Isolation of viable Babesia bovis merozoites to study parasite invasion

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Title:

Isolation of viable *Babesia bovis* merozoites to study parasite invasion

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Abstract

Babesia parasite invades exclusively red blood cell (RBC) in mammalian host and induces alterations to host cell for survival. Despite the importance of *Babesia* in livestock industry and emerging cases in humans, their basic biology is hampered by lack of suitable biological tools. In this study, we aimed to develop a synchronization method for *Babesia bovis* which causes the most pathogenic form of bovine babesiosis. Initially, we used compound 2 (C2), a specific inhibitor of cyclic GMP-dependent protein kinase (PKG) and a derivative of C2, ML10. While both inhibitors were able to prevent *B. bovis* egress from RBC and increased percentage of binary forms, removal of inhibitors from culture did not result in a synchronized egress of parasites. Because using PKG inhibitors alone was not efficient to induce a synchronized culture, we isolated viable and invasive *B. bovis* merozoites and showed dynamics of merozoite invasion and development in RBCs. Using isolated merozoites we showed that BbVEAP, VESA1-export associated protein, is essential for parasite development in the RBC while has no role in invasion. Given the importance of invasion for establishment of infection, this study paves the way for finding novel antigens to be used in control strategies against bovine babesiosis.

Introduction

Babesia bovis is a tick-borne intracellular protozoan parasite causes the most pathogenic form of bovine babesiosis. *B. bovis* has a complex lifecycle with sexual and asexual replication in tick vector and asexual multiplication in cattle as the intermediate host [1,2]. Asexual replication inside red blood cell (RBC) is responsible for the parasite pathogenesis. Infection starts with the invasion of parasite sporozoites released from tick salivary glands followed by parasite growth, DNA replication, parasite multiplication, and finally egress from RBC. The egressed merozoites invade new RBCs and this cycle continues. These changes are driven by stage-specific gene expression in the parasite [3]. Currently, several *Babesia* species including *B. bovis* could be cultured *in vitro* [4]. Following invasion, young merozoite is seen as ring (ring or trophozoite stage) and become paired or binary form following DNA replication which is considered as mature stage of the parasite. Parasite multiplication happens through binary fission which results in production of two daughter cells [5]. Tetrad or maltese form which results from two rounds of DNA replication is not frequently seen in *B. bovis* and accounts for less than one percent of parasites in *in vitro* culture. Therefore, unlike *Babesia divergens* which makes a complex population structure [6], the majority of *B. bovis* parasites are seen as ring or binary forms.

The parasite multiply asynchronously *in vivo* and in the culture with appearance of single, binary and free merozoites simultaneously [2]. In order to study parasite developmental stages such as egress or invasion which happens within minutes, it is needed to artificially induce synchrony in the culture. *Babesia* parasites increase RBC permeability and change its density that could be used for enrichment and purification of infected RBCs (iRBCs) using percol-sorbitol or Histodenz density gradient centrifugation [7,8]. However, given that the parasite's erythrocytic cycle is quite short (1-2 cycles per day for *B. bovis*), it is impossible to separate ring from binary forms using

these techniques. Synchronous culture could be produced using free merozoites isolated from culture supernatant [9,10], released by electroporation [11], or cold treatment [12]. However, all these methods have drawbacks such as the need for large scale culture volume due to their low efficiency or parasite damage in case of using electroporation.

Inhibition of egress using chemicals targeting cyclic GMP-dependent protein kinase (PKG) was used to synchronize *Plasmodium falciparum* and *P. knowlesi*, the causative agents of human malaria [13-15]. In this study, initially we used compound 2 (C2) and a derivative of C2, ML10, the specific inhibitors of PKG, and showed that they can prevent egress and increase proportion of binary forms. However, removal of these compounds did not result in a synchronous egress of parasites. Therefore, we used filtration to mechanically release merozoites from erythrocytes and used these free merozoites to study the kinetics of RBC invasion by *B. bovis*.

Results

1. Time-lapse imaging of *B. bovis*

To visualize the events during *B. bovis* development inside RBC and estimate one erythrocytic cycle, we performed time-lapse imaging using GFP-expressing parasites. This parasite line was produced by replacing *tpx-1* open reading frame with *gfp* and was shown to have no growth defect in the culture [16]. We used confocal microscopy by taking images of growing parasites with 30 seconds interval over 24 h (video 1). We followed up the parasites from invasion into and egress from RBCs. As shown in Fig.1, the average of one complete cycle was 12.4 h (n=10; S.D. ± 2.6 h). We were able to observe transition state from ring to binary form at 2.9 h post invasion (n=10; S.D. ± 1.6 h). Additionally, some ring forms did not develop to binary forms during imaging time which may indicate the existence of gametocytes or dormant *B. bovis* that needs future validation (Video 1, bottom left).

