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ASSESSMENT OF IVERMECTIN THERAPEUTIC EFFICACY ON THIRD-STAGE LARVAE OF *Lagochilascaris minor* IN MICE EXPERIMENTALLY INFECTED

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SUMMARY

In this study we evaluated the potential action of ivermectin on third-stage larvae, both at migratory and encysted phases, in mouse tissues after experimental infection with *Lagochilascaris minor*. Study groups I and II consisted of 120 mice that were orally administered 1,000 parasite eggs. In order to assess ivermectin action upon migratory larvae, group I (60 mice) was equally split in three subgroups, namely I-A, I-B, and I-C. On the 7th day after inoculation (DAI), each animal from the subgroup I-A was treated with 200 µg/Kg ivermectin while subgroup I-B was given 1,000 µg/Kg, both groups received a single subcutaneous dose. To assess the drug action on encysted larvae, group II was equally split in three subgroups, namely II-A, II-B, II-C. On the 45th DAI each animal was treated with ivermectin at 200 µg/Kg (subgroup II-A) and 1,000 µg/Kg (group II-B) with a single subcutaneous dose. Untreated animals of subgroups I-C and II-C were used as controls. On the 60th DAI all animals were submitted to larva search. At a dose of 1,000 µg/Kg the drug had 99.5% effectiveness on third-stage migratory larvae (subgroup I-B). Ivermectin efficacy was lower than 5% on third-stage encysted larvae for both doses as well as for migratory larvae treated with 200µg/Kg.

KEYWORDS: Ivermectin; L*agochilascaris minor;* Third-stage larvae.

INTRODUCTION

Experimental reproduction of *Lagochilascaris minor* in laboratory was first introduced by CAMPOS et al. (1989) who used mouse as the intermediate host and house cat as the definitive host. This experimental approach made possible the implementation of therapeutic assays specific for different evolutionary phases of the parasite. The egg suspension to infect the intermediate host was obtained from feces of experimentally infected cats. CAMPOS et al. (1992) reported that third-stage larvae hatch in the intestine approximately four hours pos-inoculation (PI) and migrate to the liver, reaching it at approximately six hours PI, and finally the lungs at approximately twenty-four hours PI. Third-stage larvae recovered from mouse liver and lungs are morphologically similar to those recovered by compression upon eggs that have been in culture for 30 days, missing only two cuticles. At approximately 7 days PI, the larvae are migrating in the body and, at the end of this period, larvae are mainly found in skeletal muscle and subcutaneous tissues where they are later involved by a typical host tissue reaction. When cats are fed with carcasses of infected mice, parasites development to adult stages can be noticed from the 9th to 15th day after infection in tissues of the oropharynx and cervical region. Although external ulceration of abscesses may occur, lesions and abscesses generally ulcerate to the oral cavity and thus, a large quantity of eggs are found in feces of hosts experimentally infected (BARBOSA et al., 1996).

An auto-infecting cycle occurs in cats, mice, and men. In an auto-infecting cycle, the parasite reproduces locally in the lesion, resulting in all evolutionary phases simultaneously, including eggs in various phases of segmentation, larvae in different maturation stages, and adult worms. The auto-infecting cycle is one of the strongest factors to difficult efficient treatment of infected individuals.

Our approach was to use an anti-parasitic with a large spectrum, potent against nematodes. Our choice was ivermectin (IVM), an avermectin B (22,23-dihydro) derivative, which is a macrocyclic lactone, active in low doses. IVM is produced by the actinomycete *Streptomyces avermectilis* (CAMPBELL, 1983).

IVM efficacy was demonstrated while treating pulmonary infections by worms of ovine, equine, and swine (CAMPBELL, 1985), and dogs infected with *Filarioids osleri* (CLAYTON, 1983). Satisfactory results were observed when different doses of IVM were used to treat cats infected with the following parasites: Acarids : *Notoedris cati* - 1,000 µg/Kg (QUINTAVALLA et al., 1985), *Otodectes cynotis* - 200 a 1330 µg/Kg CHAUVE et al., 1984); Helmynthes: *Toxocara cati* - 200 µg/Kg e *Aerolostrongylus abstrusus* -400 µg/Kg (KIKPATRICK et al., 1987).

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In addition, IVM was shown to be highly active against *Strongyloides westeri* and other horse intestinal nematodes. In rats, IVM efficacy was demonstrated by removing *Siphacia muris* from their intestinal tract (BATTLES et al., 1987). In mice, this drug was found to have excellent anti-helminth action, specially against filaria larvae and both *Strongyloides ratti* and *S. stercoralis* adult worms (GROVE, 1983).

