

Validation of a modified fluorimetric assay for the screening of trichomonacidal drugs

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A fluorimetric microassay that uses a redox dye to determine the viability of the flagellate Trichomonas vaginalis has been optimised to provide a more sensitive method to evaluate potential trichomonacidal compounds. Resazurin has been used in recent years to test drugs against different parasites, including trichomonadid protozoa; however, the reproducibility of these resazurin-based methods in our laboratory has been limited because the flagellate culture medium spontaneously reduces the resazurin. The objective of this work was to refine the fluorimetric microassay method previously developed by other research groups to reduce the fluorescence background generated by the media and increase the sensitivity of the screening assay. The experimental conditions, time of incubation, resazurin concentration and media used in the microtitre plates were adjusted. Different drug sensitivity studies against T. vaginalis were developed using the 5-nitroimidazole reference drugs, new 5-nitroindazolinones and 5-nitroindazole synthetic derivatives. Haemocytometer count results were compared with the resazurin assay using a 10% solution of 3 mM resazurin dissolved in phosphate buffered saline with glucose (1 mg/mL). The fluorimetric assay and the haemocytometer counts resulted in similar percentages of trichomonacidal activity in all the experiments, demonstrating that the fluorimetric microtitre assay has the necessary accuracy for high-throughput screening of new drugs against T. vaginalis.

Key words: *Trichomonas vaginalis* - antitrichomonal agents - drug screening assay - resazurin - high-throughput screening assays

Trichomoniasis is one of the most common sexually transmitted diseases, with a prevalence of 180 million infections worldwide per year, which is higher than the number of reported cases of syphilis, chlamydia and gonorrhoea (Aggarwal & Shier 2008). This cosmopolitan parasite can cause a wide range of clinical symptoms in the genitourinary tract, including vaginitis, cervicitis, endometritis and vulvitis in women and urethritis and prostatitis in men (Ali & Nozaki 2007). However, the infection in males is generally asymptomatic and they are typically considered silent carriers of the disease (Cudmore et al. 2004).

Metronidazole and related 5-nitroimidazole derivatives are potent drugs used to treat infections caused by anaerobic or microaerophilic protozoa and bacteria, although resistance to these drugs has been demonstrated both in *Trichomonas vaginalis* isolates sampled from patients refractory to treatment (Wendel & Workowski 2007, Aggarwal & Shier 2008, Kissinger et al. 2008) and in laboratory-developed resistant isolates (Dunne et al. 2003). When the trichomonacide metronidazole was first introduced in 1959, its reported cure rate was approximately 95%, but within two years of its introduction, the first

case of metronidazole resistance was reported in Canada (Lossick 1990). Clinical resistance has now been reported in most areas of the world (Dunne et al. 2003, Wendel & Workowski 2007). Metronidazole is classified by the Food and Drug Administration as pregnancy category B, therefore some clinicians have used clotrimazole as an alternative treatment for trichomoniasis in pregnant women concerned about the possible foetal damage caused by trichomoniasis (Swygard et al. 2004).

Recently, our group developed a quantitative structure-activity relationship model for the *in silico* prediction of new trichomonacidal products. However, experimental corroboration *in vitro* relies on the haemocytometer count (Marrero-Ponce et al. 2009), which is a laborious, time consuming, insensitive and subjective procedure.

Alamar Blue[®] has been used as a rapid, efficient and sensitive method for the antibacterial screening of new compounds against *Mycobacterium tuberculosis*, *Bacillus* spp and *Staphylococcus* spp (Barrow et al. 2007, Daryae et al. 2009, Flemming et al. 2009), the determination of antifungal activity (*Candida albicans*, *Aspergillus fumigatus*) (Bowman et al. 2002, Repp et al. 2007), the screening of antiviral compounds (influenza virus) (Mo et al. 2008) and the screening of antiprotozoal drugs (*T. vaginalis*, *Giardia duodenalis*, *Trypanosoma brucei*, *Leishmania* spp, *Acanthamoeba* spp) (Raz et al. 1997, Mikus & Steverding 2000, Campos Aldrete et al. 2005, McBride et al. 2005).

