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Improvement of SYBR Green I–based fluorescence assay reading procedure for anti–babesial drugs screening *in vitro*

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ABSTRACT

Drug susceptibility of the *Babesia* parasite *in vitro* has been used to assess the anti-babesial activities of new candidates. Recently, the *in vitro* drug susceptibility assay has been performed by SYBR Green I (SG I) -based fluorescence assay. Herein, we investigated the validity of a fluorescence assay for the shipping process under various culture conditions. The highest fluorescence signal was observed in freeze-thaw of *Babesia* and *Theileria* parasites cultures incubated with lysis buffer containing SG I for a half hour. This result indicates the ability of this additional step to lysis of infected RBCs completely with releasing of parasite nucleic acid. Such that, the nucleic acid can interact with SG I stain. All tested drugs showed potential anti-babesial activity and slightly high selective index against the *in vitro* growth of the *Babesia* and *Theileria* parasites. The IC_{50} values generated using the fluorescence assay with additional freeze-thaw step is comparable with those of IC_{50} values obtained from the unmodified fluorescence assay and microscopy method.

Keywords: Fluorescence; *Babesia*; SYBR Green; Freeze–thaw

INTRODUCTION

Babesiosis is a tick-borne protozoan disease caused by genus *Babesia*. The disability and the poverty associated with this disease constitute large burdens on the health and economic development of low- and middle-income countries in the world. Strategies to control this disease are based on surveillance, early diagnosis, vector control, and treatment. So far, there are a few drugs on the market to treat parasitic disease, and they are not universally available in the affected areas. Currently available drugs are inefficiency, toxicity, prolonged treatment schedule, and high cost. Therefore, there is an urgent need for new treatments

which are safe, effective, cheap, and easy-to-administer, and for a lead compound with novel mechanisms of action.

The SYBR green I (SG) dye-based fluorescence-based assay is a fast, inexpensive, readily growth assessment of the parasite and broadly applicable for use in drug screening. The SG dye binds to the double-stranded DNA of parasites in infected erythrocytes and the calculated fluorescence signals used as a measure of parasite growth and propagation or growth inhibition by antiparasitic drugs into the culture (Smilkstein et al., 2004; Rizk et al., 2015).

Although, fluorescence assay was employed for mass drug screening against the *in vitro* growth of *Plasmodium* (Smilkstein et al., 2004) and *Babesia* parasites (Rizk et al., 2015) is reported to have inherent limitations such as low fluorescence reading. It seems to be the presence of hemoglobin in the parasite culture correlated with the level of fluorescence as previously reported for a similar fluorochrome (Quashie et al., 2006). Therefore, there is a need to improve SG assay to overcome these shortages.

Recently, Rizk et al., (2015) optimized fluorescence assay with corresponding HCTs without daily replacement of the culture medium for *Babesia* and/or *Theileria* parasites, which offers a new approach for simplicity, robust performance and rapid detection of the parasites as well as large-scale screening of anti-babesial drugs in an *in vitro* culture. However, the method is not valid for the shipping process. Therefore, in the current study, we have modified the method to be consistence for the shipping process. To achieve this purpose, the effect on fluorescence when the incubation period of culture with lysis buffer containing SG was extended up to 24 h at fixed time intervals and the effect of freeze-thaw on fluorescence intensity was ascertained. Further, this study investigated the anti-babesial and cytotoxic activities of four candidate compounds compared to the standard anti-babesial drug (diminazene aceturate) *in vitro* using fluorescence assay with or without freeze-thaw effect and traditional microscopy method.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine, in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine.

Chemical reagents

SYBR Green I (SG) nucleic acid stain (Lonza, 10.000x) was stored at -20°C until use. A lysis buffer consisting of Tris (130 mM; pH 7.5), Ethylenediaminetetraacetic acid (EDTA) (10 mM), saponin (0.016%; W/V), and TritonX-100 (1.6%; V/V) was prepared in advance and stored at 4°C. Three histone deacetylase inhibitors namely, apicidin, trichostatin A, and valproic acid, and medical plant, cepharanthine (Sigma-Aldrich, Japan) were used as tested

drugs prepared as 10 mM stock solutions. Standard drugs, diminazene aceturate (DA) and fluorouracil (Sigma-Aldrich, Japan) were prepared as 100 mM stock solutions.

Parasites

The Texas T_2Bo strain of *B. bovis* (Hines et al., 1992), Argentine strain of *B. bigemina* (Hotzel et al., 1997), and U.S. Department of Agriculture (USDA) strains of *B. caballi* (Avarzed et al., 1997) and *T. equi* (El-Sayed et al., 2015) were maintained in bovine and equine red blood cells (RBCs) with medium using a microaerophilic stationary–phase culture system (Munkhjargal et al., 2012).

Determination of the relationship between parasite DNA and SYBR Green I fluorescence

DNA was extracted from the *B. bovis*-, *B. bigemina*-, *B. caballi*-, and *T. equi*-infected erythrocytes using a commercial DNeasy Blood and Tissue Kit (Qiagen). The concentration of the purified DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). After determining the concentration of the parasite DNA it was serially diluted with RPMI1640 medium to generate a concentration range. One hundred microliters of each concentration was dispensed into a 96-well plate followed by addition of 100 µl lysis buffer containing 2×SG. Control wells consisting of 100 µl RPMI1640 and an equal volume of lysis buffer containing SG were also set up. Plates were incubated in the dark at room temperature for 1 h and the fluorescence intensity was measured at 485 nm excitation and 528 nm emission using a fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific). Fluorescence units were then plotted against parasite DNA concentration. Each data point was performed in triplicate and three independent experiments were performed.

Determination of the effect of hemoglobin on SYBR Green I fluorescence

Bovine and equine uninfected blood was centrifuged and washed in RPMI 1640 to remove the plasma and buffy coat. The pellet was diluted with culture medium to generate a hematocrit (HCT) range of 0.03-10 %. Each hematocrit was spiked with parasite DNA (prepared above) at a fixed concentration (0.2 ng/µl). A hundred microliters of each mixture was dispensed into the wells of a microtiter plate and an equal volume of lysis buffer containing 2×SG was added. The plate was incubated in the dark for at least 1 h and fluorescence was measured as previously described (Dery et al., 2015). Fluorescence readings were plotted against HCT and the correlation between them was determined.

