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A new quantitative *in vitro* microculture method for *Giardia duodenalis* trophozoites

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Abstract

A reliable, rapid and low-cost method for drug sensitivity determination of *Giardia duodenalis* trophozoites (WB-strain) was developed in a 96-well plate. Using a standard inoculum of 5×10^4 trophozoites per well (300 μl), good growth was obtained after sealing the plate with an air-tight adhesive tape and incubation at 37 °C for 72 h in modified TYI-S-33 medium. Viable burdens were quantified using the formazan dyes MTT (100 μg/well) and XTT (20 μg/well) and the fluorescent substrate resazurin (2.5 μg/well). Prior removal of the culture medium is required since it causes spontaneous reduction of the substrate. Resazurin proved to be far superior to MTT and XTT with a level of sensitivity of about 3×10^4 trophozoites. Inhibitory concentrations (IC_{50}) of several anti-giardial reference drugs were within the range of published values: metronidazole 2.25 μM, tinidazole 1.75 μM, albendazole 0.10 μM, furazolidone 2.00 μM and quinacrine 0.32 μM. The broad-spectrum antibiotics chloramphenicol, rifampicin, penicillin G+streptomycin and gentamycin were devoid of any inhibitory activity and are considered suitable for decontamination during excystation experiments.

Keywords: *Giardia duodenalis*; Trophozoites; *In vitro* microculture; Resazurin; Drug susceptibility assay

1. Introduction

The protozoan parasite *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) is one of the more common gastrointestinal parasites worldwide with growing clinical relevance in public health and veterinary medicine (Savioli et al., 2006). In general, diagnostic and epidemiological studies focus on the 'cyst' form. However, for drug discovery, resistance monitoring and fundamental research, *in vitro* culture of trophozoites remains the standard laboratory tool. Trophozoites must be grown under conditions of reduced oxygen tension and several methods to achieve microaerophilic conditions have been proposed (Upcroft and Upcroft, 2001). Because of their 'amitochondriate' nature (Adam, 1991), spectrophotometric determination of viable burdens poses an additional challenge (Ali and Nozaki, 2007).

Current *in vitro* assays rely on viability assessment of trophozoites and are generally performed in 'large volume' air-tight culture tubes. However, these tube assays are laborious, time-consuming and above all low-throughput. Microtiter plate assays are the better alternative, but are problematic due to the need for standardized low-oxygen environment in every well of the plate. To accommodate this, incubation in air-tight modular chambers and flushing with nitrogen (Bell et al., 1991), low-oxygen gas mixtures (Adagu et al., 2002) or application of commercial anaerocult™ systems (Upcroft and Upcroft, 2001) have been proposed. Another problem is the lack of a standard method for quantifying total viable trophozoite burdens, whereby a wide range of endpoint parameters have been used, precluding proper comparison of data from different laboratories. Some methods, such as microscopic assessment of growth (Upcroft and Upcroft, 2001; Bell et al., 1991), morphology (Arguello-Garcia et al., 2004), motility (Upcroft and Upcroft, 2001) and inhibition of adherence (Cruz et al., 2003) are much too laborious to be

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practically applicable in reasonable throughput. Radiolabel incorporation (Arguello-Garcia et al., 2004; Bell et al., 1991) is still regarded as a golden standard, but requires sophisticated equipment and is burdened with radioactive waste disposal. Taking the above reflections into consideration, simple spectrophotometric methods are attractive, but do need proper cross-validation.

The aim of this study was to establish a simple, reliable, fast and low-cost *in vitro* drug susceptibility assay for *G. duodenalis* trophozoites in a 96-well microplate format. Four methods (trypan blue exclusion, MTT, XTT and resazurin) were compared to quantify viable burdens. Resazurin, widely used to determine cell viability and cytotoxicity (O'Brien et al., 2000) has yet never been evaluated for *Giardia* trophozoites viability assessment, despite recent indications that it could be used for *Trichomonas vaginalis* (Campos-Aldrete et al., 2005).

