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The establishment of *in vitro* culture and drug screening systems for a newly isolated strain of *Trypanosoma equiperdum*

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ABSTRACT

Dourine is caused by *Trypanosoma equiperdum* via coitus with an infected horse. Although dourine is distributed in Equidae worldwide and is listed as an internationally important animal disease by the World Organization for Animal Health (OIE), no effective treatment strategies have been established. In addition, there are no reports on drug discovery, because no drug screening system exists for this parasite. A new *T. equiperdum* strain was recently isolated from the genital organ of a stallion that showed typical symptoms of dourine. In the present study, we adapted *T. equiperdum* IVM-t1 from soft agarose media to HMI-9 liquid media to develop a drug screening assay for *T. equiperdum*. An intracellular ATP-based luciferase assay using CellTiter-Glo reagent and an intracellular dehydrogenase activity-based colorimetric assay using WTS-8 tetrazolium salt (CCK-8 reagent) were used in order to examine the trypanocidal effects of each compound. In addition, the IC_{50} values of 4 reference trypanocidal compounds (pentamidine, diminazene, suramin and melarsomine) were evaluated and compared using established assays. The IC_{50} values of these reference compounds corresponded well to previous studies involving other strains *of T. equiperdum*. The luciferase assay would be suitable for the mass screening of chemical libraries against *T. equiperdum* because it allows for the simple and rapid-evaluation of the trypanocidal activities of test compounds, while a simple, inexpensive colorimetric assay will be applicable in developing countries for the evaluation of the drug sensitivity of epidemic trypanosome strains.

1. Introduction

Dourine is caused by *Trypanosoma equiperdum* of the subgenus *Trypanozoon*. Unlike other trypanosomes, dourine is transmitted through coitus with an infected horse rather than insect vectors (Brun et al., 1998). Thus, dourine has been found to be distributed worldwide, beyond the tsetse belt in sub-Saharan Africa (Brun et al., 1998). Dourine is listed as an internationally important animal disease by the World Organization for Animal Health (OIE). Evidence from *in vitro* drug sensitivity assays using laboratory strains of *T. equiperdum* indicates that suramin, diminazene, quinapyramine and Cymelarsan® (melarsomine) are effective against

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T. equiperdum (Zhang et al., 1991; Brun and Lun, 1994; Gillingwater et al., 2007). Melarsomine was found to be effective against the *T. equiperdum* OVI strain in mice, and the *T. equiperdum* Dodola strain in mice and horses (Hagos et al., 2010; Habte et al., 2015). However, this has not been established as an effective treatment strategy for dourine-infected horses. Moreover, diminazene- and melarsomine-resistant *T. equiperdum* strains have already been reported (Gillingwater et al., 2007). Thus, an *in vitro* drug screening assay for *T. equiperdum* is strongly needed for efforts to seek novel trypanocidal compounds both for evaluating drug sensitivity and for establishing an effective treatment strategy for dourine.

The direct determination of the trypanosome count using a cell counting chamber is a simple method for evaluating trypanocidal activity. This assay is relatively cheap and simple to perform; however, it is time consuming to evaluate large numbers of compounds using this method (Fumarola et al., 2004). The incorporation of a radioactive nucleotide assay has been widely used to analyze the drug sensitivity of trypanosomes *in vitro* (Brun and Lun, 1994). After the incubation of the trypanosomes with the drugs and ^{3}[H] thymidine, the signals of the radioisotope in the parasite are counted using a liquid scintillation counter. This test has the disadvantage of involving radioactive nucleotides and cumbersome steps. A fluorescence-based screening system using Alamar blue™ has also been developed for an HTS assay for trypanosomes (Sykes and Avery, 2009a). This assay requires expensive equipment, such as a fluorometer for the 96-well plate and is therefore difficult to apply in developing countries, which are most affected by trypanosomosis (Fumarola et al., 2004). Intracellular ATP-based luciferase assays have also been utilized as HTS assays against *T. congolense*, *T. brucei* and *Leishmania* spp. (Sykes and Avery, 2009b; De Muylder et al., 2011; Suganuma et al., 2014). The kits, such as CellTiter-Glo®, are simple, rapid and efficient, with signal detection taking only few minutes. Intracellular ATP-based luciferase assays are more suitable for the rapid-evaluation of trypanocidal activity than Alamar blue™ based fluorescence assays, because the incubation time after the addition of the CellTiter-Glo® reagents is short. However, the expensive equipment and reagents that are necessary for this assay also constrain its application in developing countries. Colorimetric assays using tetrazolium salts, such as MTT, XTT and WST, have also been established for *T. brucei* and *Leishmania* spp. (Lu et al., 2013; Ginouves et al., 2014). These methods are based on a color reaction (through the reductive reaction of intracellular dehydrogenase activity), which leads to the formation of a colored chemical product, formazan. The results of the color reaction are measured using a relatively inexpensive and versatile microplate plate reader (ELISA plate reader). In addition, the reagents are affordable in comparison to fluorescence and luciferase assays (Fumarola et al., 2004). Thus, these colorimetric assays are more suitable than other assay systems for evaluating the drug sensitivity of trypanosomes in developing countries.