Maximum time of incubation with lysis buffer containing SG for optimum fluorescence signals and the effect of freeze–thaw

The incubation period of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* cultures with lysis buffer containing SG that yields the best fluorescence signal and the effect of freeze–thaw fluorescence intensity were ascertained (Dery et al., 2015). Briefly, 100 µl of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* cultures at an initial parasitemia of 4-8 % was serially diluted in a 96-well microtiter plate with blood at 2.5 % and 5 % HCTs for bovine and equine *Babesia* parasites, respectively (Rizk et al., 2015) to give a concentration range of 0.04-8 %

parasitemia. Direct lysis of the RBCs to release the parasite DNA was done by adding 100 µl of lysis buffer containing 2×SG (10.000x) to each well. After incubating the plates in the dark for 20 min, fluorescence signals were measured as previously described. Thereafter, the plate was kept in the dark and the fluorescence intensity was determined after 0.5, 1, 3, 6, and 24 h incubation in the dark. A second plate containing the same parasite concentration range was prepared in parallel to that described above. However, this plate was frozen immediately at -30 °C for 1 h after which the culture was thawed and 100 µl lysis buffer containing SG was added. The plate was then incubated in the dark and the emitted fluorescence signals were determined after 0.5, 1, 2, 3, 6, and 24 h. Fluorescence readings were plotted against parasitemia.

Determination of assay quality

To determine the assay quality, statistical parameters, the Z' factor (Z'), signal to noise (S/N) ratio, coefficient of variation at the maximum signal (% CV_{max}, positive control), and coefficient of variation at the minimum signal (% CV_{min}, negative control) were calculated for the fluorescence assay after freeze-thaw step according to Zhang et al., (1999).

Determination of inhibitory concentration 50 % (IC_{50}) values of anti-babesial drugs

An *in vitro* growth-inhibitory effect of apicidin, trichostatin A, cepharanthine, valproic acid, and DA on *Babesia* and *Theileria* parasites was evaluated by current and modified fluorescence-based assays as described previously (Rizk et al., 2015). DA was used as standard anti-babesial drug (0.01, 0.05, 0.1, 0.5, 1, and 5 µM). *B. bovis*-, *B. bigemina*-, *B. caballi*-, and *T. equi*-infected RBCs (iRBCs) were cultivated at 1% parasitemia in double 96-well plates using the previously published optimal HCT for each parasites (Rizk et al., 2015) and M199 or RPMI 1640 media alone or with 0.01, 0.05, 0.1, 0.5, 1, and 5 µM concentrations of each drug except of DA. For the control, cultures without drugs and cultures containing only DMSO (0.01%) were prepared. The iRBCs were cultivated for 4 days without replacement of the medium in triplicate wells for each concentration of the drug. On the fourth day of culture, a lysis buffer containing 2×SG was added into each well of the first 96-well plate and incubated in dark place for 6 h. After incubation, fluorescence values were determined using a fluorescence plate reader at 485 nm and 518 nm of excitation and emission wavelengths, respectively. A second plate containing the same drug concentration range was prepared in parallel to that described above. However, on the fourth day of culture, this plate was frozen at -30 °C for 1 h after which the culture was thawed and 100 µl lysis buffer containing 2×SG added. The plate was then incubated in the dark and the fluorescence reading was taken after 0.5 h. All experiments were repeated three times, and the mean fluorescence values were then plotted against the logarithm of drug concentrations. In addition, for all tested drug, thin blood smears were prepared on the fourth day and stained with Giemsa to calculate the parasitemia using the microscopy-based method. The IC_{50} values were calculated for each drug using the fluorescence-based method with and without freeze-thaw effect and microscopy method.

Cytotoxicity assay

Cytotoxicity of each drug was evaluated against Madin Darby Bovine Kidney (MDBK) cells using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution. Briefly, $3x10^4$ MDBK cells were seeded in double 96 well plates with or without 10, 50, 100, 500, 1000, 5000 μM concentrations of each drug and incubated at 37°C, 5 % CO_2 for 72 h. Fluorouracil was used as a positive control. Subsequently, 10 μl of MTT solution (a final concentration of 5 mg/ml) was added to each well of the first plate and incubated at 37°C for 4 h. To dissolve to crystals, the medium was subsequently replaced with 100 μl of DMSO was added, and the absorbance was measured at 570 nm with an automatic ELISA plate reader. Numbers of cells were evaluated as % of control without drugs. The selective index (SI) was calculated as the 50 % cytotoxic concentration on MDBK cells divided by the IC_{50} of the compound for *Babesia* parasites.

Statistical analysis

Data analysis was performed using GraphPad Prism ver. 5 for windows (GraphPad Software, Inc., USA) using the one-way ANOVA and an unpaired *t*-test (Rizk et al., 2015). The IC_{50} values were calculated using GraphPad Prism ver. 5. $P < 0.05$ was considered to be statistically significant.

RESULTS

Relationship between parasite DNA and SYBR Green I fluorescence

The relationship between the concentrations of parasite DNA and the fluorescence units determined after incubation with lysis buffer containing SG in the dark showed a well–correlated linear relationship with r^2 values of 0.9531, 0.9624, 0.9492 and 0.9283 for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*, respectively (Fig. 1).

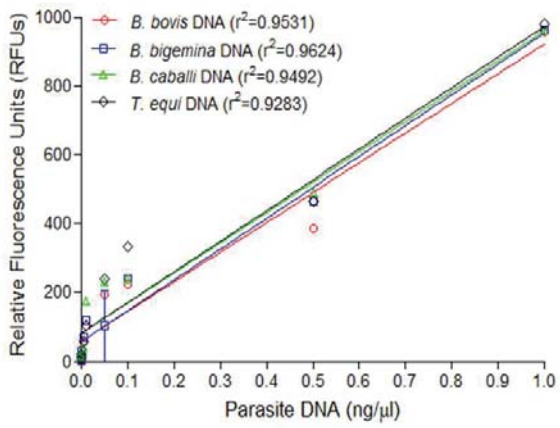

Fig. 1. DNA titration using SYBR Green I. Background fluorescence, defined as fluorescence detected in the absence of DNA, was subtracted from each data point. Fluorescence is measured as relative fluorescence units (RFUs). Data show average from three replicate experiments. Error bars indicate standard deviations.