2. Materials and methods

2.1. Axenic culture of trophozoites

Trophozoites (WB-strain [ATCC 30957] and G1-strain [source: Swiss Tropical Institute]) were grown in modified TYI-S-33 medium (Keister, 1983) at pH 7.0, supplemented with 10% heat inactivated fetal calf serum (FCSi, Gibco) and 0.05% bovine bile (Sigma) in 10 ml screw-cap culture vials (Nunc). To attain low-oxygen tension conditions, the tubes were filled to 90–95% of total volume capacity and stationary incubated at 37 °C, with subcultures (10^6 trophozoites per tube) being made three times per week. Detachment of trophozoites for preparation of inocula was achieved by chilling the cultures on ice for 20 min. Trophozoites were obtained after one washing cycle in phosphate buffered saline (PBS). The number of trophozoites was determined using 0.4% trypan blue (w/v) (Fluka) and 0.04% formol in a KOVA Glasstic® counting slide (Hycor Biomedical).

2.2. Establishment of growth conditions

5×10^4 trophozoites per well were used for all assays in 96-well microplates (Greiner) and the amount of yeast (Becton Dickinson), FCSi, bovine bile (Sigma) and ammonium ferrous citrate (Fluka) was increased by about 15% compared to the standard TYI-S-33 culture medium. The final culture volume was 300 µl/well. The plates were incubated at 37 °C, either individually sealed with an air-tight adhesive tape (Greiner) or under anaerobic (100% nitrogen) or microaerophilic (93% N_2, 4% CO_2, 3% O_2) conditions in a modular incubation chamber (Flow Labs). Trophozoite growth was determined after 72 h by microscopic counting using 0.4% trypan blue (w/v) as viability indicator. The free motile forms and the adherent fraction were counted separately in the initial experiments. At least 6 replicates were included in each test.

2.3. Spectrophotometric quantification (GENios, Tecan) of viable trophozoite burdens

The tetrazolium salts MTT and XTT (Sigma) have been used in anti-giardial drug assays (Wright et al., 1992). In the presence of the catalyst phenazine–methosulfate (PMS, Filter Service), live trophozoites reduce the salts to coloured formazan compounds that can be measured at 540 nm and 450 nm respectively. Resazurin (Sigma) is converted to a pink soluble dye by reductase activity and measured fluorimetrically (λ_{ex} 550 nm–λ_{em} 590 nm) (O'Brien et al., 2000). Since the culture medium causes spontaneous conversion of both tetrazolium salts and resazurin, prior removal of the growth medium was necessary.

A suspension of washed trophozoites was 2-fold serially diluted in PBS in a 96-well microplate (300 µl/well) within the range of 10^6–10^2 trophozoites/well. After addition of MTT (100 µg/well) or XTT (20 µg/well) and PMS (1 µg/well), the plates were sealed and further incubated at 37 °C for 90 min or 60 min respectively. The soluble XTT–formazan permits immediate reading, while MTT–formazan requires prior extraction in 100% DMSO. Since a positive effect had been demonstrated for D-glucose on the production of MTT–formazan in cells (Vistica et al., 1991), the effect of glucose addition at 1 mg/ml was also studied. A same design was used after addition of resazurin (1 µg or 2.5 µg/well) with fluorimetric reading at regular time intervals to determine the optimal endpoint reading. In parallel, microscopic counts were performed in a KOVA counting slide.

2.4. In vitro drug susceptibility assay

20 mM stock solutions of the anti-giardial drugs (Wright et al., 2003) metronidazole, albendazole, tinidazole, furazolidone and quinacrine were prepared in 100% DMSO. The series of *Giardia* reference compounds was expanded with a number of broad-spectrum antibiotics from the viewpoint of their use for cyst decontamination during *in vitro* excystation experiments (Mäser et al., 2002; Isaac-Renton et al., 1992) and included chloramphenicol, rifampicin, penicillin G+streptomycin and gentamycin. All reference compounds were obtained from Sigma and/or Gibco. The highest in-test concentration for all drugs was 64 µM, except for penicillin+streptomycin (500 U/ml+500 µg/ml) and gentamycin (1000 µg/ml). The in-test concentration of DMSO was less than 0,5%.

