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# The application of the HyPer fluorescent sensor in the real-time detection of $H_2O_2$ in *Babesia bovis* merozoites *in vitro*

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ABSTRACT

In recent years, genetically encoded fluorescent probes have allowed a dramatic advancement in time-lapse imaging, enabling this imaging modality to be used to investigate intracellular events in several apicomplexan parasite species. In this study, we constructed a plasmid vector to stably express a genetically encoded $H_2O_2$ sensor probe called HyPer in *Babesia bovis*. The HyPer-transfected parasite population was successfully developed and subjected to a time-lapse imaging analysis under *in vitro* culture conditions. HyPer was capable of sensing an increasing $H_2O_2$ concentration in the parasite cells which was induced by the administration of paraquat as a superoxide donor. HyPer fluorescence co-staining with MitoTracker Red indicated the mitochondria as the major source of reactive oxygen species (ROS) in parasite cells. The fluctuating ROS dynamics in the parasite gliding toward, attaching to, and invading the target red blood cell was visualized and monitored in real time with the HyPer expressing parasite population. This is the first report to describe the application of the HyPer probe in an imaging analysis involving *Babesia* parasites. Hyper-expressing parasites can be widely utilized in studies to investigate the mechanisms of emergence and the reduction of oxidative stress, as well as the signal transduction in the parasite cells during host invasion and intercellular development.

## 1. Introduction

Bovine babesiosis is a tick-borne disease caused by several *Babesia* species belonging to the phylum apicomplexa. These parasites produce acute and fatal disease in cattle and affect the livestock industry worldwide (Gohil et al., 2013; Florin-Christensen et al., 2014). In recent years, advances of methods to genetically manipulating apicomplexan parasites, such as *Plasmodium falciparum* and *Toxoplasma gondii*, have enabled the study of their behavior within their hosts. Among these methods, genetically encoded fluorescent probes allowed a dramatic advancement in the analysis of time-lapse imaging and facilitated the investigation of the intracellular events of the parasite cells (McGovern and Wilson, 2013; De Niz et al., 2017). However, these approaches, which may reveal important mechanisms involved in parasite invasion of and intercellular development in the host cell, have not been applied in *Babesia* parasites.

After entering the host red blood cell (RBC), *Babesia* merozoites rapidly escape from the parasitophorous vacuole (PV) formed by invagination of the host cell membrane during invasion. Once the divested parasite is established within the RBC cytoplasm, it develops to produce two merozoites by binary fission. As the merozoite actively glides and proliferates in the RBCs without protection from the PV membrane, large amounts of reactive oxygen species (ROS), which can damage biological macromolecules, are expected to be generated in the parasite cells. Mitochondria with a functional electron transport chain represent one of the major sources of ROS in parasite cells (Boveris and Chance, 1973; Munro and Treberg, 2017). However, in the case of *Babesia* parasites, it is not thoroughly understood how ROS are generated and emerge in the parasite cells during their gliding and development in the host RBCs. ROS damage the parasite cell as a harmful by-product, but at the same time, $H_2O_2$ can modify $Ca^{2+}$ signaling, which modulates key events in the parasite lifecycle by functional modification of $Ca^{2+}$ handling proteins (Booth et al., 2016).

Various methods which include chemical fluorescent dyes and genetically encoded sensor probes have been developed to monitor ROS in a living cell in real-time. Chemical fluorescent dyes detect broad range of ROS (Setsukinai et al, 2003) in living cell, however genetically encoded sensor probes can be expressed in each cellular compartment which improves their specificity for ROS monitoring (Oparka et al., 2016). One of such probes is HyPer, a fluorescent protein-based sensor,

*Abbreviations:* DHFR, dihydrofolate reductase; GFP, green fluorescent protein; IRBC, infected red blood cell; PCR, polymerase chain reaction; RBC, red blood cell; TPx-1, thioredoxin peroxidase-1

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that detects $H_2O_2$ in each cellular compartment (Belousov et al., 2006). HyPer has been widely used to analyze cellular redox status (Huang et al., 2017), and in the parasitology, one of the HyPer variants, HyPer-3 was applied to monitor transition of $H_2O_2$ level in the parasite cytoplasm under administration of antimalarial drugs in *Plasmodium falciparum* (Rahbari et al., 2017). In this study, we visualized and monitored the fluctuating ROS dynamics in the merozoite during parasite development in real-time by taking advantage of the HyPer sensor probe as a genetically encoded $H_2O_2$ indicator.

## 2. Materials and methods

### 2.1. Parasites

The Texas strain of *B. bovis* was maintained in purified bovine erythrocytes with GIT medium (Wako Pure Chemical Industries, Osaka, Japan) using a microaerophilic stationary-phase culture system (Bork et al., 2005). In brief, the parasite was cultured in 1 ml of culture medium containing 10% bovine RBCs in 24-well culture plates (Corning, NY, USA). The culture medium was replaced every day and parasitemia was monitored daily by observing thin blood smears that were stained with Giemsa solution.