Effect of hemoglobin on fluorescence

The effect of hemoglobin on fluorescent readings was tested by mixing different amounts of lysed erythrocytes (0.03-10% HCT) with a fixed amount of DNA. The fluorescence emission of SG-DNA adducts, shown in Figure 2, was highly quenched in the presence of red cells. Control samples, containing DNA without hemoglobin, rendered fluorescence readings around 20-30 fold as compared with samples containing blood, suggesting a heavy interference of hemoglobin. Additionally, the Z' factor, S/N ratio, % CVmax, and % CVmin were calculated in comparison with the fluorescence assay method without freeze-thaw effect (Rizk et al., 2015). Our results for bovine Babesia parasites revealed that S/N ratios showed the highest values at 2.5% HCT with freeze-thaw step (Table 1a). Regarding the equine *Babesia* and *Theileria* parasites, S/N ratios showed the highest values at 5%HCT for both parasites with freeze-thaw step (Table 1b). These results revealed the accuracy of the fluorescence-based assay with freeze-thaw effect at 2.5% and 5% HCTs for bovine and equine parasites, respectively.

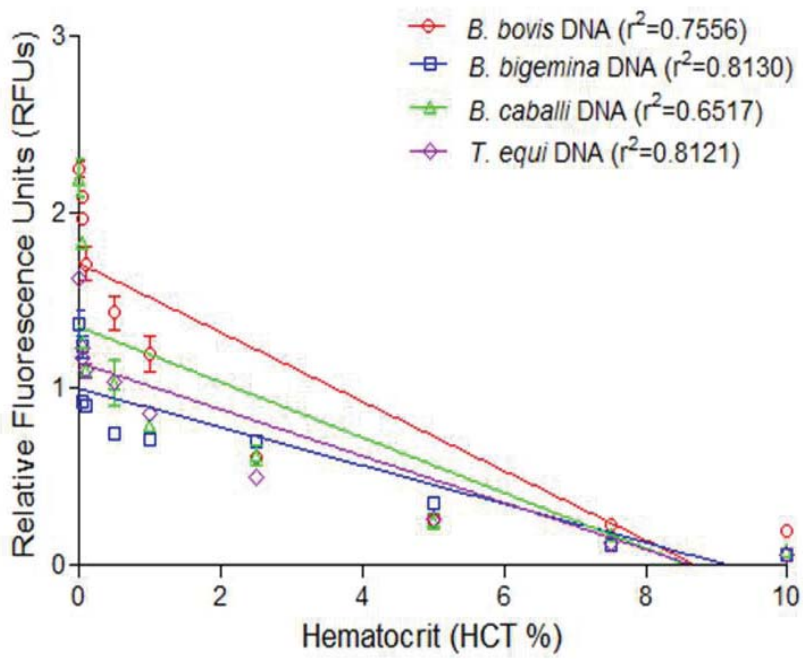

Fig. 2. Effect of hemoglobin on fluorescence emission. Fluorescence emission of SG of 0.2 ng/ml parasite DNA, in the presence of different amounts of bovine or equine RBCs. Fluorescence at 0.1 % hematocrit was obtained from a 0.2 ng/ml solution of parasite DNA in the culture medium.

Table 1a. Statistical parameters for determining the quality of the high–throughput screening (HTS) assay in bovine *Babesia* parasites with different percentages of HCTs without and with freeze–thaw step.

Parameters	*B. bovis*						*B. bigemina*					
	without freeze–thaw step			with freeze–thaw step			without freeze–thaw step			with freeze–thaw step		
	HCT %			HCT %			HCT %			HCT %		
	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
Z' factor	-1.1	-0.79	-0.4	0.9	0.43	-0.99	-19.4	-7.9	1.04	0.9	0.35	-12.4
S/N ratio	2.9	5.2	5.7	34.1	13.8	4.9	0.76	0.6	-152.9	76.5	6.8	0.25
% CV_{max}	2.5	5.6	5.2	0.7	3.3	8.4	1.5	2.9	2.3	1.04	1.9	2.3
% CV_{min}	2.7	3.1	3.8	2.1	2.8	4.5	6.24	1.9	0.5	1.02	5.6	25.1

Table 1b. Statistical parameters for determining the quality of the high–throughput screening (HTS) assay in equine *Babesia* and *Theileria* parasites with different percentages of HCTs without and with freeze–thaw step.

Parameters	*B. caballi*						*T. equi*					
	without freeze–thaw step			with freeze–thaw step			without freeze–thaw step			with freeze–thaw step		
	HCT %			HCT %			HCT %			HCT %		
	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
Z' factor	0.62	0.47	0.28	0.81	0.84	0.45	0.46	0.75	0.76	0.75	0.87	0.63
S/N ratio	16.2	12.8	10.6	38.5	37.1	88.4	6.6	18.9	72.9	16.03	24.4	10.3
% CV_{max}	0.52	2.12	3.5	0.58	0.43	6.03	0.2	0.9	0.99	1.4	2.6	0.7
% CV_{min}	0.54	2.1	3.03	0.48	0.51	0.61	1.1	2.2	0.9	2.8	0.4	3.5

Maximum time of incubation with lysis buffer for optimum SYBR Green1 fluorescence and effect of freeze-thaw cycle

The high fluorescence intensity was observed in freeze-thaw cycle followed by incubation with SG for 0.5 h gave the highest fluorescence intensity (Fig. 3). The results showed a well-correlated linear relationship (r^2 values of 0.9183, and 0.9874 for *B. caballi*, and *T. equi*, respectively) for freezing the samples for 0.5 h followed by thawing before the addition of lysis buffer containing SG (Fig. 3C, and D, respectively). However, a correlation was found between the emitted fluorescence signals and freezing the sample for 0.5 h followed by thawing before the addition of lysis buffer containing SG with 0.8861 and 0.8749 goodness of fit (r^2) for *B. bovis* and *B. bigemina*, respectively (Fig. 3A and B, respectively). Though there was a significant increase in fluorescence signal when the incubation time was at 0.5 h, there appears to be not much gain in fluorescence when the incubation time was extended 1 h to overnight. A similar trend was observed in the fluorescence assay without freeze-thaw step (Fig. 4). The difference between fluorescence signal for freezing samples for at least 1 h followed by a 0.5 h incubation with lysis buffer containing SG and incubating the samples 6 h after the addition of lysis buffer containing SG without freezing was significant ($P < 0.0001$ and F values for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 47.8, 108.2, 55.3, and 23.6, respectively) (data not shown). This trend was consistently observed in 3 independent experiments.