In humans, IVM was considered to be effective for the treatment of strongyloidiasis, ascariasis, trichiuriasis, enterobiasis (NAQUIRA et al., 1989), onchocerciasis (SCHULTZ-KEY et al., 1983; TAYLOR et al., 1986; BENNETT et al., 1988), and filariasis (AZIZ et al., 1982; KUMARASWAMI et al., 1988; GUERRERO, 1983).

The positive results with the use of ivermectin against several helminthiasis, the increasing knowledge of the life cycle of *L. minor* in our laboratory, and finally the frequent reports on the failure benzimidazolic derivatives in the treatment of human lagochilascariasis, impelled our current work. Here our main objective was to assess IVM effectiveness on third-stage larvae during both the migratory stage as well as while encysted in mouse tissues, experimentally infected with *Lagochilascaris minor*.

MATERIALS AND METHODS

Egg culture

Parasite eggs were obtained after feces sedimentation from cats experimentally infected. The egg suspension in 1% formaldehyde solution was kept at room temperature in sedimentation bottles. Daily oxygenation was obtained by handshaking the bottles. In average, the egg suspension was maintained for approximately 40 days (eggs with L3 inside).

Animals

As total of 120 isogenic C57B1/6 mice, from both sexes, weighting approximately 20 g each, and aged from 60 to 90 days were used. Mice were obtained from the IPTSP/UFG animal care service.

Inoculum characterization and experimental inoculation

Following the 40-day egg culture, the egg suspension was centrifuged at 3,500 rpm and washed twice with phosphate-saline solution. After egg counting using a microscope, an egg concentration containing approximately 1,000 eggs was prepared in order to orally inoculate the mice via esophagus intubation.

Necropsies

Animals were sacrificed by either ether or chloroform inhalation. Abdominal, thoracic, and oral cavities, as well as cervical regions and heads were thoroughly examined, searching for any stage of the life cycle of the parasite.

Drug

We used ivermectin, a B22,23 dihydro avemectin derivative, commercially known as Ivomec. IVM was diluted with propylene glycol and administered subcutaneously to the animals.

Estimation of infection index, disparasitation, and parasite elimination.

1. We estimated the infection index (e.g., the percentage of recovered larvae to the number of inoculated eggs) to assess egg infectivity. Infection index was calculated using the equation:

$$I.I. = \frac{L \times 100}{N}\text{, where}$$

I.I. = Infection index;
L = Number of recovered larvae;
N = Number of inoculated eggs.

2. We estimated the disparasitation index (e.g., the percentage of animals that totally eliminated the parasite from their body) caused by ivermectin. We used the percentage of animals with parasitic infection in the control group and calculated the index using THOMPSON & REINERSTON'S (1952) equation:

$$I.D. = \left(\frac{A-B}{A}\right) \times 100\text{, where}$$

I.D. = Disparasitation index;
A = percentage of animals in the control group with parasitic infection;
B = percentage of animals in the study group with parasitic infection.

3. We also estimated the index of parasite elimination (e.g., the percentage of eliminated helminthes in the treated group) using as a comparative parameter the helminthic population of the control group (PUMAROLA et al., 1987). The index of parasite elimination was estimated using the following equation:

$$I.E.E. = \left(\frac{C-D}{C}\right) \times 100\text{, where}$$

I.E.E. = Index of estimated parasite elimination;
C = Total number of parasites in the control group;
D = Total number of parasites in the study group.

EXPERIMENTAL APPROACH I - IVM action against migratory third-stage larvae

In order to assess the drug action against migratory third-stage larvae (Figure 1), group I, consisting of 60 mice, was inoculated with a 1,000 eggs per animal and subsequently split into three subgroups of 20 animals each (I-A, I-B, and I-C).

Each animal in both subgroups I-A and I-B was treated with a single dose of IVM at 200 and 1,000 µg/Kg, respectively, on the 7th day after inoculation (7th DAI) - a period which corresponds to the larva migration through the host's organs. Animals from subgroup I-C received no treatment and comprised the control group. Sixty days after inoculation (60 DAI), all animals were sacrificed and necropsy was performed as to search the host body for any parasitic form and to estimate the number of larvae. To assess drug efficacy, we used the infection (I.I.), disparasitation (I.D.), and estimated parasite elimination (I.E.E.) indexes.