Alamar Blue[®] and its major component, resazurin, are blue and non-fluorescent dyes that become pink and fluorescent (resorufin) due to the chemical reduction of oxidoreductases present in viable cells (O'Brien et al. 2000).

Financial support: AECID (A/030156/10), MICINN (SAF2009-10399), CEI-Campus Moncloa

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Received 21 December 2011

Accepted 19 March 2012

The fluorescence method with Alamar Blue® was first described by Campos Aldrete et al., in 2005. More recently, a resazurin-based method was described by Duarte et al. (2009b). We have been able to reproduce this resazurin-based method, but have observed interference between our culture media and the resazurin dye. Thus, we have modified the experimental conditions to increase the sensitivity of the method. Different drugs have been screened to validate the new protocol.

The aim of this study was the development and validation of a simple, rapid (one-step), sensitive and reliable method for use in our laboratory to screen new trichomonacidal drugs based on the fluorescence emitted by the redox indicator (resazurin) as it is reduced by viable protozoa. The results presented here enable the replacement of microscope-based methodologies with spectrofluorimetric assays.

SUBJECTS, MATERIALS AND METHODS

T. vaginalis - *T. vaginalis* (isolate JH31A #4, ref. 30326; ATCC, MD, USA) was cultured in vitro in trypticose-yeast-maltose (TYM) media (6.4 pH) supplemented with 10% (v/v) heat-inactivated bovine serum. Cultures were incubated at 37°C in 5% CO₂ and subcultured every two-three days. Samples of the isolate were conserved in liquid N₂ using TYM containing 10% heat-inactivated foetal bovine serum and 10% of the cryoprotectant dimethyl sulfoxide (DMSO) (Sigma Chemical Co, St. Louis, MO).

Drugs - The drugs assayed during the method validation were the 5-nitroimidazoles metronidazole and secnidazole; clotrimazole, an antifungal compound used by some clinicians if the reference drugs, are contraindicated and new synthetic products. The compounds were dissolved in DMSO to measure their effectiveness against *T. vaginalis* via different methods. The final DMSO concentration in the culture media never exceeded 0.2% (v/v). Previous studies with 0.2% (v/v) DMSO in *T. vaginalis* cultures were performed to confirm the absence of cytotoxic effects of the dissolvent in the cultures. All cultures (with and without DMSO) had the same growth rate (GR) (data not shown). The synthetic compound G-1 was previously evaluated by our laboratory (Meneses-Marcel et al. 2008).

Resazurin preparation - Resazurin sodium salt (Sigma-Aldrich, St. Louis, MO, USA) was stored at 4°C and protected from light until use. Several resazurin stock solutions (1, 2, 3 and 4 mM) were prepared in 0.1% phosphate buffered saline/glucose (PBS/G) (Bénére et al. 2007) and filter-sterilised prior to use. The reduction of resazurin by reductase activity was measured by fluorimetric readings taken in a plate fluorometer (Infinite 200, TECAN) at $\lambda_{\text{excitation}}$ 535 nm and $\lambda_{\text{emission}}$ 590 nm after performing the Alamar Blue® Assay (US Patent 5,501,959). Because carbohydrates are the main source of energy in *T. vaginalis*, glucose was added to the PBS/G solution.

Assay optimisation - Before further optimisation, the spontaneous reduction of the resazurin in the culture media was demonstrated. Our results corroborate those

described by other authors (Bénére et al. 2007, Borra et al. 2009). TYM media without *T. vaginalis* and modified media lacking the reducing compounds ascorbic acid and L-cysteine were compared with PBS/G. The fluorescence of the plate was measured at different times and compared between these different media and non-resazurin media. The effect of the addition of 1 mg/mL glucose was also studied.