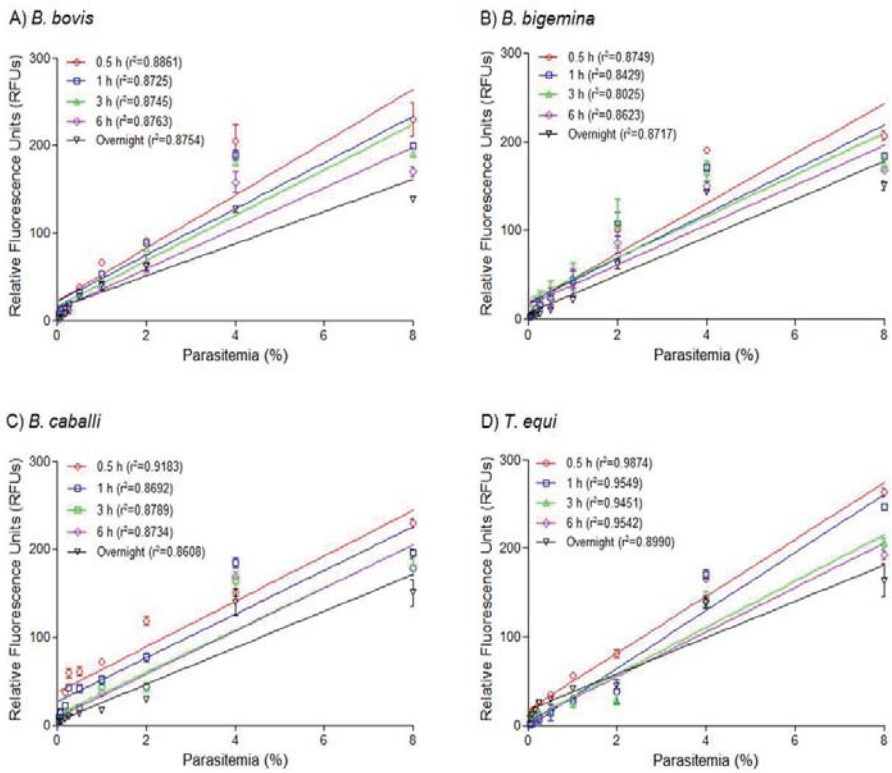

Fig. 3. Effect of length of incubation with lysis buffer containing SG after freeze-thaw effect on the intensity of fluorescence signal. Fluorescence at 2.5 % and 5 % hematocrit for bovine and equine *Babesia* parasites, respectively, to give a concentration range of 0.04-8 % parasitemia in the culture medium. Fluorescence was measured at 485 nm excitation and 528 nm emission.

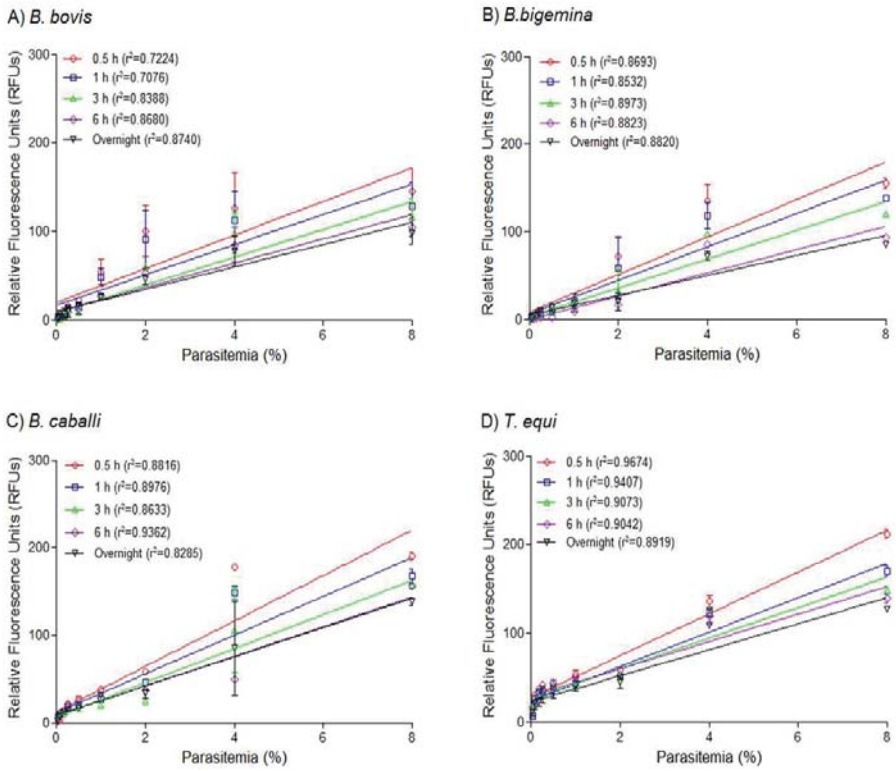

Fig. 4. Effect of length of incubation with lysis buffer containing SG without freeze-thaw step on the intensity of fluorescence signal. Fluorescence at 2.5 % and 5 % hematocrit for bovine and equine *Babesia* parasites, respectively, to give a concentration range of 0.04-8 % parasitemia in the culture medium. Fluorescence was measured at 485 nm excitation and 528 nm emission.

