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Improvement of 96-well microplate assay for estimation of cell growth and inhibition of *Leishmania* with Alamar Blue

María Jesús Corral, Elena González, Montserrat Cuquerella, José María Alunda *

Department of Animal Health (ICPVet Group), Faculty of Veterinary Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain

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ABSTRACT

The value of resazurin-based Alamar Blue redox indicator to determine multiplication of *Leishmania* promastigotes in 96-well microtiter plates was examined. In addition, assay was validated with amphotericin B (AmB) and allicin. The method was tested on *L.donovani* and *L.infantum* promastigotes under different culture conditions (variable air-phase, presence of phenol red, initial cell density, incubation time, use of Hepes buffer). Results showed that the gas-phase of promastigote cultures was critical. The method yielded consistent results with initial plating cell densities of 2.5×10^5 promastigotes/well, up to 72 h incubation and 5% CO_2 atmosphere or reduced air availability (sealed plastic bags, film-sealed microplates). Detection of low numbers of promastigotes and earlier results could be obtained using fluorimetry instead of spectrophotometry. The addition of 20 mM Hepes improved the results. Fluorescence intensity correlated to promastigotes number in both *Leishmania* spp. Inhibitory concentration (IC_{50}) values for AmB and allicin using cell counting and fluorimetry were comparable. Under these conditions this one-step, low-cost redox indicator can be used in drug sensitivity assays and studies of differential proliferation rates of *Leishmania* isolates or strains in a 96-well format.

1. Introduction

Visceral leishmaniasis is a parasitic disease caused by *Leishmania donovani* and *L. infantum* (= *L.chagasi*) (Kinetoplastida). The infection affects both humans and dogs in large areas of the world (i.e. India, Mediterranean Basin, and South America) and it is fatal unless treated. Current first-line chemotherapy of leishmaniasis relies on a rather limited arsenal of drugs, most of which have serious side-effects including nephro- and hepatotoxicity and teratogenicity. Therefore, the identification of new molecules or formulations is an urgent need and has been recognised by WHO as one of the research areas where a sustained effort has to be made (Alvar et al., 2006).

It is assumed that in vivo models have superior predictive value than in vitro models, and that screening using intracellular amastigotes are more convenient than axenic amastigotes and promastigotes (Sereno et al., 2007; Vermeersch et al., 2009; De Muylder et al., 2011; Gupta and Shakya, 2011). In spite of the limitations the promastigote stage is currently used and screening with this parasitic stage has been exploited as a first-step to identify "hit" and "lead" anti-leishmanial compounds in undirected massive screening [High Throughput Screening (HTS)] of chemical libraries (Sharlow et al., 2009; Siqueira-Neto et al., 2010; Walker et al., 2011).

There are several in vitro systems available to determine promastigotes proliferation of *Leishmania* spp (i.e. reporter gene assays, enzymatic determinations, H^3 -thymidine incorporation, colorimetric methods). Among colorimetric methods, resazurin-based Alamar Blue entails several advantages. First, it is simple to use as it requires only a one-step procedure. Other benefits reported are its low cost, environmentally friendly composition and transferability to field sites if necessary (Räz et al., 1997). Unlike other assays, this redox indicator is relatively non-toxic to cells and can be used with long incubation periods (up to 72 h) (Fumarola et al., 2004). This indicator has been extensively used in the related genus *Trypanosoma* (Räz et al., 1997; Rolón et al., 2006; Sykes and Avery, 2009) and in some *Leishmania* spp (Mikus and Steverding, 2000; de Oliveira-Silva et al., 2008; Shimony and Jaffe, 2008; Kulshrestha et al., 2013). Recently, this method has been adapted using HTS with *Leishmania* and two different platforms and a 384-well format (Sharlow et al., 2009; Siqueira-Neto et al., 2010).