2. Egress arrest using PKG inhibitors

To validate application of PKG inhibitors, C2 and ML10, for synchronization of *B. bovis*, we first calculate EC_{50} of these compounds. The wild type parasites were cultured in the presence of C2 or ML10 in a 72 h growth inhibition assay. The EC_{50} of C2 and ML10 was 172±29.4 nM and 69.9±18.7 nM, respectively (Fig. 2). To investigate the proper concentration of drugs and the length of exposure time, we incubated the parasites with different concentrations of ML10 or C2 for 4, 12 or 24 h (Fig 3a). 0.5 µM of ML10 for 4 and 12 h arrested the egress of parasites and increased proportion of binary forms (Fig 3a, b). Exposure to 1 or 2 µM of ML10 for 4 h also prevented egress and increased percentage of binary forms. However, longer exposure to 12 or 24 h with 1 or 2 µM of ML10 affected viability of parasites and significantly decreased parasitemia (Fig 3a).

In regards to C2, 1, 2 or 5 μM of drug for 4 or 12 h was able to prevent egress and increased the proportion of binary parasites up to 95% which was higher than ML10 (Fig 3a, b). Exposure to 2 and 5 μM of C2 for 24 h decreased the viability of parasites seen as a significant decline in parasitemia (Fig 3a). While both drugs were able to arrest egress and increase binary forms, we decided to validate whether this arrest is reversible and how long the arrested parasites remain viable. Following exposure to different concentrations of drug for 4, 12 or 24 h, the iRBCs were pelleted and incubated in fresh medium and parasitemia was calculated at 24 h post drug removal. Incubation with 0.5 μM ML10 up to 24 h or 1 μM C2 for 4 h and 12 h had negligible effect on the parasite viability (Fig 3c). Similarly, exposure to 1 μM of ML10 up to 12 h had minor effect on parasite growth while longer incubation time or increasing drug concentrations significantly declined parasite viability (Fig 3c). We decided to further validate whether 0.5 μM of ML10 or 1 μM of C2 could be used for *B. bovis* synchronization.

The parasites were incubated in the presence of ML10 or C2 for 4 or 12 h. 0.5 μM of ML10 or 1 μM of C2 did not cause a noticeable change in the parasite's morphology (Fig 4a). The drugs were removed and the cultures were monitored for further 36 h. Following drug removal, the parasitemia increased gradually indicating that the egress arrest was reversible (Fig 4b). While incubation with C2 or ML10 increased the proportion of binary forms, drug removal resulted in a gradual and not synchronized egress of parasites (Fig 4c). These results indicate while PKG is important for egress of *B. bovis*, other factors such as calcium-dependent protein kinase (CDPK) and cAMP-dependent kinase (PKA) may contribute [3,17]. Thus, PKG inhibitors alone is not sufficient to synchronize *B. bovis* in the culture.

3. Isolation of viable and invasive *B. bovis* merozoites

Because PKG inhibitors alone were not effective to prepare a synchronized culture, we decided to

mechanically release the merozoites with filtration to synchronize parasites at free merozoite stage and study the parasite invasion. Considering 1.5-1.9 μm size of *B. bovis* merozoite [18], we decided to use 2 μm filter for merozoite purification. Given that following one-time filtration some intact RBCs were seen in Giemsa-stained smear, we performed double filtration which reduced the number of intact noninfected and iRBCs (Sup Fig.1). Next, we investigated the parasite invasion capacity following filtration. Initially, we explored invasion of the purified merozoites following incubation with noninfected bovine RBCs over 1 h time course (Fig 5a). The invasion of *B. bovis* merozoites increased gradually over time and most invasion events happened within 30 min (~80% of total invasion) while merozoites kept their invasion capacity up to 1 h.

The growth of *B. bovis* merozoites following invasion was monitored by Giemsa staining for a course of 36 h (Fig 5b, c). In the *in vitro* culture, the majority of parasites exists as ring or binary forms. Following merozoite invasion, all parasites were ring from up to 4 h and gradually binary form parasites start to appear from 6 h post invasion. The proportion of binary forms gradually increased and became dominant in the culture at 26 h when the parasitemia reached more than 5% (Fig 5b, c). These results suggested that purified merozoites were viable and grow normally in the culture and could be used to study RBC invasion.

4. Induced knockdown of BbVEAP did not affect RBC invasion but arrested parasite development

Recently, we found a novel spherical body protein, BbVEAP, that is exported to RBC during parasite development [8]. Because the export of VESA1, Variant Erythrocyte Surface Antigen 1, as a ligand for cytoadhesion was dependent on the expression of BbVEAP, this protein was named as VESA1-export associated protein, BbVEAP [8]. Additionally, conditional knockdown of

BbVEAP reduced *B. bovis* growth suggesting an important role of this protein for parasite development [8]. To investigate whether this growth defect is due to decreased invasion and/or parasite development in the RBC, we used *bbveap glmS*-myc tagged parasites [8]. The parasites were treated with 5 mM of Glucosamine (GlcN) for 24 h, merozoites were purified, invasion assay was performed, and parasites growth was monitored for 24 h post invasion (Fig. 6). BbVEAP knockdown following GlcN treatment was confirmed by Western blot analysis (Fig 6a). While VEAP knockdown parasites kept their invasion ability similar to control parasites, they showed a significant lower parasitemia at 24 h post invasion with a higher proportion of ring forms, and a decreased percentage of binary forms (Fig 6b, c). Indirect immunofluorescence antibody test at 24 h post invasion confirmed specific knockdown of BbVEAP while the expression of control protein, spherical body protein 4 (SBP4) was unchanged (Sup Fig. 2). Altogether, these results suggest that lower growth rate of BbVEAP knockdown parasites is due to developmental defect and not impaired invasion ability.