A similar experimental design was used after the addition of resazurin (1, 2, 3 and 4 mM/ 20 μ L/ well) with fluorimetric readings at regular time intervals to determine the optimal endpoint reading. A suspension of washed trophozoites (log-phase culture) was serially diluted two-fold in PBS in a 96-well microplate (Costar, NY, USA) (200 μ L/ well) at a concentration between 6×10^4 - 4×10^6 trophozoites/mL. Then, the plates were incubated at 37°C (5% CO₂) for 30 min, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h. At each time point, a fluorescence intensity reading was performed for the experimental media and the PBS/G with resazurin control. The variation, correlation coefficients and other statistical parameters were calculated to adjust for the number of parasites, time of reading and resazurin concentration.

In vitro assay and experimental corroboration - The in vitro activity of metronidazole, secnidazole, clotrimazole, G-1 and the synthetic derivatives of 5-nitroindazoles and 5-nitroindazolinones were compared via a microscope-based count in a Neubauer chamber and by the fluorescent method using resazurin. The trichomonacidal activity of metronidazole and secnidazole was studied at 0.0625, 0.125, 0.25, 0.5, 1 and 2 μ g/mL. G-1 was studied at 5, 10 and 20 μ g/mL. Clotrimazole was studied at concentrations ranging from 0.625-10 μ g/mL and the synthetic drugs were screened at 1, 10 and 100 μ g/mL.

A logarithmic phase culture of *T. vaginalis* was seeded in 15 x 150-mm glass tubes. After 6-h incubation (0 h), the compounds were added to cultures containing 10⁵ protozoa/mL in a final volume of 2 mL at the indicated concentrations. Three replicate experiments for each drug concentration were performed. A non-treated control (100% growth) and culture medium control (0% growth) were also used. After incubating for 24 h at 37°C in a humidified 5% CO₂ atmosphere, 200 μ L from each tube was seeded in sterile 96-well flat-bottomed microplates and centrifuged at 300 g for 5 min to eliminate the media. The parasites were resuspended in 200 μ L PBS/G and 20 μ L resazurin (stock solution at 3 mM). After 1 h of incubation, the fluorescence was measured (λ_{ex} 535 nm - λ_{em} 590 nm). The microscope-based haemocytometer count of viable flagellates was simultaneously assessed. At least three independent experiments were performed for each in vitro assay.

Expression of antiprotozoal activity - The percentage of growth inhibition (% GI) was calculated over GR, which is the relation between the number of viable protozoa at 24 h and the number counted at 0 h (Muelas-Serrano et al. 2000):

$$\% \text{ GI} = [1 - (\text{GR drug} / \text{GR control})] \times 100$$

RESULTS

Spontaneous reduction of TYM media - A spontaneous reduction of resazurin and an accompanying increase in the fluorescence intensity of the TYM media used for the culture of *T. vaginalis* were observed (Fig. 1). Even TYM media without the reducing compounds L-cysteine and ascorbic acid (modified media) was fluorescent. The fluorescence units value recorded in the wells containing media was higher than those with viable parasites. The wells from which media had been removed had lower fluorescence than the wells containing trophozoites in PBS/G (Fig. 2). After taking these results into account, the protocol was changed for the subsequent experiments. Under the new protocol, after 24 h of drug exposure, 200 µL of each culture was added into the wells and the media was removed before the addition of the resazurin solution. No fluorescence variation was noted at any resazurin concentration when using PBS/G (1 mg/mL) to replace the media. These results indicate that PBS is useful as an alternative, non-fluorescent medium.

Optimisation of the assay - Resazurin is an oxidation-reduction dye that turns into a fluorescent compound (resorufin) after a reduction process caused by viable trophozoites. The intensity of fluorescence measured is proportional to parasite growth and can be quantified by fluorescence spectroscopy.

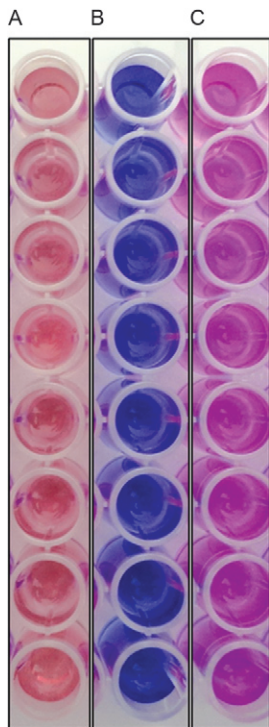


Fig. 1: comparison of colours in wells without trophozoites using trypticose-yeast-maltose (A) and phosphate buffered saline/glucose (PBS/G) (B) as medium after incubation with resazurin. C shows the pink resorufin colour obtained after incubation of *Trichomonas vaginalis* (100.000 trophozoites/mL) in PBS/G.