Our laboratory has been engaged on the study of the anti-leishmanial antiproliferative effect of different molecules. While Alamar Blue could be easily employed to determine the cytotoxicity for the murine cell line J774 (Wert et al., 2011), results obtained with *Leishmania* promastigotes were inconsistent, since resazurin reduction did not correlate with cell counts. Given the lack of experimental details given in the available literature dealing with *Leishmania*, our aim was to examine the value of Alamar Blue to determine the multiplication and growth inhibition of *L. donovani* and *L. infantum* promastigotes under different culture conditions (variable air-phase, cell density and incubation time). Results showed that optimal conditions of Alamar Blue assay with promastigotes in 96-well microtiter plates included a 5% CO_2 atmosphere, the presence

* Corresponding author at: Dpt. Animal Health, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain. Tel.: +34 91 3943701; fax: +34 91 3943908.
E-mail address: jmalunda@ucm.es (J.M. Alunda).

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on 20 mM Hepes in the culture medium and an initial concentration of promastigotes of ca. 2.5×10^5/mL. With these conditions, reduced resazurin (resorufin) measured by fluorimetry provided an accurate estimation of promastigotes multiplication and could be used for drug screening and IC_{50} estimation.

2. Material and methods

2.1. Parasites

An autochthonous isolate of *L. infantum* (UCM 9), obtained from affected dogs in the area of Madrid (Spain) by the Clinical Service of the Department of Animal Health, Faculty of Veterinary Medicine (Universidad Complutense), and Khartoum 1246 isolate from *L. donovani*, provided by Dr. Toraño (Department of Immunology, Instituto de Salud Carlos III, Madrid) were routinely maintained as promastigotes in RPMI 1640 medium (Lonza Group, Basel, Switzerland) at 26 °C supplemented with heat inactivated (30 min, 56 °C) foetal bovine serum (FBS) (Sera Laboratories International, Horsted Keynes, UK) and 100 U/mL penicillin + 100 μg/mL streptomycin (BioWhittaker, Verviers, Belgium) in 25 mL culture flasks.

2.2. Chemicals

Alamar Blue was purchased from AbD Serotec (Oxford, UK). Allicin (2-Propene-1-sulfinothioic acid S-2-propenyl ester) was obtained as liquid Allisure® from Allicin International Ltd (Rye, East Sussex, UK) at a concentration of 5000 ppm and kept at a temperature of −80 °C until used. Amphotericin B (AmB) was obtained as fungizone (Sigma, St. Louis, USA).

2.3. Promastigote assays

Depending on the experiment promastigotes were cultured in flat-bottomed 96-well cell culture microtiter plates with lid (Costar, Corning, NY, USA), in microtiter plates wrapped with Parafilm®, or in plates sealed with Thermal adhesive film for PCR plates (Simport, Beloeil, Canada). For comparative purposes promastigote cultures were also done in 1.5 mL eppendorf ® tubes. Cultures were carried out at 26 °C in aerated culture chamber or incubated in a 95% air/5% CO_2 humidified atmosphere. Culture media (RPMI 1640) with and without additional 20 mM Hepes were employed depending on the experiment.

2.4. Alamar Blue assay

Concentration of resorufin, the product of reduction of resazurin, in the *Leishmania* cultures was determined following the manufacturer's recommendations by reading the absorbance (A) at 570 and 600 nm, and fluorescence (550 nm excitation wavelength, 590 nm emission wavelength) in a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) fluorimeter. Fluorescence intensity was expressed as arbitrary units (A.U.). Briefly, mid-log phase promastigotes were added to the wells of microtiter plates or eppendorf tubes up to a volume of 200 μL/well. After 24 h, 20 μL Alamar Blue (10% v/v) was added and the cultures were kept for 24, 48 or 72 additional hours. Absorbance and fluorescence intensity were determined every 24 h. Promastigote counts were carried out in Neubauer improved chambers and cell viability was assessed by trypan blue exclusion staining. Untreated cultures, wells without cells and the maximal concentration of the drugs, and wells with culture medium and Alamar Blue (10% v/v) were included as controls. All experiments were performed at least in triplicate.