Discussions

Dissecting the developmental stages of *B. bovis* inside RBC is hampered by the lack of high throughput synchronization methods. Chemical arrest of egress using PKG inhibitors was successfully used to synchronize *P. falciparum* and *P. knowlesi* [15]. PKG in malaria parasites is responsible for initiation of a signaling cascade that results in the lysis of parasitophorous vacuole membrane (PVM) and subsequently RBC membrane and parasite egress [14]. C2 and ML10 were used to reversibly block PKG and prevent parasite egress [19]. Removal of these inhibitors results in merozoite release within minutes [15]. *B. bovis* has a single PKG with a conserved gatekeeper T618 (BBOV_I004690). In our study, C2 and ML10 were able to prevent parasite egress and increase the proportion of binary forms. While the arrested parasites were able to grow following inhibitor removal, the egress from RBCs was gradual and took several hours. This difference in egress of *B. bovis* with *Plasmodium* could be due to the lack of PVM and the difference in signaling cascade responsible for egress. *B. bovis* PVM is ruptured few minutes after invasion [20] which may cause a difference in egress signaling cascade in comparison with malaria parasites that maintain PVM during development in the RBC. Additionally, in our study, long exposure of *B. bovis* to PKG inhibitors did not result in induction of multiple fission [5] and production maltese form or iRBCs with more than two parasites. This is similar to *Plasmodium* that longer exposure of PKG inhibitors decline the viability and did not increase merozoite numbers per schizont [15]. Treatment of *B. bovis* with a bumped kinase inhibitor, RM-1-152, resulted in egress arrest and an increase in number of parasites per RBC [21] which is similar to egress block in *Toxoplasma gondii* [22]. However, the targeting kinase by RM-1-152 is unclear to delineate the egress block phenotype in *B. bovis* and needs further investigation [3]. Egress and growth arrest of *B. bovis* in the presence of PKG inhibitors

confirmed PKG as a promising target for treatment babesiosis and these compounds could be used to study egress in these parasites.

Because application of PKG inhibitors did not produce a synchronized *B. bovis* culture, we decided to mechanically release the merozoites from RBC. Isolation of free merozoites have been used for culture synchronization and studying invasion of malaria parasites and *B. divergens* [6,23-25]. The purified *B. bovis* merozoites were able to invade even up to 1 hour after mixing with bovine RBCs. However, 80% of total invasion happened within 30 min. This ability to keep the invasion capacity for long duration is similar to *B. divergens* and *P. knowlesi* [6,25] and different from *P. falciparum* merozoites which are viable for few minutes [23]. To have a shorter window of invasion and a tighter synchronized culture, it is possible to prevent further invasion using invasion inhibitors such as heparin [26]. Purified *B. bovis* merozoites started growing upon invasion and lost their synchronicity within one cycle (Fig 5c). This is partially due to the short lifecycle of *B. bovis* and their dynamic growth. However, simplicity and high throughput efficiency of merozoite purification in this study and our established time-lapse imaging can make this method as a routine and valuable tool to study *B. bovis* invasion and its development in the RBC.

We used purified merozoites to study the function of BbVEAP, a recently identified secreted protein into the RBC cytoplasm [8]. Previously, we have shown that BbVEAP is involved in ridge formation, VESA1 export and expression on the surface of iRBC, and cytoadhesion of iRBCs to endothelial cells [8]. While induced knockdown of BbVEAP reduced parasitemia, whether this reduction is due to invasion defect is not known. Here we have shown that BbVEAP knockdown merozoites have no defect in invasion ability and reduction of parasitemia is due to impaired development in the RBC. This gene is conserved across piroplasmida and is upregulated in *B. bovis* blood stage [27] which suggests a conserved important function for BbVEAP during

development in the RBC. Identification and characterization of interacting proteins with BbVEAP may shed light on BbVEAP role during parasite development.

In conclusion, the methodology of merozoite purification introduced in this paper is robust and could be a valuable tool to study *B. bovis* development in the RBC.

Methods

Parasite culture

B. bovis Texas strain was maintained in culture using a microaerophilic stationary-phase culture system composed of bovine RBCs at 10% hematocrit and GIT medium (Wako Pure Chemical Industries, Japan). For time-lapse imaging, a hybriwell chamber with 13 mm diameter and 0.15 mm depth (Grace BioLabs, USA) was used. Cells with 3% hematocrit from parasite culture were loaded, the chamber was sealed, and cells were viewed at 37 °C using a confocal laser-scanning microscope (A1R; Nikon, Japan). The images were taken using DIC and laser 488 nm for GFP at 30 s interval over 24 h.

Measurement of EC_{50} of PKG inhibitors

C2 and ML10 were received from LifeArc, dissolved in DMSO at 2 mM concentration, and kept at -30 °C. *B. bovis* wild type parasites were cultured in the absence or presence of different concentrations of C2 or ML10. The initial parasitemia was 0.05% and parasites were cultured in triplicate for 3 days with daily culture medium change. Parasitemia was calculated by examining at least 10,000 RBCs on thin smears prepared on day 3.