Table 2a. Comparison of inhibitory concentration 50 % (IC_{50}) values obtained from fluorescence assay without and with freeze−thaw effect and microscopy method. IC_{50}s of all tested drugs for bovine *Babesia* parasites

Drugs	IC_{50} values (nM)[a]									
	B. bovis					*B. bigemina*				
	Fluorescence			Microscopy	*P* value[c]	Fluorescence			Microscopy	*P* value[c]
	without freeze−thaw	with freeze−thaw	*P* value[b]			without freeze−thaw	with freeze−thaw	*P* value[b]		
Apicidin	42±6.7	43±5.4	0.9959	32.1±5.6	0.9333	19.8±8.9	16.8±10.2	0.9061	12.5±8.2	0.5077
Trichostatin A	37.4±5.2	36.5±8.1	0.9671	35.5±4.9	0.9290	68.4±35	68.5±33.6	0.9810	54.9±30.2	0.7293
Cepharanthine	45±20.5	49.2±20.1	0.9295	40.2±18.2	0.7933	19.5±11.5	20.1±12.1	0.8902	22.1±9	0.9494
Valproic acid	87.3±46.6	92.5±52.2	0.8678	110.2±78.2	0.4852	240.5±180	191.2±156	0.4049	265±160.2	0.5536
Diminazene aceturate	46.4±14.1	47.2±19	0.9064	35.1±12.1	0.9403	25.9±18	22.6±20.2	0.6626	25±10.2	0.9275

Table 2b. Comparison of inhibitory concentration 50 % (IC_{50}) values obtained from fluorescence assay without and with freeze−thaw effect and microscopy method. IC_{50}s of all tested drugs for equine *Babesia* and *Theileria* parasites.

Drugs	IC_{50} values (nM)[a]									
	B. caballi					*T. equi*				
	Fluorescence			Microscopy	*P* value[c]	Fluorescence			Microscopy	*P* value[c]
	without freeze−thaw	with freeze−thaw	*P* value[b]			without freeze−thaw	with freeze−thaw	*P* value[b]		
Apicidin	43±25.4	30±23.5	0.5104	29.1±21.4	0.7940	28.4±11.4	21.1±13	0.7907	25.5±9.8	0.9520
Trichostatin A	96.7±25.1	93.1±28.4	0.9978	84.8±26.9	0.9204	21.7±9.2	15.7±10.4	0.7575	14.7±7.1	0.6882
Cepharanthine	75.5±15.2	59.3±14	0.8390	55.5±10.1	0.9154	15.4±5.8	11.2±5.5	0.7259	18.2±6.1	0.6272
Valproic acid	468±155	453.1±162	0.9869	430.2±149.4	0.9381	70.6±55.4	72±59	0.8259	75.5±46.6	0.8052
Diminazene aceturate	35±8.4	25.4±9.7	0.8133	34.5±6.8	0.8002	33±29	37±34	0.2137	42±24	0.8098

[a]IC_{50}s for each drug were calculated on the fourth day based on the growth inhibitions determined using fluorescence− and microscopy− based methods in three separate experiments. [b]The differences between the IC_{50}s calculated using current and modified fluorescence assay were not statistically significant for all drugs analyzed. [c]The differences between the IC_{50}s calculated using present fluorescence− and microscopy-based methods were not statistically significant for all drugs analyzed.

The quality of fluorescence assay after a freeze−thaw step

To evaluate the quality of the assay, the Z' factor, S/N ratio, % CV_{max}, and % CV_{min} were calculated for previously optimized HCTs (2.5 % and 5 %) (Rizk et al. 2015). The Z' factor of additional freeze-thaw plus 0.5 h incubation was higher than 0.5 at a fixed HCT for each parasite (Table 1). Additionally, after freeze-thaw step, the assay exhibited high S/N ratios in bovine *Babesia* and *T. equi* parasites. On the contrary, S/N ratio showed low value in *B. caballi* for the assay after freeze-thaw step (Table 1).

Comparison of IC50 values obtained by fluorescence assay with or without freeze−thaw step

The correlation of the drug-induced growth inhibition and emission of fluorescence signal after freeze-thaw step was confirmed by the commonly used anti-babesial drug, DA which was inversely correlated with the emission of fluorescence signals in a dose-dependent manner (Fig. 5). The *in vitro* growth of the *Babesia* and *Theileria* parasites including *B. bigemina, B. caballi*, and *T. equi* was significantly inhibited ($P < 0.05$) by a 0.01 µM DA treatment (Fig. 5). While 0.05 µM DA significantly inhibited the *in vitro* growth of *B. bovis* (Fig. 5). Notably, a similar growth inhibition for bovine *Babesia* and equine *Babesia* and *Theileria* parasites was obtained by the fluorescence assay with and without freeze-thaw step and microscope (Fig. 6).

Further, the outcome of the *in vitro* test of susceptibilities of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* to apicidin, trichostatin A, cepharanthine, and valproic acid using the fluorescence method with or without freeze-thaw step is showed in Figs. 7. Data showed that fluorescence values were inversely correlated with drug concentrations in *in vitro* parasite cultures with and without freeze-thaw step (Figs. 7). After the freeze-thaw step, the numbers of fluorescence units slightly increased without substantive changes in the IC_{50} values. The *in vitro* growth of the three parasites, *B. bigemina, B. caballi*, and *T. equi* was significantly

inhibited ($P < 0.05$) by a 0.01 μM apicidin treatment (Fig. 7). Whilst, the *in vitro* growth of *B. bovis was* significantly inhibited by 0.05 μM apicidin (Fig. 7). The growth of the cultured parasites was significantly inhibited ($P < 0.05$) by cepharanthine at 0.01 μM for *B. bigemina* and 0.05 μM for *B. bovis* and *B. caballi*, and *T. equi*. Whereas, 0.01 μM treatment by trichostatin A was significantly inhibited ($P < 0.05$) the *in vitro* growth of *B. bovis* and *T. equi*. 0.05 μM of trichostatin A was sufficient for the significant inhibition ($P < 0.05$) of *B. bigemina and B. caballi* (Fig. 7). In contrast, the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* was significantly inhibited by 0.05, 0.1, 0.5, and 1 μM valproic acid, respectively. Similar findings observed in *in vitro* parasite culture with freeze-thaw step (Fig. 7).

