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Isolation, excystation and axenization of *Giardia lamblia* isolates: *in vitro* susceptibility to metronidazole and albendazole

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From 53 samples of human faeces containing *Giardia lamblia* cysts, 18 isolates were successfully excysted *in vitro*, and cultivated axenically in TYI-S-33 modified medium. The *in vitro* effects of metronidazole and albendazole on these isolates were evaluated by the trophozoite adherence inhibition method. The IC_{50} was between 2.4 and 11.5 μM for metronidazole and 0.027 and 0.192 μM for albendazole. These IC_{50} values were similar to those found for the ATCC 30888 and 30957 reference isolates. All isolates were susceptible to the antiparasitic drugs tested. These results suggest that resistance of *G. lamblia* to metronidazole and albendazole does not seem to be a significant problem in our population.

Keywords: *Giardia*, susceptibility assays, metronidazole, albendazole

Introduction

There is a lack of information about *Giardia lamblia* strains, which parasitize different populations. The difficulties in isolating and cultivating *G. lamblia* trophozoites *in vitro* are probably the main reason. In the 1970s, in Portland, *G. lamblia* was isolated and cultured axenically for the first time (ref. P-1), and was kept as a reference in the American Type Culture Collection (ATCC 30888).[1] Most *in vitro* studies of *G. lamblia* have used the P-1 or the WB isolate (ATCC 30957), which was obtained from a patient with prolonged symptomatic giardiasis, probably acquired in Afghanistan.[1] The introduction of methods for the axenic cultivation of *G. lamblia*, improvements in culture media and development of techniques for the *in vitro* excystation of the parasite have made it possible to obtain many axenic isolates from various geographical regions.[2,3] Isolation can also be performed using trophozoites obtained directly from duodenal aspiration or from excystation *in vivo*.[2,4]

There is only a limited range of drugs available for treatment of giardiasis. These drugs comprise the nitroimidazoles, quinacrine and furazolidone. Metronidazole is the drug of choice, but alternatives, such as albendazole, are available.[5–7]

Some clinical reports suggest the appearance of drug resistance to explain treatment failure in giardiasis.[7]

The establishment of *G. lamblia* axenic cultures in Portugal made it possible to evaluate the susceptibility of isolates to antiparasitic drugs. Giardiasis is considered an important parasitic disease in Portugal, but there is a lack of information regarding the antimicrobial susceptibility profile of the strains, which parasitize the Portuguese population. In the present study, we describe the isolation and axenic cutting of *G. lamblia* parasites from human faeces obtained in the Porto region and report on the susceptibility of these isolates to metronidazole and albendazole using the inhibition of adherence method.

Materials and methods

G. lamblia cyst isolation

Fifty-three human faecal samples (1–3 days old) from different patients, containing abundant *G. lamblia* cysts, were obtained

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from an epidemiological survey of the school children population and clinical laboratories in the Porto region.

G. lamblia cysts were purified and concentrated from faeces combining the sucrose flotation method with a simplified sucrose gradient method.[4] The cysts, after being washed twice in distilled water, were resuspended in distilled water and stored at 4°C for a maximum of 3 days prior to use.

Excystation and axenization

The excystation procedure was a modification of the Bingham & Meyer technique performed by Schupp *et al.*[2] The isolation procedure involved three steps: the concentration and cleaning of cysts by centrifugation in sucrose gradients performed from 1 to 3 days after collection, the induction of excystation performed in acid solution from 1 to 5 days after cleaning cyst suspensions, and the culture and axenization in modified TYI-S-33 medium.

Cryopreservation of *G. lamblia* trophozoites was performed using the method of Hautus *et al*.[4]

Reference organisms

The control reference isolates were *G. lamblia* ATCC 30888 and ATCC 30957.

Chemotherapeutic agents

The chemotherapeutic agents used were metronidazole and albendazole (Sigma). Stock solutions were prepared in phosphate-buffered saline (10 mM in phosphate, pH 7.4), for metronidazole, and DMSO for albendazole. The final DMSO concentration in the culture tubes was always <0.5% (v/v).

