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Comparison between two common methods for measuring *Giardia lamblia* susceptibility to antiparasitic drugs in vitro

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Abstract

In this study a comparison between two different methods for measuring the susceptibility of *Giardia lamblia* trophozoites to metronidazole and albendazole is performed. Modifications of Meloni's method, based on the loss of adherence of parasites to surfaces, and the Hill method, based on the loss of parasite division capacity, are compared. A logistic model was used to calculate the inhibitory concentrations IC_{10}, IC_{50} and IC_{90} that were further compared using the respective standard errors. The results obtained, after contact of parasites with the antiparasitic drugs for 24 h, show that the adherence method is more sensitive than the multiplication method for low and moderate inhibitory concentrations of albendazole. Conversely for metronidazole the multiplication method seems to be more sensitive for high inhibitory concentrations of the drug. For screening the IC_{50}, both methods seem to be effective, however, the inhibition of adherence method have even better performance for the benzimidazole like drugs.
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1. Introduction

Giardia lamblia is a common enteric parasite spread all over the world. It is responsible for diarrhea, malabsorption and growth disturbances in children. Giardiosis is normally difficult to treat being, metronidazole and more recently albendazole drugs of choice (Ortega and Adam, 1997). Metronidazole needs to be reduced to an active cytotoxic form. The antimicrobial activity of reduced metronidazole results from the reactivity and dismutation of short-lived intermediates that kill the trophozoites by interacting with various cellular components such as RNA, DNA, proteins and membrane components resulting in irreparable cellular damage (Freeman et al., 1997). On the other hand, albendazole has the microtubules as target. It seems that it binds to parasite β-tubulin, inhibiting polymerization into microtubules with a

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cascade of other metabolic effects resulting from this (Reynoldson et al., 1992; Upcroft and Upcroft, 2001).

Some evidence suggests the existence of drug-resistant *G. lamblia* strains (Kulda and Nohyhová, 1995; Barat and Bloland, 1997), which have contributed to the development of drug susceptibility tests in order to define the resistance rate of the strains that infect human population. The most commonly used methods are the one described by Meloni et al. (1990), that checked the trophozoites viability by measuring the adherence inhibition of parasites, and the method firstly described by Hill et al. (1986) for measuring growth inhibition of trophozoites.

It is important to have a method that can be easily applied in routine laboratory tests to check the drug sensibility of *G. lamblia* strains and for screening new antigiardial agents. In this work, our objective was the comparison of the two methods referred above in order to choose the more convenient, in the sense of it being less costly, easy to apply and less prone to error measurements. This will be the method of choice to be routinely applied in our laboratory for further studies with *G. lamblia* isolates.

2. Materials and methods

2.1. Chemotherapeutic agents

The chemotherapeutic agents used were metronidazole (Sigma) and albendazole (Sigma). Stock solutions were prepared in phosphate buffer saline (10 mM; pH 7.4), for metronidazole, and dimethyl sulphoxide for albendazole, with a concentration that never exceeded 0.5%.

2.2. Parasites

G. lamblia trophozoites (strain WB, ATCC 30957) were routinely cultivated at 37 °C in Diamond's modified TYI-S-33 medium (Keister, 1983), supplemented with 10% heat inactivated bovine serum (Biochrom) and gentamicin (50 μg/ml), penicillin G (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Trophozoites in the mid-logarithmic phase were harvested by cooling on water-ice for 20 min and counted in a hemocytometer (Newbauer cell-counter chamber).

2.3. Susceptibility assays

Two different methods were used to access trophozoites viability. A modification of the method described by Meloni et al. (1990), who checked the trophozoite viability measuring the adherence inhibition of parasites resulting from the presence of chemotherapeutic agents. In this assay 5×10^5 trophozoites were added to 9 ml of modified TYI-S-33 medium and incubated for 45 min at 37 °C. This step is needed for adherence of trophozoites into polystyrene screw cap wall tubes (Nunk). Appropriate drug dilutions, in order to obtain 0.5–4000 μM of metronidazole and 6×10^{-3} to 400 μM of albendazole, were added and the volume completed to 10 ml with medium. After incubation for 24 h at 37 °C, the adherent trophozoites/field were counted in a inverted microscope.