Microscopy- and SG-based fluorescence method with or without additional freeze-thaw step was used to determine the IC_{50} values of apicidin, trichostatin A, cepharanthine, valproic acid, and DA (Table 2). Importantly, the IC_{50} values obtained from the fluorescence assay with and without freeze-thaw effect were significantly concordant with those obtained by traditional microscopy method ($P < 0.05$) in all tested drugs for the bovine *Babesia* and equine *Babesia* and/or *Theileria* parasites (Table 2).

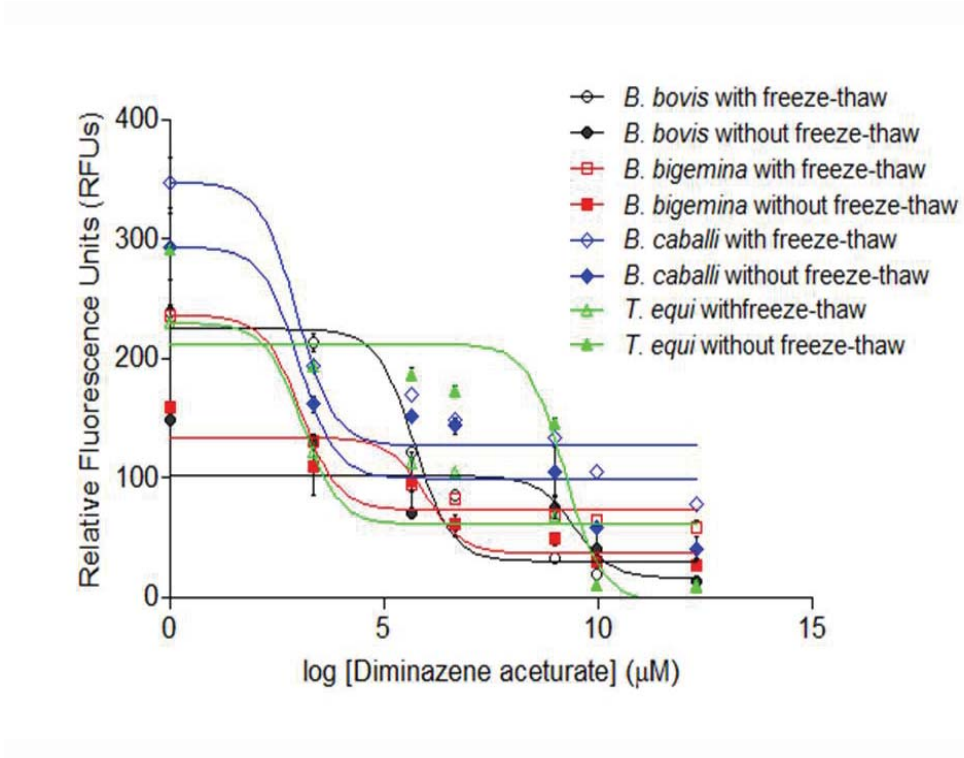

Fig. 5. Correlation between diminazene aceturate concentrations and RFUs on *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* at 0.5 and 6 h incubation with and without freeze-thaw step, respectively. Each value represents a mean of triplicate wells after subtraction of the background fluorescence for non-parasitized RBCs. Statistically, significant differences are indicated by asterisks (*P<0.05) between the drug-treated cultures and the control cultures.

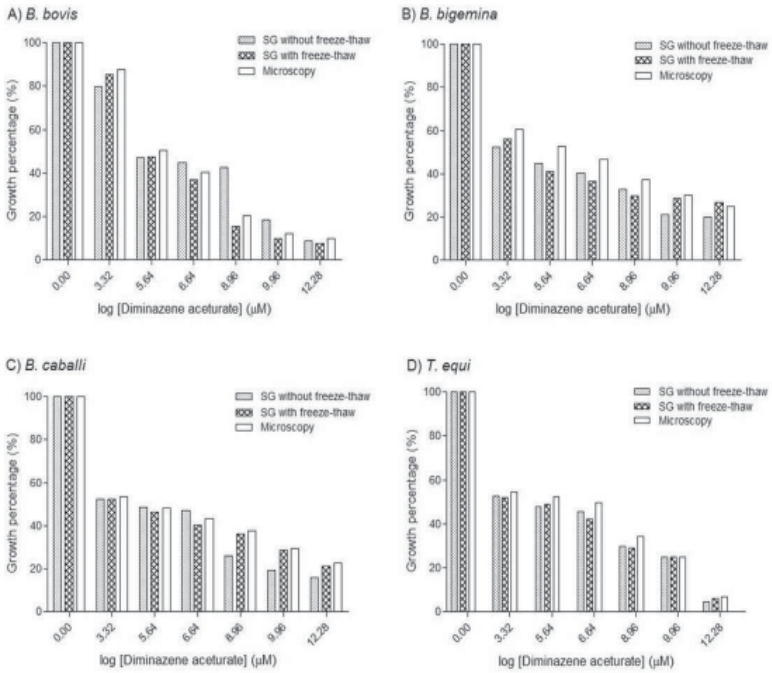

Fig. 6. Comparison of the inhibitory effects of diminazene aceturate on bovine and equine *Babesia* and *Theileria* parasites by fluorescence- and microscopy-based methods. Growth percentage of the parasite (y-axis) and log concentration of drugs (x-axis). Each value is represented as the mean of three triplicate wells ± SD.