### 2.2. The development of HyPer-expressing parasites

A schematic diagram of the plasmid that was used in the present study is shown in Fig. 1-a. The plasmid was constructed by cloning the HyPer coding sequence (hyper) between the 5′-flanking region of the elongation factor-1α gene (5′-ef-1α) and the 3′-flanking region of rhoptry associated protein-1 gene (3′-*rap-1*) into our previously developed DHFR-gfp plasmid (Asada et al., 2012b). Once transfected, the plasmid confers the parasite a stably expresses HyPer $H_2O_2$ indicator (Belousov et al., 2006) under a WR99210/human dihydrofolate reductase (*dhfr*) selection system. The *hyper* gene was amplified from the monomeric *hyper* expression plasmid (pHyPer-cyto vector, Evrogen, Moscow, Russia) by a PCR using the following primer pairs: 5′- CCG GAT ATC ATG GAG ATG GCA AGC CAG -3′ and 5′- CCG GAT ATC TTA AAC CGC CTG TTT TAA AAC -3′ (the EcoRV sites are underlined). The constructed plasmid was purified using a Qiagen plasmid midi-kit (Qiagen, MD, USA) in accordance with the manufacturer's instructions and the DNA sequence was confirmed before transfection. The circular plasmids were transfected into the *in vitro*-cultured *B. bovis*-infected red blood cells (IRBCs) using a Nucleofector device (Amaxa Biosystems, Cologne, Germany) as described previously (Asada et al., 2012b). Briefly, 100 µl of IRBCs (at 5–8% parasitemia) was transfected with 10 µg of the plasmid DNA in 100 µl of AMAXA nucleofector human T-cell solution using the v-024 program. Following electroporation, the transfected parasite population was selected using 10 nM WR99210 and the parasite population that expressed yellow fluorescence was cloned by limiting dilution.

### 2.3. Mitochondrial staining

To stain the mitochondria of HyPer-expressing *B. bovis* merozoites, MitoTracker Red CM-H2XRos (Molecular Probes, OR, USA) was used as described previously (Asada et al., 2012a). The fluorescent probe was diluted to 200 nM in GIT medium, and mixed with an equal volume of IRBCs. The mixture was incubated for 30 min at 37 °C and washed with GIT medium 3 times before observation.

### 2.4. Imaging of HyPer expressing parasites

The *in vitro*-cultured HyPer-expressing *B. bovis* IRBCs were observed under a TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 40 × oil immersion objective lens. The HyPer probe was excited at 488 nm and fluorescence was detected in the 500 nm to 520 nm range (Belousov et al., 2006).

The effect of paraquat, a superoxide donor, on the parasite cells was analyzed by time-lapse imaging. A suspension of IRBCs was loaded on a 35-mm glass-bottomed dish (MatTek, MA, USA) that was coated with

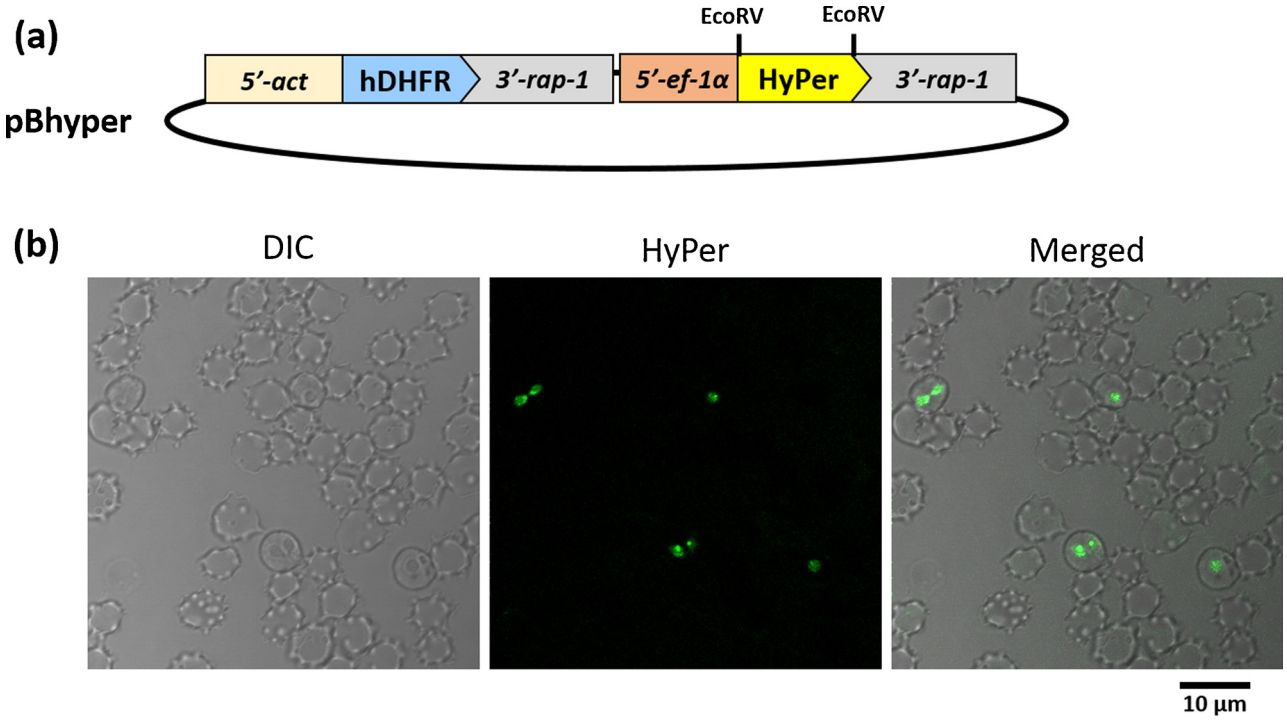


**Fig. 1.** The development of HyPer-expressing *B. bovis*. (a) A schematic diagram of the construction of the HyPer expressing plasmid (pBhyper). pBhyper was constructed based on the DHFR-gfp plasmid (Asada et al., 2012b). hDHFR, human dihydrofolate gene; 5′-*act*, 5′-flanking region of the actin gene; 3′-*rap*, 3′-flanking region of the rhoptry associated protein-1 gene; 5′-*ef-1α*, the 5′-flanking region of the elongation factor-1α gene; and HyPer, the HyPer coding region which was cloned into the EcoRV site of the backbone construct. (b) A live fluorescence image of HyPer-expressing *B. bovis* merozoites. Bar, 10 µm.