The method described by Hill et al. (1986), is based on the inhibition growth of *G. lamblia* parasites. In this assay 5×10^5 log-phase trophozoites were added to 9 ml of modified TYI-S-33 medium and incubated for 45 min at 37 °C. Metronidazole and albendazole were added to the media in the same concentrations used in the previous method. After incubation for 24 h at 37 °C, the adherent cells were resuspended in the media after cooling on water-ice for 30 min. Then, 100 μl of this trophozoite suspension were diluted in 1500 μl of TYI-S-33 modified medium and incubated for 48 h at 37 °C. After inactivation of parasites with 2% formalin, the cells were counted in a Newbauer chamber and compared with control cultures.

For each drug and assay five independent experiments were conducted. Within a single experiment three independent determinations of the number of viable cells were taken at each drug concentration, 15 different concentrations ranging from 0 (control) to 4000 μM for metronidazole and 18 different concentrations ranging from 0 (control) to 400 μM for albendazole. For each experiment the number of cells at different concentrations was expressed as a percentage of the average number of viable cells in the respective control culture.

2.4. Data analysis

A logistic model was fitted to the data for each assay using the method of least squares. The model used

(Guilhermino et al., 1999) can be described as:

$$Y = \frac{K}{1 + (X/\mathrm{IC}_{50})^b}$$

where Y is the square root of the number of viable cells after exposure to a concentration X for a given time period, IC_{50} represents the concentration that produced a 50% reduction in the number of viable cells, b the rate constant and K is the square root of the expected number of viable cells in the control ($X = 0$). This model can be parameterized in terms of any concentration, such as IC_{10} and IC_{90}, since for $X = \mathrm{IC}_{10}$, Y should be equal to 90% of K and for $X = \mathrm{IC}_{90}$, Y should be equal to 10% of K. For each assay three models were fitted to the data to obtain the estimates of these concentrations as the actual parameters:

$$Y = \frac{K}{1 + ((1 - r)/r)(X/\mathrm{IC}_r)^b}$$

where b, K have the same meaning as before and r is the square root of the proportion of reduction in the number of viable cells.

This model was fitted to the global data for the five experiments within each assay. Assumptions of randomness and normality of residuals were tested for data validation and identification of outliers. The number of viable cells, as defined by the parameters in the two models—K, b and the inhibitory concentrations IC_{10}, IC_{50}, IC_{90}, were compared using the t-statistic. This statistic is the ratio of the difference between the parameters estimated to the square root of the sum of square standard errors associated with the separate parameter estimates, since the number of data points used to obtain each parameter estimate is similar. This value was compared with the critical value of the t-distribution with the degrees of freedom equal to the sum of observations in the two experiments minus 6, which is the sum of the number of parameters estimated in the two models.

3. Results

3.1. Metronidazole

The logistic model was suitable to represent the data obtained in each assay, after exclusion of one outlier in the adherence model and three outliers in the multiplication model. The different patterns of decrease in the number of viable cells for the two models is apparent from the estimates for the parameters based on these models and respective standard errors shown in Table 1. The shape parameter b is higher for the multiplication model than for the adherence model, which is reflected in a steepest decrease in the number of viable cells in the first model, reaching a 90% reduction at a concentration of 8.4 μM whilst similar reduction in the adherence model was experienced for a concentration of 21.9 μM. Despite this fact, the inhibitory concentrations IC_{10} and IC_{50} were not significantly different for the two models (Table 1).

Table 1
Metronidazole IC_{10}, IC_{50} and IC_{90} estimates for the logistic adherence and multiplication models

Parameters	Adherence model (μM) ($n = 224$)	Multiplication model (μM) ($n = 222$)	t-statistic
K	10.3 (0.17)[a]	9.65 (0.15)	2.90**
b	1.86 (0.07)	2.58 (0.12)	4.99***
IC_{10}	0.54 (0.06)	0.71 (0.07)	1.91[b]
IC_{50}	2.63 (0.22)	2.26 (0.15)	1.41[b]
IC_{90}	21.9 (1.8)	8.40 (0.48)	7.03***

[a] Standard error.
[b] $P > 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3.2. Albendazole

Since there were no viable cells for concentrations higher than 1.0 μM, the fit involved only 135 data

Table 2
Albendazole IC_{10}, IC_{50} and IC_{90} estimates for the logistic adherence and multiplication models

Parameters	Adherence model (μM) ($n = 134$)	Multiplication model (μM) ($n = 135$)	t-statistic
K	9.59 (0.11)[a]	9.48 (0.10)	0.72[b]
b	4.04 (0.35)	5.89 (0.52)	2.94**
IC_{10}	0.059 (0.004)	0.080 (0.004)	3.82***
IC_{50}	0.098 (0.003)	0.113 (0.003)	3.26**
IC_{90}	0.15 (0.005)	0.15 (0.005)	0.39[b]

[a] Standard error.
[b] $P > 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

points. With exclusion of one outlier in the adherence model, the logistic curve provided an adequate description of the data for both methods. There was evidence of a different pattern of decrease in the number of viable cells for the two methods, reflected in the shape parameter and in the inhibitory concentrations IC_{10} and IC_{50} (Table 2). These concentrations are significantly lower in the adherence model than in the multiplication model. Moreover no significant differences were found in the concentration needed for a 90% reduction in the number of viable cells for both models.

