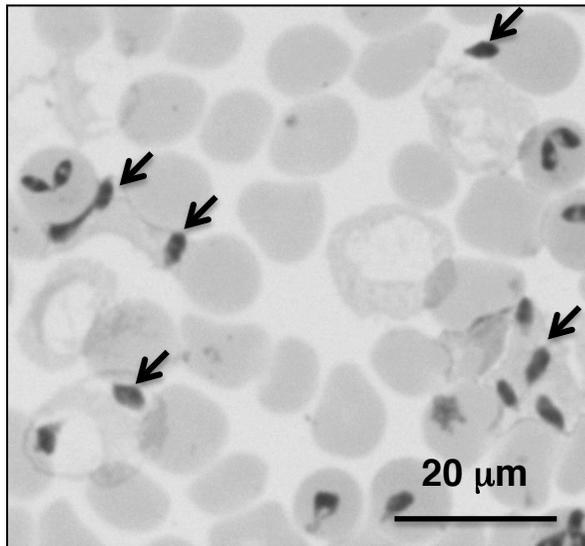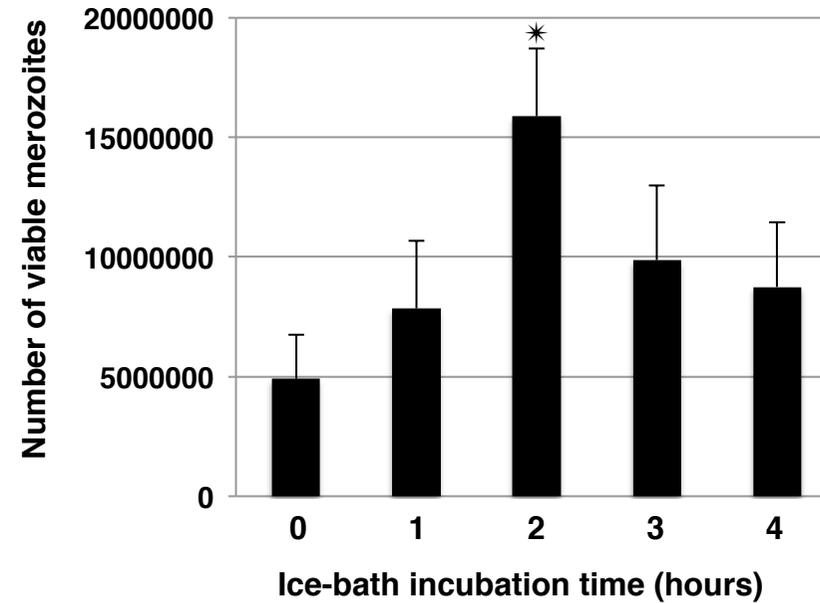
Fig. S1. Cold-treatment-induced release of free viable *B. bovis* merozoites from infected RBCs. Panel A: A light microscopic image of a Giemsa-stained thin blood smear prepared from an *in vitro* culture of *B. bovis* that was subjected to cold treatment in an ice bath for 2 h, showing the release of free merozoites. Arrows indicate free merozoites found inside and outside the RBC ghosts. **Panel B**: Total number of free viable merozoites purified with Percoll-gradient centrifugation from 100 ml of RBCs from *in vitro* cultures of *B. bovis* after incubation in an ice bath for 1, 2, 3, or 4 h. The asterisk indicates that the highest number of free viable merozoites was isolated from the culture incubated in the ice bath for 2 h, compared with those incubated for 0, 1, 3, or 4 h ($P < 0.05$).