Merozoite purification

IRBCs from culture with 5% parasitemia or more were filtered twice at room temperature through a filter unit with a 2 μm pore size and 25 mm diameter (Isopore ™), spun down and washed with PBS (1000 g, 5 min, RT). The merozoites were quantified using a hemocytometer and used for invasion assay with a ratio of 1:10 to bovine RBCs. Merozoites and bovine RBCs at 10% hematocrit was mixed at 250 rpm for 10 min at 37 °C and transferred to culture incubator.

Western blotting

IRBCs were treated with 0.2% saponin and proteins were extracted using 1.0% Triton-X 100 (w/v) in PBS containing protease inhibitor cocktail (Complete Mini, Roche) at 4°C for 1 h. The protein fractions were separated by electrophoresis using 5–20% SDS-polyacrylamide gradient gel (ATTO, Tokyo, Japan) in a reducing condition and transferred to polyvinylidene difluoride membranes (Clear Blot Membrane-P, ATTO, Tokyo, Japan). The membrane was probed with rabbit anti-myc polyclonal antibody (1:500; ab9106, Abcam, UK) or rabbit anti-SBP4 polyclonal antibody (1:1000) [28] at 4°C overnight. The membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1:8,000; Promega, USA) as secondary probe. Protein bands were visualized using Immobilon™ Western Chemiluminescent HRP substrate (Merck Millipore) and detected by ImageQuant LAS 500 (GE healthcare).

Indirect immunofluorescence antibody test

Thin blood smears from cultured parasites were prepared, air-dried and fixed in a 1:1 acetone:methanol mixture at -30°C for 5 min [29]. Smears were blocked with PBS containing 10% normal goat serum (Invitrogen) at 37°C for 30 min and immunostained with mouse anti-myc monoclonal antibody (9B11, Cell Signaling Technology) at 1:500 dilution in PBS supplemented with 0.05% Tween-20 and incubated at 4°C overnight. Double immunostaining of smears was done with rabbit anti-SBP4 at 1:1000 dilution. The smears were incubated with Alexa fluor® 488-conjugated goat anti-mouse or Alexa fluor® 594-conjugated goat anti-rabbit IgG antibody (1:500; Invitrogen) at 37°C for 30 min. Nuclei were stained by incubation of smears with 1 μg/mL Hoechst

33342 solution. The smears were examined using a confocal laser-scanning microscope (CS-SP5, Leica Micro-system, Wetzlar, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. The parasitemia and proportion of parasite stages were plotted and evaluated using unpaired *t*-test. The values were considered significantly different if *P*-value was below 0.05.

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Competing interests

We declare that we have no competing interests.

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Figure legends

Fig 1. Time-lapse imaging of *B. bovis*. The GFP-expressing *B. bovis* was observed over 24 h period and events following parasite egress and subsequent invasion were witnessed (n=10).

Fig 2. Dose-response curve of C2 and ML10 for *B. bovis*. The parasites were cultured in presence of different concentrations of C2 or ML10. Data are shown as mean ± SEM of triplicate culture.

Fig 3. Impact of ML10 and C2 concentration and length of exposure on egress block and parasite viability. a) Parasites were cultured in presence of 0.5, 1 or 2 μM of ML10 or 1, 2 or 5 μM of C2 for 4, 12 or 24 h. The initial parasitemia was ~ 1% and parasitemia in presence of drugs were calculated. The data are shown as mean ± S.D. of triplicate culture. Statistical comparisons were done between each group and initial parasitemia. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; determined by unpaired *t* test). **b)** Proportion of ring, binary, and multiple stages in presence of ML10 or C2 for 4, 12 or 24 h. The data are shown as mean ± S.D. of triplicate culture. **c)** Cultures that were exposed to different concentrations of ML10 or C2 for 4, 12 or 24 h were washed and allowed to grow in fresh medium for 24 h. The statistical significance of the difference between each group and parasite treated for 4 h of 0.5 μM of ML10 or 1 μM of C2 determined by unpaired *t* test. (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). The data are shown as mean ± S.D. of triplicate culture.

Fig 4. *B. bovis* growth and egress following removal of C2 and ML10. a) The Giemsa-stained

smears were prepared before drug removal to validate the effects of drugs on parasite morphology. Scale bar = 10 μm. **b)** Parasites that had been arrested in the culture in the presence of 0.5 μM of ML10 or 1 μM of C2 for 4 or 12 h were washed and transferred to fresh medium to allow egress and invasion of new RBC for 36 h. The data are shown as mean ± S.D. of triplicate culture. **c)** Proportion of ring, binary and multiple stages in initial parasites, at the time and following removal of ML10 or C2 were calculated for 36 h (mean ± S.D. of triplicate culture).

Fig 5. Invasion kinetics of *B. bovis* filter isolated merozoites. a) The proportion of invaded merozoites over time is shown as % of invasion after 60 mins (mean ± S.D. of three independent experiments in triplicate culture). **b)** Parasite growth over 36 h time course. Smears were prepared every 2 h and data are shown as mean ± S.D. of triplicate culture. **c)** Proportion of ring, binary and multiple stages following invasion (mean ± S.D. of triplicate experiment).