A 300 µl standard inoculum containing 5×10^4 trophozoites (WB and G1 strain) was added to 4 replicate wells containing the pre-diluted test compounds (10 µl). A non-treated control (100% growth) and culture medium control (0% growth) were included on each plate. The culture plates were sealed and incubated at 37 °C for 72 h. Only the adherent fraction of trophozoites was considered since the growth medium must be removed before addition of the substrates. This will not affect the outcome of the test since the free motile and adherent fraction are interchanging continuously during culture and are considered one dynamic population (Ghosh et al., 2001). After addition of resazurin, MTT, XTT or trypan blue in PBS+1 mg/ml glucose total viable trophozoites burdens were determined spectrophotometrically and by microscopic counting. Each test was repeated on 3 separate occasions.

2.5. Statistics

In all protocols, in-test and inter-test replicates were included. Probit analysis was used to calculate the concentration of drug

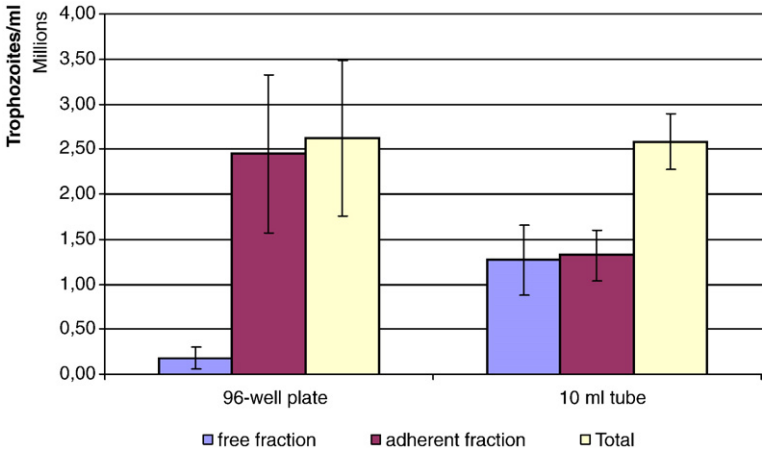

Fig. 1. Comparative *in vitro* growth of *G. duodenalis* trophozoites (WB-strain) in 10 ml and 300 µl culture volumes after 72 h incubation (microscopic counting of 6 independent replicates: means ± SD).

that inhibited trophozoite viability by 50% (StatView® statistical software, V.5.0.1).

3. Results

3.1. Optimal growth conditions in microplates

Open microplate cultures in a modular incubation chamber did not support adequate growth under strict anaerobiosis (100% N_2) nor after flushing with the microaerophilic gas mixture (93% N_2–4% CO_2–3% O_2). Sealing of the plates with an air-tight adhesive seal resulted in good growth, however, with the requirement to fill up the well completely with 300 µl, leaving a minimal residual air bubble. The latter entailed careful handling during manipulations and incubation to avoid spillage and/or cross-contamination. Lower volumes (200 µl and 250 µl) did not support optimal growth. In contrast, growth decreased with almost 100% and 50% (data not shown). Since a *Giardia* trophozoite culture is composed of an adherent fraction and a free motile fraction, their relative contribution to the total viable burden was determined for both the standard 10 ml tube culture and the 300 µl plate culture by microscopic counting (Fig. 1). In the 10 ml culture (total count: 2.58×10^6/ml), the adherent and the free fraction each contributed for about 50% with minimal variation between the replicate counts. In the micro-culture (total count: 2.62×10^6/ml), the free fraction was negligible (approx. 8%). However, the microscopic counts were slightly more variable (Fig. 1), related to the overall smaller sample in the replicate micro-well samples.