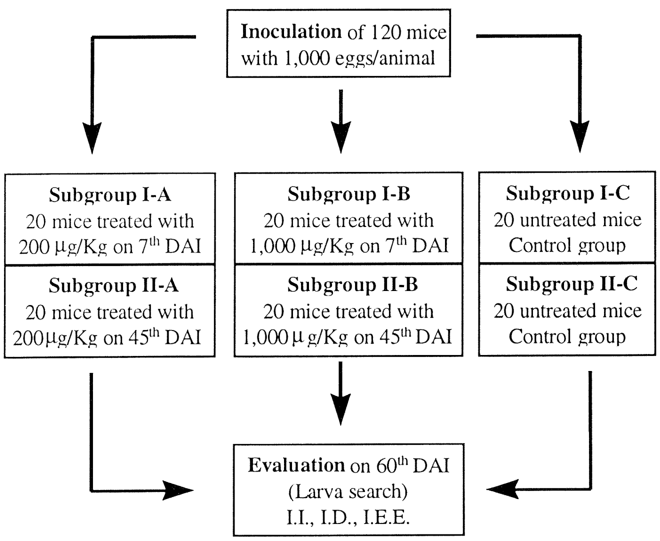

Fig. 1. IVM therapeutic assay against migratory and encysted third-stage larvae of *Lagochilascaris minor.*

EXPERIMENTAL APPROACH II - IVM action against encysted third-stage larvae

In order to assess the drug action against encysted third-stage larvae (Figure 1), group II, consisting of 60 mice, was inoculated with a 1,000 eggs of *L. minor* per animal and subsequently split into three subgroups of 20 animals each (II-A, II-B, and II-C).

Each animal in both subgroups II-A and II-B was treated with a single dose of IVM at 200 and 1,000 µg/Kg, respectively, on the 45th day after inoculation (45th DAI) - a period necessary to find encysted larvae. Animals from subgroup II-C received no treatment and comprised the control group.

Statistical Analysis. We used the "t" test to perform the statistical analysis of our data. The t value with 38 degrees of freedom in a two-tail test, including 5% significance level, was 2.02. The following equation was used to perform the statistical test:

$$t = \frac{\bar{x}_1 - \bar{x}_2 - (n_1 - n_2)}{\sqrt{(n_1 - 1) s_1^2 + (n_2 - 1) s_2^2}} \cdot \sqrt{\frac{n_1 \cdot n_2 (n_1 + n_2 - 2)}{n_1 + n_2}}$$

RESULTS

EXPERIMENTAL APPROACH I - IVM action against migratory third-stage larvae

The number of recovered larvae from tissues of mice inoculated with *L. minor* and submitted to IVM action on the 7th DAI at doses of 200 and 1,000 µg/Kg can be found in Table 1. We noted that there is no significant difference between the number of larvae recovered from tissues of untreated animals (I-C) and animals treated with 200 µg/Kg (I-A).

TABLE 1

Total number of recovered larvae per group and average number of larvae per animal, 60 DAI in mice infected with 1,000 eggs of *L. minor*. Mice were treated with 200 and 1,000 µg/Kg on the 7th DAI for subgroups I-A and I-B, respectively.

	Animals treated with IVM 200 µg/Kg (I-A)	Animals treated with IVM 1,000 µg/Kg (I-B)	Untreated control animals (I-C)
Total number	1680	9	1753
Average	84	0.45	87.65
S.D.*	47.01399568	1.394538218	41.1125221
t test	I-A, I-C	0.26	t value = 2.02

*S.D. - Standard deviation of the mean

TABLE 2

Infection, disparasitation, and estimated parasite elimination indexes in mice inoculated with 1,000 eggs of *L. minor* and treated with IVM doses of 200 and 1,000 µg/Kg on the 7th DAI.

Animal group	IVM Treatment	I.I	I.D	I.E.E
I-A	200 µg/Kg	8.40%	0	4.2%
I-B	1,000 µg/Kg	0.90%	85.0%	99.5%
I-C	untreated	8.76%	-	-

Table 2 depicts no significant difference between the infection index of untreated animals (I-C) and of animals treated with dose of 200µg/Kg (I-A). Mice treated with IVM at 1,000 µg/Kg (I-B) had an infection index of 0.90%.

Using the equation reported by THOMPSON & REINERSTON (1952), no disparasitation in animals treated with IVM at 200 µg/Kg was observed (Table 2). However, when animals were treated with IVM at 1,000 µg/Kg , a disparasitation index of 85% was observed.

The estimated parasite elimination index for animals receiving IVM at 1,000 µg/Kg (subgroup I-B) showed that IVM has 99.5% efficacy to eliminate migratory larvae. However, the same index is only 4.2% for animals that received IVM at 200 µg/Kg.

EXPERIMENTAL APPROACH II - IVM action against encysted third-stage larvae

Mice were inoculated with *L. minor* following subsequent treatment with IVM at 200 and 1,000 µg/Kg, forty-five days after inoculation. The number of larvae recovered from mouse tissues is depicted in Table 3.