Fig 6. BbVEAP knockdown did not affect parasite invasion. a) Western blot analysis of myc-*glmS* expressing *B. bovis* in the presence or absence of glucosamine (GlcN). Anti-SBP4 antibody was used to detect SBP4 protein as a loading control. The image is representative of three independent experiments. **b)** The myc-*glmS* expressing merozoites in the presence or absence of GlcN for 24 h were filter isolated and invasion assay was performed. Parasitemia was measured at 1 h and 24 h after invasion. The data are shown as mean ± S.D. of three independent experiments in triplicate culture. (ns, not significant; **, $P < 0.01$ determined by unpaired *t* test**).** Scale bar = 10 μm. **c)** Proportion of ring, binary and multiple stages in parasites in the absence or presence of GlcN at 1 h or 24 h following invasion (mean ± S.D. of three independent experiments in triplicate culture. **, $P < 0.01$; ***, $P < 0.001$ determined by unpaired *t* test).

Authors contribution

H.H. designed the study, performed the experiments, and wrote the manuscript. H.H., M.A., and S.K. analyzed the results. M.A., T.I., and S.K. contributed to methodology. All authors read, edited, and approved the final version of the manuscript.

Figures

Fig. 1

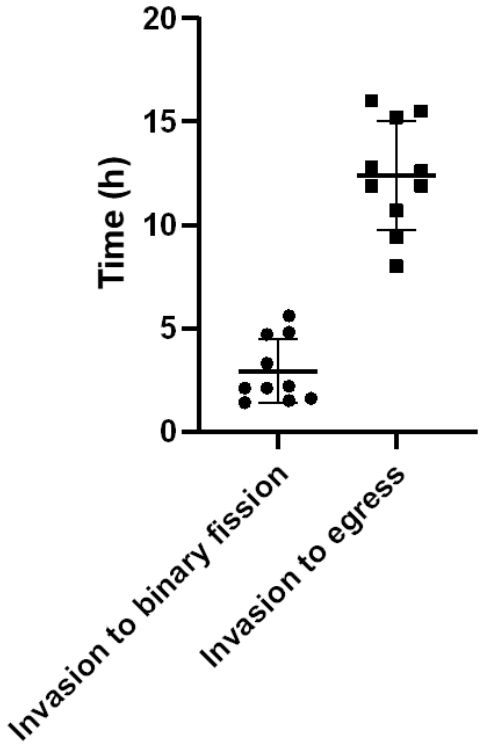

Figure 1

Time-lapse imaging of B. bovis. The GFP-expressing B. bovis was observed over 24 h period and events following parasite egress and subsequent invasion were witnessed (n=10).

Fig. 2

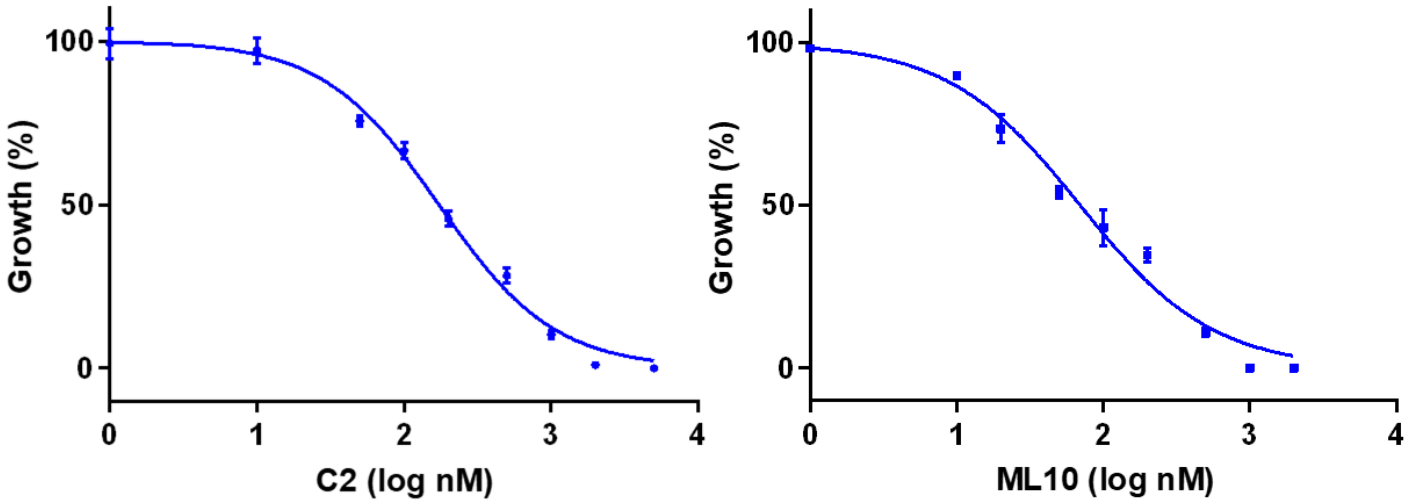

Figure 2

Dose-response curve of C2 and ML10 for B. bovis. The parasites were cultured in presence of different concentrations of C2 or ML10. Data are shown as mean ± SEM of triplicate culture.