3.2. Spectrophotometric quantification of viable trophozoite burdens

Application of MTT and XTT to a two-fold dilution of washed trophozoites in PBS revealed rather low sensitivity for both formazan salts (Fig. 2). Specific absorbance above background levels occurred only at concentrations exceeding 5×10^5 trophozoites, thereby making this colorimetric technique less suitable for viability determination of low trophozoite burdens. Addition of glucose at 1 mg/ml did not have any marked influence (data not shown). Addition of resazurin at either 1 µg or 2.5 µg/well and fluorimetric reading after about 16 to 24 h incubation resulted in an adequate response and easy endpoint reading. The addition of 1 mg/ml glucose showed some enhancing effect with a

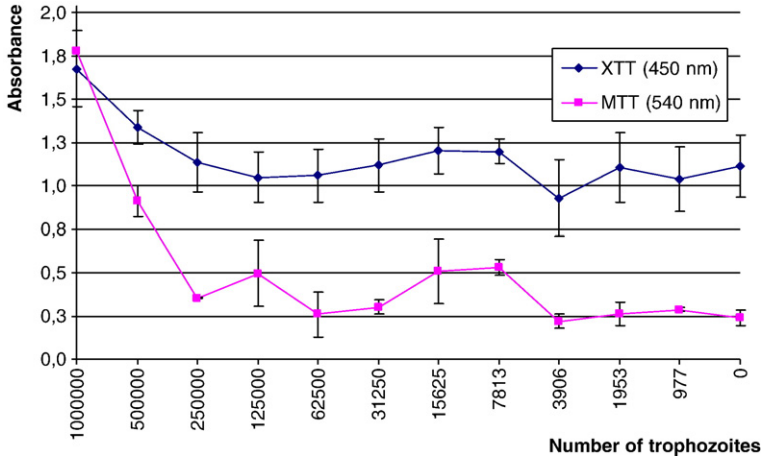

Fig. 2. Spectrophotometric quantification of *G. duodenalis* trophozoites (WB-strain) with the formazan salts MTT and XTT (4 independent replicates: means ± SD).

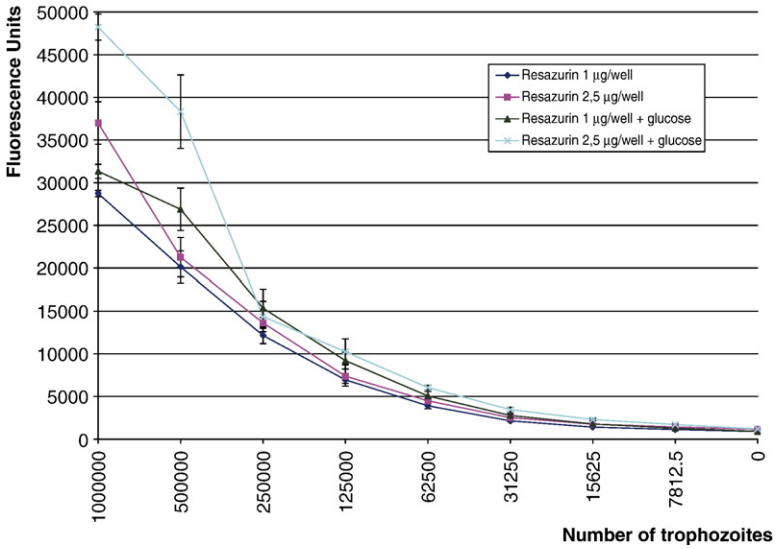

Fig. 3. Spectrophotometric quantification of *G. duodenalis* trophozoites (WB-strain) with resazurin (4 independent replicates: means±SD).

sensitivity level of about 3×10^4 trophozoites (e.a. absorbance>mean+2 SD) (Fig. 3). Spontaneous reduction of XTT, MTT and resazurin occurred in complete medium, necessitating its prior removal. The responsible medium constituents are Vit-C, L-cystein and yeast extract.