No significant difference was found in the number of recovered larvae among the three study groups (Table 3).

There is no statistically significant difference for the infection index for the three study groups (Table 4). Similar results were found when comparing the disparasitation index for the two groups treated with IVM. Moreover, ivermectin was not found effective against encysted larvae. The estimated parasite elimination index was 4.6% when animals were treated with a single dose of 1,000 µg/Kg. Further analysis of encysted larvae showed no difference at both macroscopic and microscopic levels of specimens recovered from treated mice (200 and 1,000 µg/Kg) as well as from the control group.

DISCUSSION

The auto-infecting cycle is frequent in human lagochilascariasis. In that, the parasite is found reproducing locally in the lesion with the simultaneous occurrence of all possible life forms (PAWAN 1926, 1927; OOSTBURG & VARMA, 1968, ARTIGAS et al., 1968; OOSTBURG 1971; BORGO et al., 1978; LEÃO et al., 1978; CORRÊA et al., 1978; CAMPOS et al., 1983; MORAES et al., 1986; SOUZA et al., 1986; TELLES FILHO et al., 1987, CAMPOS et al., 1989). In experimental lagochilascariasis, the presence of all life forms in the abscess has also been reported (CAMPOS et al., 1992; BARBOSA et al., 1996). The presence of all evolutionary forms of the parasite in a lesion is a major factor to reduce treatment effectiveness and difficult therapeutic strategies in patients infected with *L. minor.* Therefore, the development and improvement of therapeutic assays specific against each individual evolutionary phase of the parasite is justified.

Several anti-helminthic drugs have been used for the treatment of human lagochilascariasis, such as diethylcarbamazine, thiabendazole, mebendazole, levamisole, cambendazole, and albendazole. However, most of the therapeutic approaches have failed. There are several reports of relapse episodes, following an initial period of apparent cure (DRAPER et al., 1963; OOSTBURG et al., 1968; BORGO et al., 1978; LEÃO et al., 1978; MORAES et al., 1983; BACARAT et al., 1984; ROCHA et al., 1984; ORIHUELA et al., 1987, CAMPOS et al., 1991, OOSTBURG et al., 1992).

Ivermectin has been shown to be effective against a number of parasites of the intestinal tract, including *Haemonchus contortus, Ostertagia ostertagi, Ostertagia circuncicta, Trichostrongylus columbriformis, Dictyocaulus viviparus, Dictyocaulus filaria, Cooperia curticei, Oesophagostomun columbianum, Strongyloides westeri, Ancylostoma sp,* and *Toxocara cati* (EGERTON et al., 1980;

TABLE 3

Total number of recovered larvae per group and larva average per animal. Larvae were recovered sixty days after inoculation in mice infected with 1,000 eggs of *L. minor*. Animals were treated with IVM at dose of 200 and 1,000 µg/Kg on the 45th DAI.

	Animals treated with IVM 200 µg/Kg (II-A)	Animals treated with IVM 1,000 µg/Kg (II-B)	Untreated control animals (II-C)
Total number	2204	2070	2169
Average	110,288	103.5	108.45
S.D.*	39.02576747	37.85915727	40.22106675
t test	II-A, II-C II-B, II-C	0.14 0.40	t value = 2.02

*S.D. - Standard deviation of the mean

TABLE 4

Infection, disparasitation, and estimated parasite elimination indexes in mice inoculated with 1,000 eggs of *L. minor* and subsequently treated with IVM doses of 200 and 1,000 µg/Kg on the 45th DAI.

Animal group	IVM Treatment	I.I	I.D	I.E.E
II-A	200 µg/Kg	11.02%	0%	-
II-B	1,000 µg/Kg	10.40%	0%	4.6%
II-C	untreated	10.80%	-	-

BLAGBUM et al., 1987; KIRKPATRICK et al., 1987; BOGAN et al., 1988) and of the respiratory tract in ovine, equine, and swine (CAMPBELL, 1985). MAK et al. (1987) reported that, in monkeys, IVM was effective against the filaria *Brugia malayi* and *Dipetalonema vitae*. IVM was found effective against *Siphacia muris* and *Strongyloides ratii*, eradicating these worms from the intestinal tract of rats (BATTLES et al., 1987; GROVE, 1983).