2.5. Statistical analysis

Results were expressed as means ± standard deviation. Data were compared by analysis of variance (one- and two-ways ANOVA) and GLM analysis using GraphPad Prism5. Differences were considered significant when $p < 0.05$. Figures were also prepared with GraphPad Prism5.

3. Results

3.1. Determination of optimal cell density

Different concentrations (10^4, 2.5 x10^4, 5×10^4, 7.5×10^4, 10^5, 2.5×10^5, 5×10^5, 7.5×10^5, 10^6) of mid-log phase promastigotes of *L.donovani* and *L.infantum* were added in a final volume of 200 μL/well in 96-microtiter plates. Cultures were carried out at 26 °C in plates with lid under a 5% CO_2 atmosphere or in film-sealed plates. After 24 h incubation, Alamar Blue was added and the plates were kept for 24, 48 or 72 h. For each time determination a plate was used. Resazurin was effectively reduced to resorufin in the medium, evidenced by the higher levels of absorbance and, particularly, fluorescence related both to the initial promastigotes density and the time of culture. Absorbance determinations, especially with low initial promastigote concentration ($<10^5$ promastigotes/well) were more variable (not shown). This variability was not observed when cultures were kept in the CO_2 atmosphere. Results were more consistent when resorufin concentration was determined by fluorimetry. Fluorescence was significantly higher (ca. 2 times) when cultures were exposed to 5% CO_2 (Fig. 1A) as compared to those performed in film-sealed plates (Fig. 1B). Highest levels of fluorescence were obtained with initial inoculums of 2.5 to 5×10^5 promastigotes/well from both *Leishmania* species, after 72 h and exposition to CO_2.

3.2. Correlation between Alamar Blue reduction and promastigotes multiplication

Preliminary results obtained in our laboratory allowed the use of the redox indicator to determine the proliferation of *Leishmania* promastigotes in culture tubes. However, as shown above, for a given initial number of promastigotes and time of incubation, significant differences ($p < 0.05$) were found in the concentration of resorufin estimated by absorbance and, particularly, fluorimetry depending on the exposition to CO_2 or to a limited air phase in the sealed microtiter plates. To rule out the possibility of resazurin reduction being an inaccurate estimation of *Leishmania* multiplication, promastigotes (2.5×10^5/well) were cultured in 96-well plates under a CO_2 atmosphere or under air phase. For comparative purposes parallel cultures were done in eppendorf tubes. In all cases cell multiplication was estimated by fluorimetry and cell counting of viable *Leishmania* in Neubauer chamber. A set of cultures was employed for each time determination (24, 48, 72 and 96 h).

Fig. 2 shows that no significant differences ($p > 0.05$) were found between cultures in the experiment irrespective of the exposition to 5% CO_2 or to an unlimited air phase. After 96 h, all cultures reached values ca. 2.7×10^6 promastigotes/mL in both *Leishmania* spp (Fig. 2A). However, resazurin concentration determined by fluorimetry was strongly dependent on the culture conditions (Fig. 2B). Highest levels of fluorescence were seen in cultures exposed to CO_2 and the lowest values were present using standard culture conditions with air atmosphere. Cultures in eppendorf tubes, with a limited amount of air, displayed intermediate values. These results were consistent with the absence of colour change in the standard microtiter plates in spite of the active multiplication. These results suggested the importance of the air phase of the cultures in the usefulness of Alamar Blue method to determine *Leishmania* proliferation and also the need of standardization of the assay.

3.3. Effect of atmosphere and buffer on resazurin reduction by promastigotes of Leishmania

Promastigotes of *L.infantum* and *L.donovani* were cultured in 96-well standard microtiter plates exposed to 5% CO_2, in standard microplates, in microplates wrapped with Parafilm, in microplates in sealed plastic