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Research paper

Evaluation of high-throughput assays for *in vitro* drug susceptibility testing of *Tritrichomonas foetus* trophozoites

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ABSTRACT

Tritrichomonas foetus is a sexually transmitted protozoan parasite that causes abortions in cattle and results in severe economic losses. In the United States, there are no safe and effective treatments for this parasite and infected animals are typically culled. In order to expedite drug discovery efforts, we investigated *in vitro* trophozoite killing assays amenable to high-throughput screening in 96 well plate formats. We evaluated the reduction of resorufin, incorporation of propidium iodide, and a luminescence-based ATP detection assay. Of these methods, reduction of resorufin was found to be the most reliable predictor of trophozoite concentrations. We further validated this method by conducting dose-response experiments suitable for calculation of EC_{50} values for two established compounds with known activity against trophozoites *in vitro*, namely, metronidazole and ronidazole. Our results demonstrate that the resorufin method is suitable for high-throughput screening and could be used to enhance efforts targeting new treatments for bovine trichomoniasis.

1. Introduction

Tritrichomonas foetus is a flagellated obligate protozoan parasite of the bovine reproductive tract and the etiologic agent of bovine trichomoniasis. In adult cows, infection results in early embryonic death and increased culling rates (Bondurant, 2005). Venereal transmission of *T. foetus* occurs when an infected bull breeds a susceptible cow or an infected cow is serviced by a susceptible bull. Cows typically clear infections, however, this may take up to 22 months (Bondurant, 2005). Bulls harbor chronic infections that are typically asymptomatic. In the United States, there is no approved treatment and control measures rely on testing and culling infected bulls.

There is a need to develop chemotherapy for bovine trichomoniasis, but current procedures for drug susceptibility testing involve cumbersome microscopic evaluation of cell cultures. This approach has the disadvantage that is it not amenable to high-throughput screening procedures. In order to improve therapeutic approaches targeting *T. foetus*, there is a need to develop more rapid methods for evaluating the activity of synthetic and natural compounds against the parasite.

Fluorescence assays have been proposed to be reliable indicators of cell viability for *Trichomonas vaginalis*, a related protozoan parasite that infects humans. Initial reports indicated that resazurin (Alamar Blue) could be used to detect viable trophozoites (Duarte et al., 2009; Natto et al., 2012). However, ensuing studies demonstrated that resazurin was rapidly metabolized to resorufin and resulted in discrepancies when experiments were repeated (Natto et al., 2012). This led to the discovery that *T. vaginalis* trophozoites reduce fluorescent resorufin to a colorless dihydroresorufin and this change could be used in drug susceptibility assays (Natto et al., 2012). Propidium iodide, a fluorescent intercalating agent that permeates cells with compromised membranes, has also been used to evaluate *T. vaginalis*. Propidium iodide stains *T. vaginalis* permeabilized with digitonin and the corresponding fluorescence signal can be used to estimate trophozoite concentrations (Natto et al., 2012).

We hypothesized that fluorescence-based assays described for *T. vaginalis* could be applied to rapid, high-throughput *in vitro* susceptibility testing of *T. foetus* trophozoites. In the present study, we evaluate the use of resorufin and propidium iodide as well as a commercial luminescence-based ATP detection kit. The present studies tested the suitability of these indicators as tools for drug

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susceptibility testing of *T. foetus* trophozoites in a 96-well plate format.

2. Methods

2.1. Parasites

T. foetus (BP-4 Beltsville strain, ATCC® 30003™) was maintained in trypticase-yeast-maltose (TYM) medium which is also referred to as modified diamonds medium (Clark and Diamond, 2002). Media was supplemented with 10% adult bovine serum, XX penicillin, and XX streptomycin. Cultures were maintained at 32 °C and regularly sub-cultured to maintain cell concentrations of 4×10^5 trophozoites/mL. The cultures were maintained in sterile 15 mL centrifuge tubes filled completely with media and capped tightly to create an anaerobic environment.

2.2. Chemicals

Metronidazole, ronidazole, and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO). Resorufin was purchased from Marker Gene Technologies (Eugene, OR). Celltiter glo reagent was purchased from Promega (Madison, WI). Test compounds were dissolved in TYM culture medium under sterile conditions. All drug and resorufin solutions were made fresh for each assay. Propidium iodide and digitonin solutions were prepared and stored at −20 °C until required.

2.3. Fluorescence assays

Drug assays were conducted by culturing *T. foetus* trophozoites in black walled 96-well sterile tissue culture plates (Corning, Corning NY). Trophozoites (5×10^4/mL) were incubated under anaerobic conditions by placing plates into a GasPak™ container containing BD EZ anaerobe sachets (BD Biosciences, Franklin Lake, NJ). Dilutions of test compounds, prepared in TYM culture medium, were co-incubated with trophozoites for 24 h at 32 °C under anaerobic conditions. All experiments were repeated a minimum of three times in duplicate.