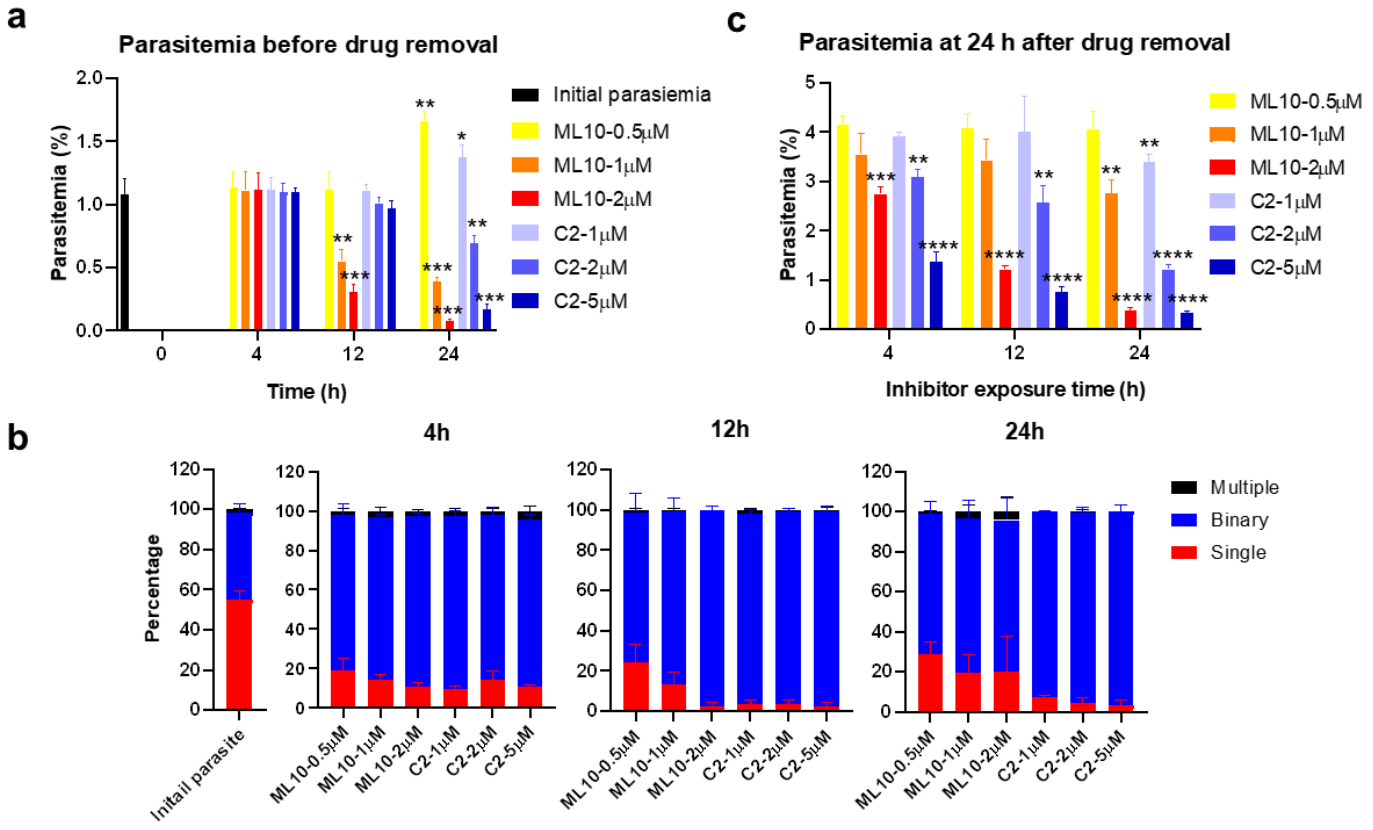

Figure 3

Impact of ML10 and C2 concentration and length of exposure on egress block and parasite viability. a) Parasites were cultured in presence of 0.5, 1 or 2 µM of ML10 or 1, 2 or 5 µM of C2 for 4, 12 or 24 h. The initial parasitemia was ~ 1% and parasitemia in presence of drugs were calculated. The data are shown as mean ± S.D. of triplicate culture. Statistical comparisons were done between each group and initial parasitemia. (*, P < 0.05; **, P < 0.01; ***, P < 0.001; determined by unpaired t test). b) Proportion of ring, binary, and multiple stages in presence of ML10 or C2 for 4, 12 or 24 h. The data are shown as mean ± S.D. of triplicate culture. c) Cultures that were exposed to different concentrations of ML10 or C2 for 4, 12 or 24 h were washed and allowed to grow in fresh medium for 24 h. The statistical significance of the difference between each group and parasite treated for 4 h of 0.5 µM of ML10 or 1 µM of C2 determined by unpaired t test. (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001). The data are shown as mean ± S.D. of triplicate culture.