To optimise the method, the experimental parameters, such as the resazurin concentration, media used in the probe, inoculum of parasites and time of incubation with the redox dye, were adjusted.

Different concentrations of resazurin were tested, allowing us to obtain the smallest variation coefficient (Table I). The strongest linear correlation between the fluorescence percentage and protozoan density (Fig. 3) was noted at 3 mM ($R^2 = 0.9916$).

A standard curve for *T. vaginalis* at different incubation times with a 10% solution of 3 mM resazurin is shown in Fig. 4. An optimum linearity was achieved after 1 h of incubation and the highest coefficient of linear determination ($R^2 = 0.9983$) was observed for 6×10^4 - 4×10^6 trophozoites/mL.

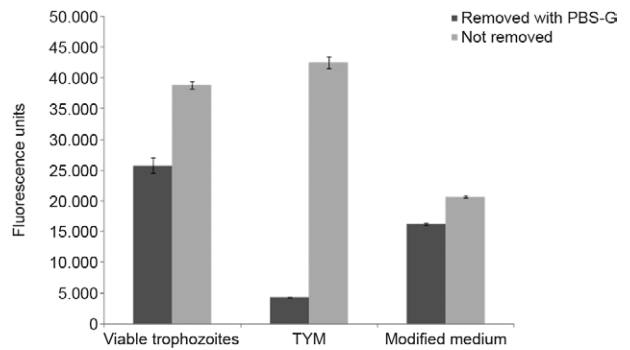


Fig. 2: evaluation of resazurin substrate reduction by different medium. Comparative fluorescence lectures of a *Trichomonas vaginalis* culture and fresh medium without trophozoites [complete trypticose-yeast-maltose (TYM) medium and modified medium without L-cysteine and ascorbic acid]. The fluorescence emitted was studied with prior removal with buffered saline/glucose (PBS/G) (dark bar) and without remove (gray bar). The fluorescence background obtained when the fresh medium without parasites is used in comparison with PBS/G (2nd group of bars) revealing PBS/G as the most suitable for using with resazurin. All experiments were performed three times. Mean and standard deviation values are shown.

TABLE I

Determination of the resazurin concentration based on the coefficient variation of fluorescence values of *Trichomonas vaginalis* cultures at different time incubation

Time (h)	4 mM	3 mM	2 mM	1 mM
0.5	10.16	4.82	7.20	7.55
1	5.28	3.06	2.60	3.20
2	3.83	1.61	1.59	2.46
3	2.86	1.53	1.47	2.29
4	2.32	1.21	1.37	2.03
5	2.20	1.05	1.41	2.00
6	2.14	1.07	1.31	1.96
Mean ± SD	4.11 ± 2.9	2.05 ± 1.4	2.42 ± 2.2	3.07 ± 2.0

SD: standard deviation.

In vitro drug susceptibility assay - To determine the reliability of this fluorescence method for the screening of new products against *T. vaginalis*, reference drugs, alternative drugs for trichomoniasis treatment (metronidazole, secnidazole and clotrimazole) and synthetic