Adherence inhibition assays

To assess trophozoite susceptibility, the adherence inhibition method was performed.[6]

The results are presented as a percentage of inhibition of adherence (%IA) according to the formula:

$$\%IA = (\text{control cell number} - \text{tested cell number})/\text{control cell number} \times 100$$

All the assays were performed in duplicate and repeated twice for each isolate and drug.

Statistical analysis

Probit analysis was used to calculate the concentration of drug that inhibited the viability by 50% of *G. lamblia* adherent trophozoites (IC_{50}).

Results

From 53 different human faecal samples containing *G. lamblia* cysts, we were able to obtain 18 *G. lamblia* axenic cultures, representing an overall success rate of 34%. A high concentration of cysts in the faeces was needed for successful excystation and axenization (Figure 1). During the process of excystation, trophozoites hatched from their cysts, underwent cytokinesis and attached to the surface of the culture tubes 1–3 h after *in vitro* excystation. In successful cultures, trophozoites began to divide and multiply within 2–3 days and subsequently formed a monolayer on the surface of the culture tube.

A significant number of purified cyst samples were not established as cultures. The three major reasons for this were: no excystation, persistent microbial contamination and failure of the trophozoites to establish the monolayer culture. In the latter case, the number of trophozoites increased during the first 24 h, but then declined.

The susceptibility of the 18 *G. lamblia* isolates to metronidazole and albendazole was evaluated by the inhibition of adherence method.[6]

The IC_{50} values calculated for each isolate after 24 h of drug exposure are presented in Table 1. For metronidazole the IC_{50} values varied from 2.38 to 11.50 μM (mean 4.92 μM; S.D. 2.56 μM), representing a range of variation of 4.8-fold in susceptibility. For albendazole, the range of IC_{50} values varied from 0.027 to 0.192 μM (mean 0.088 μM; S.D. 0.035 μM),

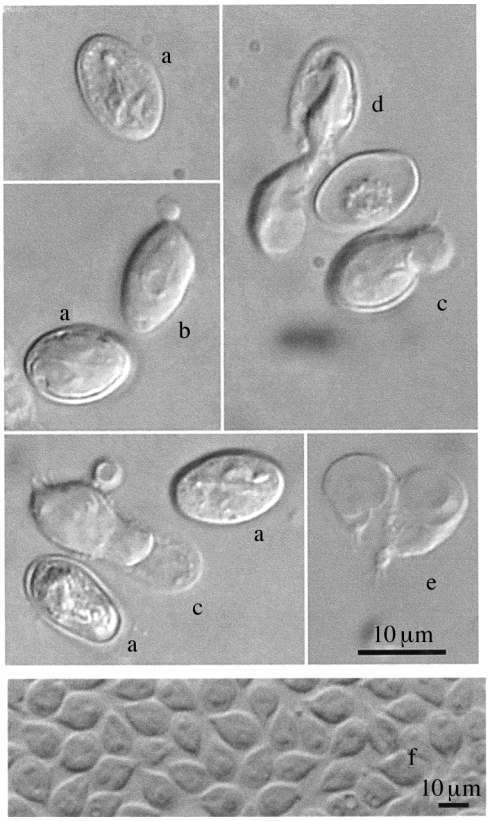

Figure 1. Differential interference contrast photomicrographs of excystation and axenic culture of *G. lamblia* in TYI-S-33 modified medium. (a) Cysts prior to excystation; (b–e) excystation phases; (f) axenic monolayer.