3.3. In vitro drug susceptibility assay

The inhibitory effect of metronidazole was titrated on WB-strain trophozoites, comparing all four methods for endpoint reading (Fig. 4). The IC_{50}-values were: 1.50±0.44 µM for resazurin, 2.67±0.58 µM for XTT, 3.01±0.11 µM for microscopic reading and 5.50±1.53 µM for MTT, which proved to be the least sensitive with the largest variation. Resazurin was subsequently used in a drug susceptibility validation assay including additional standard anti-giardial drugs (Table 1) and a number of antibiotics that could be used for decontamination during cyst excystation experiments. None of the antibiotics showed any inhibitory effect at the highest in-test concentration (data not shown).

4. Discussion

Over the last decades, the 96-well microplate format has become the standard in a wide range of laboratory applications because of the practical advantage of using small volumes of reagents and easy implementation of automation, throughput and endpoint reading. In that respect, cultivation of *Giardia* trophozoites remains problematic due to the need for a low-oxygen environment, causing several practical shortcomings to current plate methods (Upcroft and Upcroft, 2001). Although different microculture methods have been proposed in the literature, the present study clearly indicated that optimal growth can be obtained by using the near-total well volume with air-tight adhesive sealing of the plate. Careful handling of the plates is required to avoid contamination. The need for a small residual air bubble follows the current view that *Giardia* trophozoites are obligate microaerophilic (Brown et al., 1998; Lane and Lloyd, 2002) rather than strictly anaerobic, which is confirmed by the fact that no growth at all was noted after flushing with 100% N_2. The observation that the low-oxygen

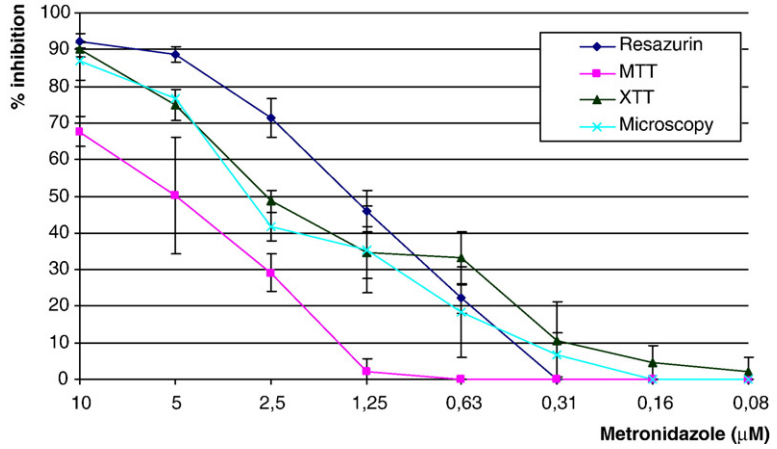

Fig. 4. Inhibitory effect (IC_{50}-value) of metronidazole on *G. duodenalis* trophozoites (WB-strain) using different endpoint parameters. (3 independent replicates: means±SD).

Table 1
IC_{50}-values of different anti-Giardial reference compounds in a microplate assay, using resazurin for viability measurement

Giardia strains	Reference strain — WB	Lab. strain — G1	Literature data[a] reference strain — WB	
	Measured IC_{50} (μM)±SD		Mean IC_{50} (μM)	IC_{50} range (μM)
Metronidazole	2.25±0.96	2.70±0.95	1.6	0.54–2.90
Tinidazole	1.75±0.46	0.90±0.11	0.78	0.30–1.26
Albendazole	0.10±0.04	0.54±0.44	0.12 (tube assay)	0.055–0.39 (tube assay)
Furazolidone	2.00±0.76	1.40±0.55	1	0.97–1.03
Quinacrine	0.32±0.05	0.31±0.04	0.04	0.01–0.06

[a] From: Arguello-Garcia et al., 2004, Bell et al., 1991, 1993, Cruz et al., 2003, Katiyar et al., 1994.

gas mixture (93% N_2, 4% CO_2, 3% O_2), normally used for cultivation of *Plasmodium* (Trager, 1982) and *Helicobacter* (Bury-Mone et al., 2006) did not support adequate growth, was unexpected as it contradicts previous literature data (Adagu et al., 2002).