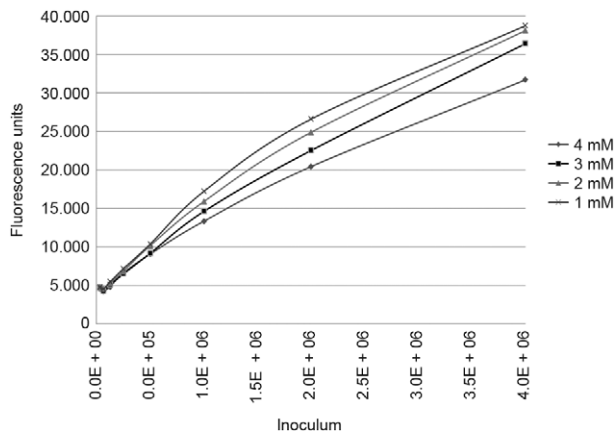


Fig. 3: change in resazurin reduction with dye concentration and plating density of *Trichomonas vaginalis* after 1 h of incubation time with 20 μ L of different resazurin solution. The concentrations studied were 1 mM, 2 mM, 3 mM and 4 mM showing a correlation coefficient of 0.9728, 0.9841, 0.9916 and 0.9728 respectively. The optimal value ($R^2 = 0.9916$) is shown at 3 mM concentration. Each signal is the average of six individual experiments.

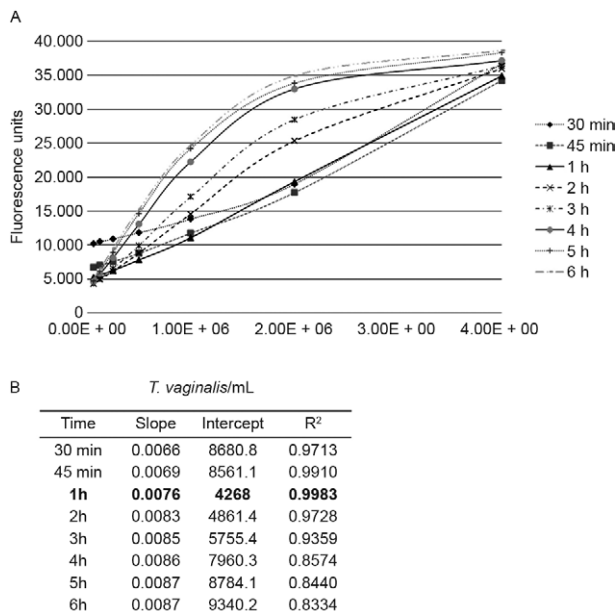


Fig. 4: correlation between resazurin reduction (3 mM) and the number of *Trichomonas* per millilitre after different incubation periods. A: graphic representation of standard curves of *Trichomonas vaginalis* cultures at different incubation times; B: correlation data between the different standard curves. The time of incubation with best correlation ($R^2 = 0.9983$) between fluorescent and viable trophozoites has been 1 h.

compounds (G-1, 5-nitroindazole and 5-nitroindazolone derivatives) were tested using the resazurin assay and the microscope-based counting method simultaneously. No relevant difference between the % GI was observed when comparing both methods (Tables II, III). An analysis of variance (ANOVA) was performed using SPSS software (v.20, IBM) on the data collected using both methods. There was no statistically significant difference ($p > 0.05$) between the resazurin and the microscope-based assays. The quality of the resazurin-based method presented here for use in high-throughput screening assays has been estimated using the Z'-factor (Zhang et al. 1999). The obtained result [$Z' = 0.8$ ($1 > Z' \geq 0.5$)] suggests that the resazurin assay has excellent potential for screening.

Each plate should include control wells without parasites to avoid false negative results that could occur if any of the assayed products reduce the resazurin or produce fluorescence. In addition, growth and metronidazole control wells should be included.

DISCUSSION

Our results demonstrate that the resazurin-based fluorimetric method is a fast, non-subjective, reliable, sensitive and affordable assay for *in vitro* drug screening against the human protozoan *T. vaginalis*.