In humans, ivermectin was found effective against strongyloidiasis (88%), ascariasis (100%), trichiuriasis (85%), and enterobiasis (85%) [NAQUIRA et al., 1989]. IVM has been used for the treatment of human onchocerciasis since 1981 with good results (SCHULTZ-KEY et al., 1983; DOW et al., 1988). Moreover, this drug shows no severe adverse reactions, making it ideal to be used in a large scale approach (GREENE, 1985; TAYLOR, 1986; BENNETT et al., 1988; GUERRERO, 1993). Ivermectin was also chosen as the most suitable drug for the treatment of Bancroft's filariasis (KUMARASWAMI et al., 1988). In human lagochilascariasis, BENTO et al., 1993 reported a case sucessfully treated with ivermectin in two cycles of four doses of 0.2 mg/kg at weekly intervals followed by a month without therapy.

In our experimental approach, ivermectin (Ivomec) was administered subcutaneously and was chosen over Oromec, which is generally administered orally, because it reaches higher plasmatic concentrations. BOGAN et al. (1988) reported that the subcutaneous administration of IVM at 200 µg/Kg in ovine led to higher plasmatic concentrations. Besides the drug had longer plasmatic half life when compared to oral administration to the same host. Following subcutaneous administration, IVM plasmatic concentration peaked at 32 ± 3.2 ng/ml and was detected up to the 21st day after drug administration. Oral administration of the drug peaked only at 11.7 ± 0.7 ng/ml and could only be detected up to the 7th day after administration.

It has been reported that ivermectin showed no effectiveness to eradicate helminthic infection when used at lower concentrations in host such as rats, mice, and hamsters. RAJASEKARIAH et al. (1989) reported that a reduction of 100% of *Necator americanus* in hamsters experimentally infected requires IVM dose of 18 mg/Kg. On the other hand, in humans it has been demonstrated that an IVM dose as little as 50 µg/Kg was sufficient to eliminate ancylostomids (AZIZ et al., 1982) and doses lower than 200 µg/Kg were effective against *Enterobius vermicularis* (NAQUIRA et al., 1989). Moreover, BLAIR et al. (1982) determined ivermectin effectiveness against *Ancylostoma caninum* in dogs treated with a dose of 200 µg/Kg. Finally, BATTLES et al. (1987) reported a therapeutic approach of 5 x 200 µg/kg as an ideal treatment protocol for satisfactory eradication of *Syphacia muris* in infected rats.

In our work, ivermectin showed no effectiveness against migratory third-stage larvae at a dose of 200 µg/Kg. However, when infected mice were treated with IVM at 1,000 µg/Kg, a reduction of 99.5% in larva load was observed. Our results suggest that the parasite response to ivermectin depends upon host intrinsic factors as well as drug dose.

In mice inoculated with eggs of *Lagochilascaris minor,* and treated with ivermectin doses of 200 and 1,000 µg/Kg on the 45th DAI, larva viability was maintained while encysted inside nodules. Similar outcome was observed in the control group. We suggest that the host granulomatous reaction in response to the larvae might work as a natural barrier protecting against the drug uptake into the nodule.

RESUMO

Avaliação da eficácia terapêutica da Ivermectina sobre larvas de terceiro estádio de *Lagochilascaris minor* em camundongos infectados experimentalmente

Avaliou-se a ação da ivermectina sobre larvas de 3º estádio, tanto em fase de migração, quanto larvas encistadas em tecidos de camundongos infectados experimentalmente com *Lagochilascaris minor*. Foram utilizados 120 camundongos (grupos I e II), sendo que cada animal foi inoculado, por via oral, com 1.000 ovos do parasito. Para verificar a ação da ivermectina sobre larvas em migração, o grupo I (60 animais) foi dividido igualmente em três subgrupos: I-A, I-B e I-C. No 7º dia após a inoculação (DAI), cada animal foi tratado com ivermectina na dosagem de 200 µg/Kg (subgrupo I-A) e 1.000 µg/Kg/dose única/via sc (subgrupo I-B). Com o objetivo de verificar a ação da droga sobre larvas encistadas, os animais do grupo II foram divididos igualmente em três subgrupos: II-A, II-B e II-C; no 45º DAI cada animal foi tratado com ivermectina na dosagem de 200 µg/Kg (subgrupo II-A) e 1.000 µg/Kg/dose única/via sc, (subgrupo II-B). Os animais dos subgrupos I-C e II-C constituíram o grupo controle. No 60º DAI todos os animais foram submetidos à pesquisa de larvas. Observou-se 99,5% de eficácia da droga na dosagem de 1.000 µg/Kg (grupo IB) sobre larvas de 3º estádio em fase de migração e eficácia inferior a 5% sobre larvas de 3º estádio encistadas, em ambas dosagens, bem como sobre larvas em migração na dosagem de 200 µg/Kg.

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