Another practical consideration is that multiplying trophozoites are present as one dynamic population, interchanging between the free motile fraction and the adherent fraction covering the available substrate surface (Ghosh et al., 2001). Inoculation with 10^5 trophozoites/ml resulted in a confluent layer of adhering trophozoites after 48–72 h incubation at 37 °C, both in 10 ml tubes and 300 μl microwells, however with a difference in the relative composition of the two fractions (Fig. 1). While both fractions are about equal in the 10 ml tube cultures, the microcultures mainly consisted of adherent forms (approx. 92%), which can be ascribed to the proportionally larger surface area and the smaller volume of medium. Removing the culture medium will therefore have no relevant influence on the outcome of a test.

One of the more basic concerns of any *in vitro* test with *Giardia* is the enumeration of viable trophozoite burdens, which is reflected by the wide range of methods that have been proposed. Microscopic counting after addition of trypan blue has been most frequently used, but it is labour-intensive and suffers from inaccuracy in the identification of death cells. Evaluation of adhesion has therefore been proposed as a more functional alternative (Cruz et al., 2003). Because of the amitochondriate nature of *Giardia*, spectrophotometric methods based on the enzymatic reduction of colorigenic (formazan salts: MTT, XTT, …) or fluorigenic (Alamar Blue™, resazurin) substrates were not addressed to the same extent as for viability assessment of cells and other aerobic (microbial) organisms (Hamid et al., 2004). There is still some speculation concerning the specific enzymes in *Giardia* that could be responsible for substrate reduction, whereby pyruvate ferredoxin oxidoreductase, α-ketobutyrate oxidoreductase and ferredoxin NAD oxidoreductase have been suggested (Brown et al., 1998). A practical disadvantage of using MTT, XTT or resazurin in *Giardia* viability testing is the fact the culture medium spontaneously reduces these substrates, due to the presence of strong reducing agents, such as ascorbic acid, L-cystein and yeast extract. Prior removal of the medium becomes therefore essential, but actually does not affect the outcome of the test since the majority of trophozoites are adherent (Ghosh et al., 2001). Titration of washed trophozoites in the presence of MTT and XTT resulted in low sensitivity with a detection level of 5×10^5 trophozoites (Fig. 2). Addition of glucose did not enhance sensitivity, a positive effect observed in cell lines (Vistica et al., 1991). A practical disadvantage of MTT is the need for an additional extraction step of insoluble formazan in an organic solvent, for example DMSO.

Addition of resazurin resulted in a reproducible and linear fluorescence and a lower detection level (3×10^4) with slightly enhanced signal after glucose addition (Fig. 3). Since resazurin and its reduced derivative resorufin are not cytotoxic (O'Brien et al., 2000), dynamic follow-up experiments remain possible. Using this experimental design, the inhibitory potencies (IC_{50}) of the anti-giardial reference compounds metronidazole, tinidazole, quinacrine and albendazole were determined and corresponded fully with published values (Table 1). It was also shown that several broad-spectrum antibiotics were devoid of any inhibitory action, making them suitable for inclusion in a decontamination cocktail for excystation experiments and axenisation of field isolates (Mäser et al., 2002; Isaac-Renton et al., 1992).

The present experiment demonstrated that resazurin can be used for viability endpoint measurement of *Giardia* trophozoites in *in vitro* drug susceptibility assays. The test is simple, rapid, sensitive, reliable and cost-effective and does not require sophisticated equipment. This microplate method offers possibilities for automation and may become an asset in screening large compound libraries for new drug discovery.

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