TABLE II

Percentage of fluorescence inhibition (% FI) and percentage of growth inhibition (% GI) of therapeutic drugs determined by resazurin and microscopic counting against *Trichomonas vaginalis*

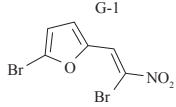
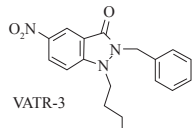
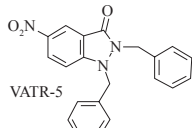
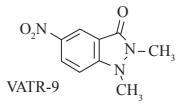
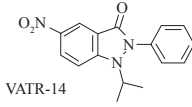
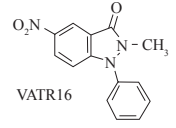
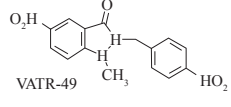
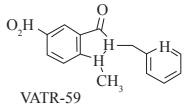
Therapeutic drugs	Concentration μ g/mL	% FI resazurin ^a	% GI haemocytometer ^a
Metronidazole	2	100 \pm 0	94.86 \pm 0.03
	1	92.1 \pm 5.58	91.5 \pm 2.77
	0.5	77.6 \pm 4.14	81.06 \pm 4.14
	0.25	46.15 \pm 9.36	51.64 \pm 9.36
	0.125	20.16 \pm 8.45	19.9 \pm 8.45
	0.0625	13.96 \pm 7.08	3.78 \pm 7.08
Secnidazole	2	99.95 \pm 0.03	100 \pm 0
	1	92.07 \pm 0.67	95 \pm 1.7
	0.5	75.37 \pm 8.46	79.71 \pm 8.3
	0.25	48.8 \pm 13.72	55.14 \pm 8.08
	0.125	30.65 \pm 0.96	45.71 \pm 10.49
Clotrimazole	0.0625	31.17 \pm 1.09	30 \pm 12.63
	10	100 \pm 0	84.63 \pm 2.01
	5	79.32 \pm 6.91	75.53 \pm 2.16
	2.5	54.86 \pm 16.95	65.54 \pm 5.23
	1.25	41.49 \pm 3.86	51.74 \pm 6.73
0.625	36.5 \pm 8.59	21.49 \pm 8.43	

a: the results obtained show no difference between methodologies ($p > 0.05$). All experiments are performed at least per triplicate. Statistical comparisons of both methods were performed by the analysis of variance test with SPSS software (v.20, IBM).

The traditional method used for testing new compounds against this flagellate is based on microscope-based counting of viable trophozoites with a haemocytometer (Muelas-Serrano et al. 2000, Gomez Barrio et al. 2004, Tiwari et al. 2008). This technique has several drawbacks: it is time consuming, laborious and a highly dependent on the observer. Several drug sensitivity assays for this parasite have been described using radiolabelled nucleotides (Adagu et al. 2002) or colourimetric assays utilising *p*-nitrophenyl phosphate (Martinez-Grueiro et al. 2003) or tetrazolium salts (Zhang et al.

2009) as chromogenic substrates. These screening tests have been proposed as alternatives to microscope-based counting; however, the fluorescent and colorimetric dye Alamar Blue® permits the development of a non-radioactive, sensitive and one-step assay (Raz et al. 1997). In the years following the development of the fluorescent technique, resazurin, which is the major constituent of the Alamar Blue® started to be used in many assays due to lower cost. Resazurin is currently being used instead of Alamar Blue® to evaluate natural and synthetic products against bacteria, fungi and parasites (Rolon et al.

TABLE III
Trichomonacidal activity of synthetic products (G-1, 5-nitroindazoles, 5-nitraindazolinones derivatives) by resazurin and haemocytometer methods

Synthetic compounds	Concentration $\mu\text{g/mL}$	% FI resazurin ^a	% GI haemocytometer ^a	Structures
G-1	20	100 \pm 0	100 \pm 0	
	10	100 \pm 0	100 \pm 0	
	5	100 \pm 0	100 \pm 0	
VATR-3	100	62.4 \pm 1.04	78.23 \pm 4.77	
	10	0 \pm 0	0 \pm 0	
	1	0 \pm 0	0 \pm 0	
VATR-5	100	46 \pm 3.64	76 \pm 2.27	
	10	0 \pm 0	0 \pm 0	
	1	0 \pm 0	0 \pm 0	
VATR-9	100	98.01 \pm 0.1	99.89 \pm 0.13	
	10	0 \pm 0	0 \pm 0	
	1	0 \pm 0	0 \pm 0	
VATR-14	100	38.7 \pm 5.28	37.58 \pm 7.43	
	10	0 \pm 0	0 \pm 0	
	1	0 \pm 0	0 \pm 0	
VATR-16	100	5.57 \pm 4.13	11.38 \pm 11.96	
	10	1.12 \pm 1.94	8.94 \pm 7.6	
	1	0 \pm 0	3.06 \pm 3.03	
VATR-49	100	95.79 \pm 0.23	96.05 \pm 0.18	
	10	33.92 \pm 1.28	35.79 \pm 8.93	
	1	0 \pm 0	0 \pm 0	
VATR-59	100	86.35 \pm 0.76	90.84 \pm 0.78	
	10	0 \pm 0	2.11 \pm 3.94	
	1	0 \pm 0	0 \pm 0	