4. Discussion

Nowadays, the increasing number of noticeable drug-resistant infect agents, like *Plasmodium* spp. and *Leishmania* spp. (World Health Organization, 2000), is one of the major public health problems. *G. lamblia* infections are frequent in children (Ortega and Adam, 1997), sometimes very hard to treat, without an apparent known reason (Kulda and Nohyhová, 1995; Zaat et al., 1997). The resistance to some of the antiparasitic drugs frequently used is one possible explanation. A routine method for the evaluation of the susceptibility of *G. lamblia* to the antiparasitic drugs is desirable. In this study we compared two of the most frequent described methods for that proposal (Hill et al., 1986; Meloni et al., 1990) using an ATCC *G. lamblia* strain. These methods use different ways to evaluate the viability of the parasitic cells, by measuring the inhibition of the trophozoite adherence (Meloni et al., 1990) and the losses of cellular division capacity (Hill et al., 1986). Since metronidazole and albendazole, most frequent drugs used to treat *Giardia* infections, have different mechanism of action (Reynoldson et al., 1992; Freeman et al., 1997; Zaat et al., 1997), it is important to check out how is the performance of those methods in the evaluation of the susceptibility of *G. lamblia* isolates to these different antiparasitic drugs.

For the comparison of the two methods the logistic function, describing a process whose relative rate of decrease is proportional to the actual number of subjects, has the desirable asymptotic behavior for low and high concentrations, useful in modeling reproduction or growth (Silva et al., 1995). The parameterization used has a direct interpretation of the biological points of interest, the inhibitory concentrations corresponding to a *r*% reduction in the number of viable cells, reducing the level of multicollinearity which led to smaller standard errors of the parameter estimates, resulting in their improved precision. Compared to the assignment of ranks to interval counts of cells used by Meloni et al. (1990) allows a more precise estimation of any specific inhibitory concentration and makes use of all dose–response data points in contrast with the graphical method based on the average number of cells obtained for each concentration used by Edling et al. (1990).

Using this statistical model our results indicate that to achieve a 10 and 50% reduction in the number of viable cells, the metronidazole concentrations needed are not significantly different for both methods. Thus it can be concluded that both methods perform equally well at low concentrations of metronidazole, IC_{10} and IC_{50}. Conversely to achieve a 90% reduction the multiplication model seems to be more sensitive than the adherence model, the values for IC_{90} obtained were 8.4 and 21.9 μM, respectively. These results suggest that for high metronidazole concentrations, *G. lamblia* trophozoites lose their capacity of division before losing the adherence capacity, which can be ascribed to the mechanism of action of this drug on the replication of the nucleic acids of the parasite by acting as an electron acceptor (Freeman et al., 1997). On the other hand, the pattern of decrease in the number of viable cells displayed as a result of albendazole action at low concentrations is different for the two methods. The drug sensitivity of *G. lamblia* is higher in the adherence model than in the multiplication model, reflected in the lower values IC_{10} and IC_{50} in the former method. In contrast with metronizadole, albendazole is more active at the citoskeleton level, provoking the disruption of the ventral disc (Chávez et al., 1992), which is responsible for the adherence of the parasite. This effect is reflected in the adherence model before a similar effect is achieved in the multiplication model. Nevertheless, for high concentrations of albendazole, both methods indicate a similar activity of the drug.

Our results indicate that the methods used are largely equivalent and the logistic model, besides providing an adequate fit to the data, provided also estimates of parameters, which could be ascertained

by the different mechanisms of action of these drugs. In terms of further use of these methods and if the screening process is to be based in the inhibitory concentration IC_{50}, the modification of Meloni's adherence method will be preferred for drugs with similar mechanism of action as albendazole, being also easier to perform and less time consuming than the Hill's multiplication method. This method seems to be more sensitive for high concentrations of drugs that have a similar mechanism of action of the metronidazole.

For screening the IC_{50}, both methods seem to be effective, however, the inhibition of adherence method have even better performance for the benzimidazole like drugs.

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