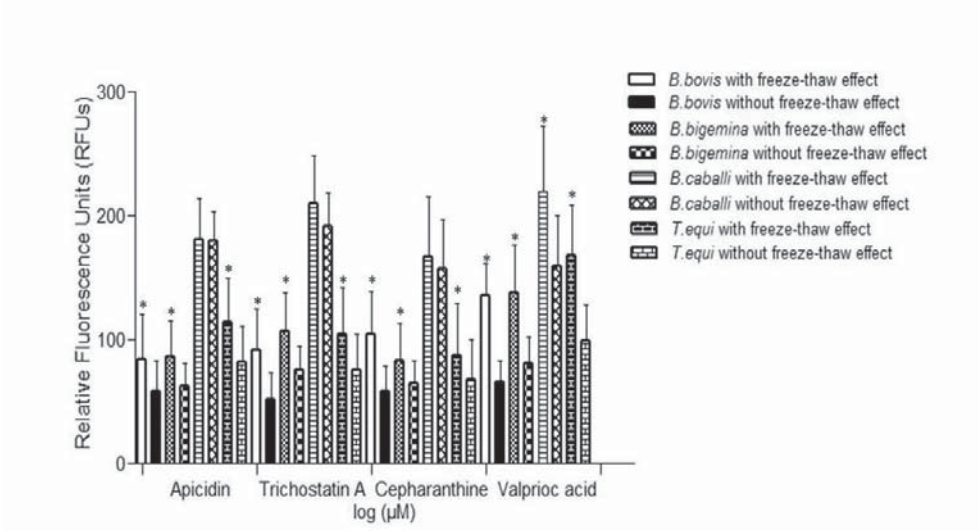

Fig. 7. Fluorescence-based monitoring of apicidin-, trichostatin A-, cepharanthine-, and valproic acid-induced growth inhibition of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* on the fourth day of treatment. Correlation between apicidin, trichostatin A, cepharanthine, and valproic acid concentrations and RFUs on *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* with and without freeze-thaw step. Each value represents a mean of triplicate wells after subtraction of the background fluorescence for non-parasitized RBCs. Statistically, significant differences are indicated by asterisks (*P<0.05) between the drug-treated cultures and the control cultures.

Cytotoxicity of anti–babesial drugs

The results of the cytotoxic activity of apicidin, trichostatin A, cepharanthine, valproic acid, and DA on MDBK cells are shown in Fig. 8. For all drugs' concentrations as high as 2400 μM, no cytotoxic effect was found which was assessed using MTT assay (Table 3). The SI was employed to compare the toxicity for mammalian cells and the activity against the parasites. The best anti-babesial activity was obtained with apicidin against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* with IC_{50} ranged between 1.3 and 35.7 nM and the SI values greater than 90 that generated by MTT assay results (Tables 2 and 4). In contrast, cepharanthine was showed activity (SI > 90 and IC_{50}s< 50) against *B. bigemina* and *T. equi*, whereas trichostatin A exhibited activity with SI=204.1 and IC_{50}s < 50 against only *T. equi* (Table 2b). Although, IC_{50}s of valproic acid great than 50 nM, SI values less than 90. Conversely, DA was less active (SI < 90 and IC_{50}s < 50) against the test all parasites compared with those of other drugs (Table 4).

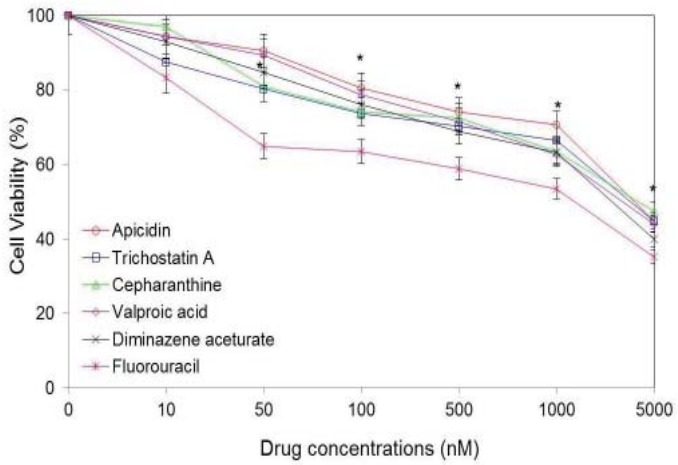

Fig. 8. Effect of apicidin, trichostatin A, cepharanthine, valproic acid, diminazene aceturate, and fluorouracil on MDBK cell viability. Cells were cultured for 48 h in the presence of different concentrations of each drug. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described in Materials and Methods.

Table 3. Cytotoxic concentration 50 % (CC_{50}) values of each drug against MDBK cells

Organism	CC_{50} values drugs (μM)[a]						
	Apicidin	Trichostatin A	Cepharanthine	Valproic acid	Diminazene aceturate	Fluorouracil	References
MDBK cells	3300±3200	3000±2800	3300±3100	2700±2400	3500±2900	1500±1300	Present study
Jurkat cells	10	0.1	ND	10.000	ND	ND	29
Esophageal squamous cell carcinoma	ND	ND	ND	2150±1020	ND	ND	30

[a] The CC_{50} was defined as the concentrations (μM) of apicidin, trichostatin A, cepharanthine, valproic acid, diminazene aceturate, and fluorouracil required for the reduction of cell viability by 50%, which were calculated by regression analysis.

Table 4. Selectivity index (SI) values for each drug against *Babesia* and *Theileria* parasites

Organism	Apicidin	Trichostatin A	Cepharanthine	Valproic acid	Diminazene aceturate
B. bovis	>90	>90	>90	>90	>90
B. bigemina	>90	>90	>90	>90	>90
B. caballi	>90	>90	>90	>90	>90
T. equi	>90	>90	>90	>90	83.3

The results were obtained from the average of three independent experiments performed in duplicate. Selectivity Index (CC_{50}/IC_{50}).

DISCUSSION

Previously, fluorescence assay using SYBR Green I for assessing the susceptibility of parasites to anti-babesial compounds was reported (Guswanto et al., 2014; Rizk et al., 2015). The assay detects the presence of parasite DNA in infected erythrocytes as a measure of parasite propagation and the inhibition in parasite growth by antiparasitic drugs. However, SG assay for testing parasite susceptibility to antiparasitic drugs needs further improvement which avoids of low fluorescence signal due to the presence of hemoglobin in the parasite culture, which could be responsible for quenching of fluorescence (Bennett et al., 2004).