Table 1. Susceptibility of *G. lamblia* isolates to the drugs tested

	Metronidazole		Albendazole	
Isolate	IC_{50}[a] (μM)	95% CI	IC_{50} (μM)	95% CI
ACPT 98004	3.38	2.23–5.13	0.084	0.071–0.100
ACPT 98005	4.25	2.25–8.01	0.055	0.041–0.074
ACPT 98006	7.49	4.40–12.80	0.192	0.096–0.385
ACPT 98013	5.52	4.10–7.45	0.079	0.072–0.087
ACPT 98012	2.38	1.77–3.21	0.027	0.021–0.034
ACPT 98014	3.60	2.52–5.14	0.120	0.100–0.145
ACPT 98018	3.84	2.42–6.07	0.068	0.044–0.105
ACPT 98019	10.09	8.37–12.17	0.102	0.075–0.138
ACPT 98020	11.50	9.69–13.64	0.087	0.039–0.198
ACPT 98021	4.28	2.26–8.10	0.051	0.048–0.054
ACCA 98007	3.01	1.91–4.73	0.062	0.051–0.077
ACCA 98010	4.92	3.49–6.93	0.109	0.095–0.126
ACPT 98008	3.33	2.21–5.01	0.076	0.057–0.101
ACPT 98009	3.34	1.93–5.78	0.075	0.070–0.080
ACPT 98011	4.46	3.53–5.64	0.087	0.075–0.101
ACPA 99015	2.52	0.98–6.45	0.088	0.073–0.105
ACPT 99017	7.18	5.64–9.13	0.103	0.092–0.115
ACPT 99016	3.41	2.09–5.56	0.117	0.093–0.147
ATCC 30888	3.10	1.97–4.86	0.083	0.074–0.094
ATCC 30957	4.27	2.73–6.68	0.064	0.055–0.074

[a]IC_{50} values were obtained after probit analysis of data from two assays with two replicates each.
CI, confidence interval.

representing a 7.1-fold variation. The isolates were ~56 times more susceptible to albendazole than to metronidazole.

The mean IC_{50} values for metronidazole and albendazole obtained for the 18 isolates in the inhibition of adherence assay are similar to the mean values obtained with the ATCC reference isolates.

Discussion

This report of the isolation, excystation and axenic culture of *G. lamblia* parasites is an important step in establishing drug susceptibility data from the Portuguese population. The methodology used simulates the intestinal passage of the cyst.[2] It includes low pH induction in a reducing environment followed by neutralization and incubation in medium and is a good alternative to duodenal aspiration or *in vivo* excystation. Duodenal aspiration is not readily accepted, or justified in most cases, particularly in asymptomatic infections. Excystation *in vivo* requires colonies of *Giardia*-free animals, normally gerbils, which in spite of the advantage of large numbers of trophozoites produced, is expensive, time-consuming and not easy to perform.

Our isolation rate of 34% is similar to rates described in the literature, varying from 21% to 44%.[4,8] Cyst viability, microbial contamination and adaptation of trophozoites to the medium have been described as the major causes for the low rate of excystation.[8] In our case, we increased cyst inoculum and the antimicrobial drug concentration to try to achieve a higher isolation rate, but these changes failed to produce higher values. It is possible that genetic variation is one important reason for the inability of excysted trophozoites to survive in TYI-S-33 medium, as suggested by Meloni & Thompson;[8] they failed to establish cultures of excysted trophozoites isolated from dogs.

The principal objective of our study was to obtain susceptibility data for metronidazole and albendazole against *G. lamblia* isolates from the Portuguese population. For this purpose, we used the adherence properties of *G. lamblia* trophozoites instead of the capacity of cells to divide. The ability to adhere to gastrointestinal mucosa is an important prerequisite for *G. lamblia* to establish a sustainable infection.[1] In the presence of active antiparasitic drugs *in vivo*, trophozoites fail to adhere to epithelial layers, thus helping terminate the infection.

Our results indicate that the 18 *G. lamblia* isolates were susceptible to metronidazole and albendazole. Several authors obtained similar levels of susceptibility in spite of differences in the methodology used, such as the time the parasites were in

contact with the drug.[3,9] The IC_{50} and dose–response curves of our isolates show small variation among the 18 isolates and these values are close to the ATCC reference isolates (Table 1). Similar results were obtained by Farbey *et al*.[3] for albendazole using 29 Australian *G. lamblia* isolates, but against metronidazole the IC_{50} values showed greater variation, in contrast to our results. Drug presence itself can be responsible for such variation, since, *in vitro*, cultivation of *G. lamblia* isolates in the presence of sublethal concentrations of metronidazole resulted in an increase in resistance to the drug.[10]

We conclude that metronidazole is effective for the treatment of giardiasis in our population and that albendazole will be a suitable alternative in the future.

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