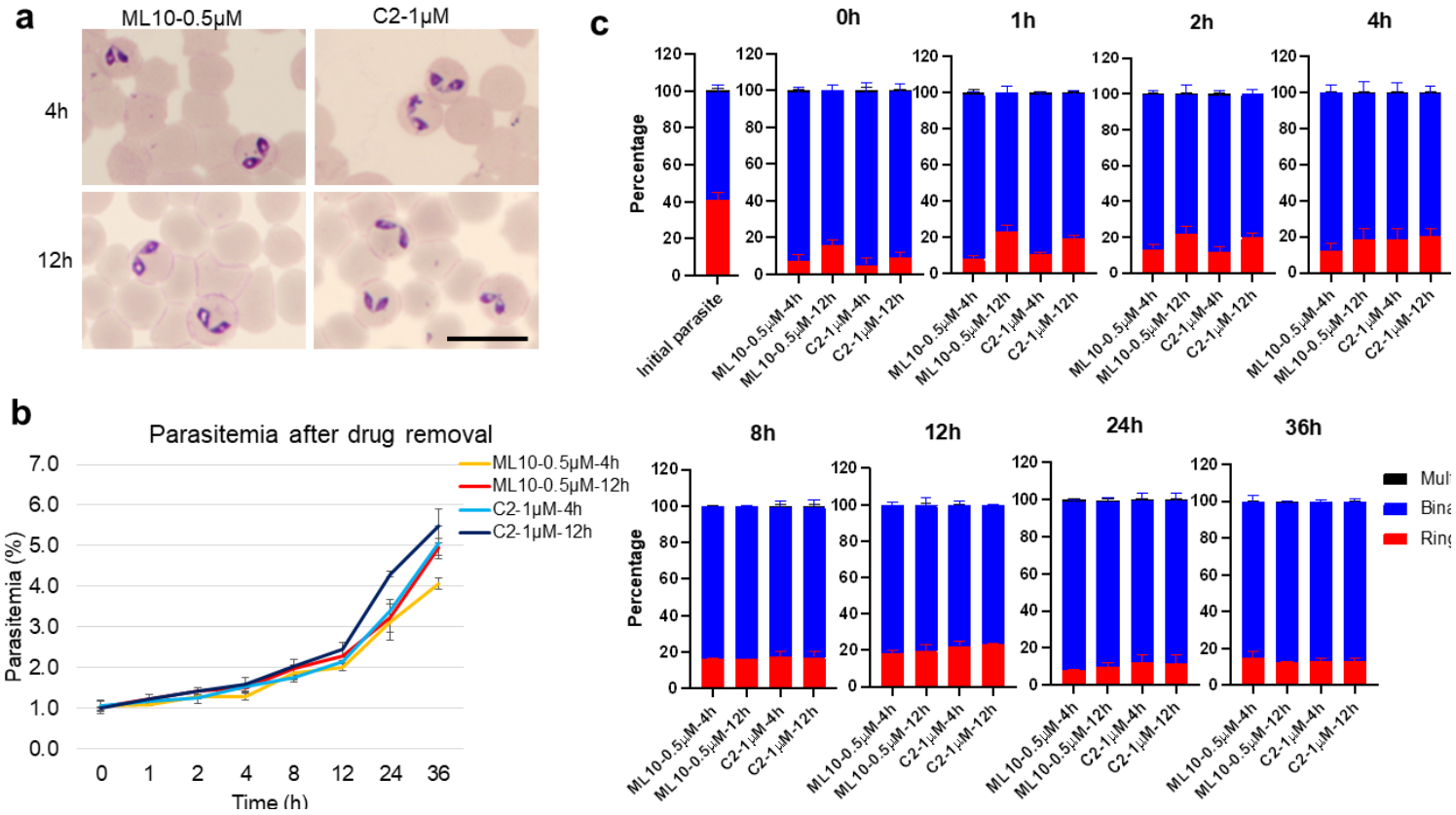

Figure 4

B. bovis growth and egress following removal of C2 and ML10. a) The Giemsa-stained smears were prepared before drug removal to validate the effects of drugs on parasite morphology. Scale bar = 10 µm. b) Parasites that had been arrested in the culture in the presence of 0.5 µM of ML10 or 1 µM of C2 for 4 or 12 h were washed and transferred to fresh medium to allow egress and invasion of new RBC for 36 h. The data are shown as mean ± S.D. of triplicate culture. c) Proportion of ring, binary and multiple stages in initial parasites, at the time and following removal of ML10 or C2 were calculated for 36 h (mean ± S.D. of triplicate culture).