Recently, cold treatment of *B. bovis in vitro* culture was found to induce an effective release of merozoites from the infected RBCs (Ishizaki et al., 2016) which may support to ensures complete lysis of the RBC and release the parasite leading to an increased availability of parasite DNA for staining with fluorescence dye. The present study reports the effect of a SG-based fluorescence assay under different culture conditions and compared the assay results to those of previously published SG method in order to determine the best fluorescence intensity. Our result showed that freezing and thawing for at least 1 h, followed by incubation with lysis buffer containing SG in the dark for 0.5 h gave the highest fluorescence signal on bovine (Table 1a) and equine *Babesia* and *Theileria* parasites (Table 1b). A similar pattern was observed in the *Plasmodium falciparum* (*P. falciparum*) culture that was frozen at -30°C for over than 1 h, followed by thawing. Moreover, additional incubation with SG for 3 h provided a significant improvement in fluorescence signal (Smilkstein et al., 2004; Dery et al., 2015). In contrary, freezing the plates before or after the addition of lysis buffer containing SG couldn't lead to a significant increase in fluorescence signal (Johnson et al., 2007). With inference to the above, the modified fluorescence-based assay with the freeze-thaw effect is considered as the best assay with the best fluorescence intensity.

Herein, linearity between the concentrations of parasite DNA and the fluorescence units was observed with high r^2 values. In contrast, a decreasing trend in fluorescence with increasing HCT at fixed parasite DNA concentration was observed in this study, suggesting that may be attributed to the quenching effect of the red blood cells as previously reported (Moneriz et al., 2009; Dery et al., 2015). The quality of the fluorescence signal was assessed by calculating the signal to noise ratio (S/N) (Makler et al., 1991; Zhang et al., 1999), based on the measurement of the fluorescence activity of parasite exposed to previously optimized

HCT (Rizk et al., 2015). Both the signal and the background were shown to remain relatively stable. Interestingly, after the freeze-thaw effect, the results of the SG assay for bovine *Babesia* and *T. equi* parasites with previously optimized HCT (2.5 % and 5 %) generated high S/N ratios. However, other studies are required to clarify the low S/N ratio generated for *B. caballi* after freeze-thaw step. Further, the suitability of the assay for high throughput screening was determined using the Z' factor, a dimensionless statistical parameter combining the difference between the positive and negative controls, and their respective variations (Bennett et al., 2004). The Z' factor value is ≥ 0.5 generally indicates a good assay (Zhang et al., 1999). Our results showed that the Z' factors of SG assay with freeze-thaw step were higher than 0.5 at 2.5 % and 5 % HCT for bovine and equine *Babesia* parasites, respectively.

Furthermore, the SG based fluorescence assay with and without additional freeze-thaw step and microscopy methods were used to determine the IC_{50} values of apicidin, trichostatin A, cepharanthine, valproic acid, and DA. The IC_{50} values obtained using the current optimized conditions with freeze-thaw effect appear to be within the expected range and comparable to those of IC_{50} values obtained using fluorescence assay without freeze-thaw effect and microscopy method. There was no significant difference ($P > 0.05$) in the IC_{50} values of all tested drugs for bovine and equine *Babesia* and *Theileria* parasites. In addition, apicidin, trichostatin A and cepharanthine showed a marked *in vitro* anti-*Babesia* and/or -*Theileria* activity, with low IC_{50} values. In contrary, valproic acid showed activity levels 2-6 times higher than those of apicidin, trichostatin A, and cepharanthine in *B. bigemina* and *B. caballi* parasites.

As view previous reports, apicomplexan histone deacetylase (HDAC) enzymes have suggested as an attractive drug target. HDAC inhibitors, apicidin, trichostatin A, and valproic acid have broad-spectrum antiviral, anticancer, antiproliferative, and antiparasitic activities against chronic myeloid leukemia cells (Park et al., 2009), hela cells (Darkin-Rattray et al., 1996), and apicomplexan parasites (Desgrouas et al., 2014). Cepharanthine, a natural compound isolated from *Stephania cepharantha* Hayata possess many pharmacological effects such as antiparasitic, anti-inflammation, anti-allergic, and anticancer (Desgrouas et al., 2014). The IC_{50} values of cepharanthine against the growth of FCM29, W2, 3D7, and K1 *P. falciparum* have been reported to be 3059 nM, 927 nM, 2276 nM, and 1803 nM, respectively (Desgrouas et al., 2014). These values were higher than those for three *Babesia* and *T. equi* parasites determined by the SG with or without freeze-thaw effect and microscopic methods. Moreover, the IC_{50} values of apicidin and trichostatin A for *Babesia* or *Theileria* parasites were higher than those calculated for *Toxoplasma gondii* (*T. gondii*) but lower than *P. falciparum* (Darkin-Rattray et al., 1996). In contrast, the IC_{50} values of valproic acid for *T. equi* and three *Babesia* parasites were lower than that calculated for *T. gondii* (Strobi et al., 2007). Interestingly, for inhibitors of HDAC, apicidin, and trichostatin A showed IC_{50} values lower than those of currently available anti-babesial drugs including; 17-DMAG, diminazene aceturate, and Atovaquone (Guswanto et al., 2018) suggested that this candidate drug might be a potent anti-babesial drug.

Finally, we determined the CC_{50} of all tested drugs by MTT assay. The results revealed that the CC_{50} values of all drugs are higher than those of IC_{50} values obtained from the present study, suggesting that drugs are no toxic. Hit activity criteria for protozoa are

considered when the IC_{50} is < 0.2 μg/ml and the SI is >100 (Nwaka and Hudson, 2006). In this study, apicidin with IC_{50} is less than 31.2 μg/ml and the SI is higher than 100 that generated by MTT assay results, met this requirement. Cepharanthine (for *B. bigemina* and *T. equi*) and trichostatin A (for *T. equi*) also classify as a hit according to the WHO Special Programme for Research & Training in Tropical Diseases (TDR).

Herein we reported that evidence obtained in this study gives further credence to the fact that the SG method is consistent and rapid for the routine assessment of parasites sensitivity to drugs with the enhanced signal intensity. Current results showed that freezing and thawing of culture, followed by incubation with SG in the dark for 0.5 h gives the highest fluorescence signal. It may be useful for attaining the full benefit of using the recently introduced SG method in the anti-babesial drug monitoring system. Importantly, there is no need to carrying delicate equipment to the field in order to perform the assay and a freezing step permits long-term storage and shipping of assay plates. Because of the test plates can be frozen and later transported to the main laboratory for fluorescence reading. This has proved to be quite beneficial and has allowed the assay to be conducted in remote regions, where access to a fluorometer is limited.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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