^a: the results obtained show no difference between methodologies ($p > 0.05$); % FI: fluorescence inhibition; % GI: percentage of growth inhibition.

2006, Bénéré et al. 2007, Sarker et al. 2007, Duarte et al. 2009a, b, Mirza et al. 2011) and to study the proliferation of mammalian cells (O'Brien et al. 2000). A fluorescence screening method for *T. vaginalis* has already been developed (Campos Aldrete et al. 2005, Duarte et al. 2009b). The main differences between the different fluorescent assays are the number of trophozoites per well, the excitation and emission wavelength for the fluorimetric determination and the choice of redox indicator. Although Campos Aldrete et al. (2005) developed a screening test using Alamar Blue®, Duarte et al. (2009b) used the more affordable resazurin dye. In both cases, the media used for the culture of the trophozoites is not removed.

In accordance with the results of a 2007 study by Bénéré et al. and a 2009 study by Borra et al., our group observed a spontaneous reduction of resazurin by trichomonadid culture media (Fig. 1). These results suggest that one or more compounds in the TYM medium reduce the fluorescent substrate. A modified medium lacking L-cysteine and ascorbic acid was studied, revealing the reductive behaviour of both components, though at a lower reduction percentage than the complete fresh media. As shown in Fig. 2, media lacking parasites had higher fluorescence than the complete fresh media with trophozoites. This effect is likely the result of the consumption of nutrients in the TYM media by viable flagellates, resulting in the reduction of resazurin. The reductive effect of yeast extract was not confirmed, because media without this component would not have sufficient nutrients for the correct maintenance and growth of the isolate. Yeast extract and vitamins are present in the different trichomonadid culture media (Petrin et al. 1998) used by different research groups (Campos Aldrete et al. 2005, Duarte et al. 2009b). Our study presents a modified protocol that removes the reductive media, guaranteeing that resorufin can only be generated by the metabolic activity of viable *T. vaginalis*. This, in turn, lowers the possibility of false negatives caused by spontaneous media-related reduction.

Standard curves for *T. vaginalis* (Figs 3, 4) indicate a correlation between the fluorescence emitted by resorufin and the quantity of viable flagellates in the logarithmic growth phase. These curves allowed the direct in vitro determination of the trichomonadid effect of different compounds added to the parasite cultures. The addition of 1 mg/mL glucose did not have any marked influence on the reduction of resazurin (data not shown) as was observed with other research groups (Bénéré et al. 2007). Different 5-nitroimidazole drugs, the antifungal clotrimazole and synthetic products were tested to confirm the effectiveness of this modified assay. The results obtained by both screening procedures (microscope-based counting vs. fluorimetric assay) corroborate the reliability of this resazurin-based assay (Tables II, III). The comparison of the population average of the two methodologies with the ANOVA test also indicated no divergence between results. Although resazurin is a fluorimetric and colourimetric redox indicator, this research was developed using fluorescence measurements. Previous studies indicate that fluorescence readings are more sensitive than spectrophotometry (O'Brien et al. 2000).

Overall, we conclude that the media used for the in vitro culture of *T. vaginalis* can reduce the fluorescent dye resazurin, thus increasing the fluorescence background of the probe. Therefore, it is essential to remove the culture media from the wells to increase the accuracy of the assay. The modified fluorimetric method described in this study could be easily automated for high-throughput screening.

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