Fig. 5

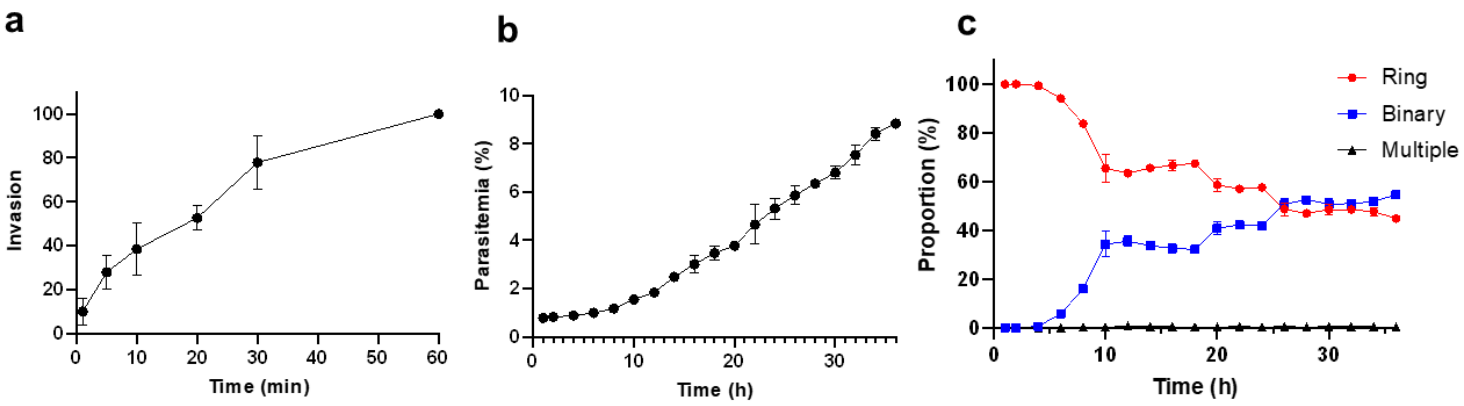

Figure 5

Invasion kinetics of B. bovis filter isolated merozoites. a) The proportion of invaded merozoites over time is shown as % of invasion after 60 mins (mean ± S.D. of three independent experiments in triplicate culture). b) Parasite growth over 36 h time course. Smears were prepared every 2 h and data are shown as mean ± S.D. of triplicate culture. c) Proportion of ring, binary and multiple stages following invasion (mean ± S.D. of triplicate experiment).

Fig. 6

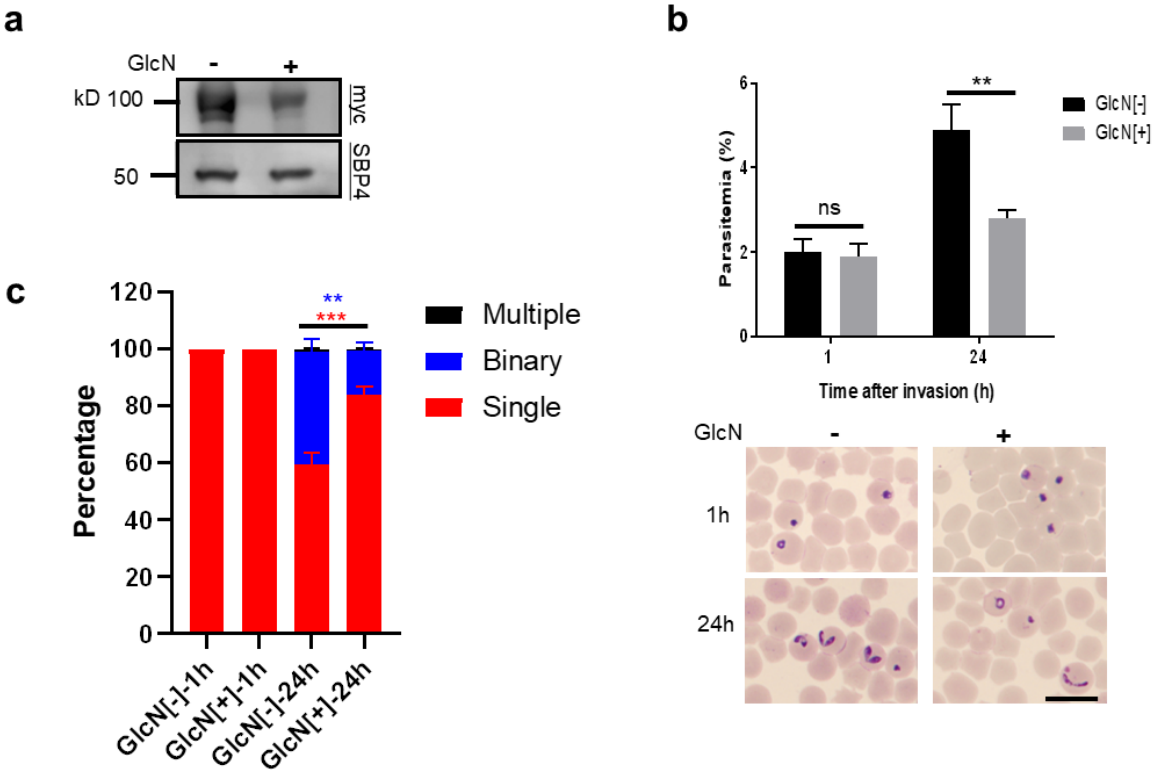

Figure 6

BbVEAP knockdown did not affect parasite invasion. a) Western blot analysis of myc-glmS expressing B. bovis in the presence or absence of glucosamine (GlcN). Anti-SBP4 antibody was used to detect SBP4 protein as a loading control. The image is representative of three independent experiments. b) The myc-glmS expressing merozoites in the presence or absence of GlcN for 24 h were filter isolated and invasion assay was performed. Parasitemia was measured at 1 h and 24 h after invasion. The data are shown as mean ± S.D. of three independent experiments in triplicate culture. (ns, not significant; **, $P < 0.01$ determined by unpaired t test). Scale bar = 10 μm. c) Proportion of ring, binary and multiple stages in parasites in the absence or presence of GlcN at 1 h or 24 h following invasion (mean ± S.D. of three independent experiments in triplicate culture. **, $P < 0.01$; ***, $P < 0.001$ determined by unpaired t test).

Supplementary Files

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