We recently isolated a new strain of *T. equiperdum, T. equiperdum* IVM-t1 from a horse in Mongolia that was confirmed to have dourine based on clinical symptoms, a positive PCR result, the serological diagnosis, and the microscopic observation of trypanosomes in a genital swab (Suganuma et al., 2016). This trypanosome strain was well adapted and propagated on the soft agarose media. In the present study, we successfully adapted *T. equiperdum* IVM-t1 that had been cultured in soft agarose media to culture in HMI-9 liquid media in order to develop a drug screening assay for *T. equiperdum*. In addition, the IC_{50} values of four trypanocidal drugs were evaluated and compared to the results obtained from established assays.

2. Materials and methods

2.1. In vitro cultivation

The *T. equiperdum* IVM-t1 strain, which was isolated in Mongolia in 2015, was used in the present study (Suganuma et al., 2016). Soft agarose culture-adapted *T. equiperdum* IVM-t1 was maintained by continuous sub-culturing once a week at 37 °C in 5% CO_2 using soft agarose media (HMI-9 (Hirumi and Hirumi, 1991) with 0.8% low gelling agarose (wt/vol) [Type VII, Sigma-Aldrich Japan, Tokyo, Japan]).

2.2. Adaptation to liquid media

Soft agarose culture-adapted *T. equiperdum* IVM-t1 parasites were transferred into HMI-9 liquid media supplemented with 0, 0.01, 0.1, 1 and 10% FCeM (vol/vol) (Nissan Chemical Industries, Ltd., Tokyo, Japan) at a parasite concentration of 1×10^4 cells/mL. The number of trypanosomes in each cell culture flask was manually counted every 24 h using a cell counting chamber. After optimizing the FCeM concentration in HMI-9, the liquid culture-adapted *T. equiperdum* IVM-t1 strain was maintained by replacing the entire culture supernatant with fresh HMI-9 supplemented with 1% FCeM every other day. A one-way ANOVA and Tukey's multiple comparison test were used for the statistical analyses of the doubling time, the daily parasite concentrations and the maximum parasite concentrations with different concentrations of FCeM. The statistical analyses were performed using the GraphPad PRISM 5 software program (GraphPad Software, Inc., CA, USA). The doubling time was calculated by counting the cells in the logarithmic growth phase of growth and using the following equation: Doubling time = (t2 − t1) x log (2)/log (q2/q1). Two measurements were performed: the initial count (q1) at time (t1) and the resultant parasite concentration after 24 h of incubation (q2, t2).

2.3. The optimization of the luciferase assay

To optimize the efficacy of the parasite detection by the luciferase assay using the CellTiter-Glo® Luminescent Cell Viability Assay reagent (Promega Japan, Tokyo, Japan), trypanosomes were prepared by two serial dilutions from $2 \times 10^6 - 1950$ cells/mL, and 100 μL aliquots were dispensed into 96-well cell culture plates in duplicate. The luminescence was measured using 12.5, 25, 50 and 100 μL of CellTiter-Glo® reagents at various time intervals after 2 min mixing, in order to evaluate the linearity between the luminescence and the trypanosome concentration.

In order to optimize the initial trypanosome concentration in the 96-well cell culture plates, 100 μL of *T. equiperdum* at a density of 5×10^4, 2.5×10^4, 1×10^4 and 5×10^3 cells/mL was inoculated on a 96-well cell culture plate and the cells were manually counted every 24 h after inoculation.

Moreover, to optimize the concentration of DMSO (which was used as a solvent) in the culture medium, the inhibitory effect of DMSO was evaluated. One hundred microliters (1×10^4 cells/mL) of *T. equiperdum* were cultivated for 72 h in HMI-9 supplemented with 1% FCeM and 0, 0.125, 0.25, 0.5, 1, 1.25, 5 and 10% of DMSO. Subsequently, the survival rate of the parasite in each concentration was evaluated by a luciferase assay using 25 μL of CellTiter-Glo® reagent. Student's *t*-test was used to analyze the differences between the control group (0% DMSO) and DMSO-treated groups.

2.4. The assessment of the trypanocidal activity with optimized assay conditions

Fifty microliters of various concentrations of reference