For resorufin assays, trophozoites were placed in black-walled 96 well sterile tissue culture plates and incubated under anaerobic conditions for 24 h at 32 °C. After 24 h, 200 μM resorufin was added to each well and plates were incubated for 10 min at 32 °C under anaerobic conditions. Fluorescence was then immediately measured with a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) (excitation: 544 nm, emission: 599 nm).

For propidium iodide assays, dilutions of trophozoites were placed in black-walled 96 well sterile tissue culture plates and incubated under anaerobic conditions for 24 h at 32 °C. After 24 h, 20 μL of 20 μM propidium iodide and 20 μM digitonin was added and plates were incubated for 1 h at 32 °C under anaerobic conditions. Fluorescence was then immediately measured with a Spectramax M2 plate reader (excitation: 544 nm, emission: 620 nm).

For CTG assays, dilutions of trophozoites were placed in black walled 96 well sterile tissue culture plates and incubated under anaerobic conditions for 24 h at 32 °C. After 24 h, 100 μL of CTG reagent was added and plates were incubated for 10 min at 32 °C under anaerobic conditions. Luminescence was then immediately measured with a Spectramax M2 plate reader.

All assays were conducted a minimum of three times in duplicate. Data was analyzed using Graphpad PRISM statistical software. Linear equations were used to model the relationship between trophozoite concentration and signal while dose-response curves were constructed using four parameter logistic regression assuming a variable slope model.

3. Results

3.1. Resorufin metabolism correlates with concentration of trophozoites

Dilutions of *T. foetus* were co-incubated with resorufin followed by fluorescence detection. Visually, live cells rapidly converted red resorufin into colorless dihydroresorufin, while dead cells and control wells maintained a stable yellow color. The fluorescence signal was proportional to the number of trophozoites for the tested range of 1–50,000 trophozoites/mL (Fig. 1).

3.2. Propidium iodide and ATP luminescence assay are poor predictors of trophozoite concentration

Dilutions of *T. foetus* trophozoites were co-incubated with propidium iodide and CTG reagent followed by fluorescence or luminescence detection, respectively. The linear relationship of trophozoites and signal in these assays was not suitable for estimation of trophozoite concentrations. For both of these assays, linear regression analysis revealed a decreased r^2 value and slope as compared to resorufin which indicated poor sensitivity (Fig. 1). Multiple attempts to optimize these assays with different incubation times and reagent concentrations were unsuccessful (data not shown).

3.3. Dose-response assays using the resorufin reduction method are useful for estimating EC_{50} values

Construction of standard curves with resorufin allowed calculation of cell concentrations and enabled performance of trophozoite killing assays (Fig. 2). Trophozoite killing assays using metronidazole and ronidazole demonstrated that the resorufin reduction method could be used to generate dose response curves suitable for comparison of EC_{50} values. The calculated EC_{50} values for metronidazole and ronidazole were 1.7 μm and 0.5 μm, respectively.

4. Discussion

T. foetus is distributed throughout the United States and bovine trichomoniasis results in substantial economic losses in the cattle industry. In humans, trichomoniasis, caused by *T. vaginalis* is treatable with metronidazole. However, this compound is banned from use in food animals due to concerns regarding its carcinogenic potential (Payne et al., 1999). In cattle, there are no antimicrobial products available that are effective against *T. foetus* and current control strategies involve testing and culling bulls. Developing therapeutic interventions for bovine trichomoniasis would dramatically reduce economic losses associated with the disease.

Currently, *in vitro* killing assays of *T. foetus* are evaluated by microscopic examination. This method is time consuming and relies on changes in motility or complete destruction of trophozoites. Methods of rapidly screening large compound libraries for anti-*T. foetus* activity would substantially enhance ongoing control efforts. In the present study, we evaluated three different methods for drug screening that can be adapted for use in 96-well plate formats.

Resorufin is a fluorescent compound that was previously shown to be reduced by *T. vaginalis* into dihydroresorufin, a colorless non-fluorescent metabolite (Natto et al., 2012). In the present study, reduction of resorufin correlated highly with the number of *T. foetus* trophozoites (Fig. 1) and standard curves could be generated to estimate concentration of trophozoites after co-incubation with test compounds. Dose-response experiments indicated the EC_{50} for metronidazole and ronidazole to be 1.7 μm and 0.5 μm, respectively. These values are similar to those reported for *T. foetus* drug testing based on microscopic observation and we expect that the