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The response of adult and free-living stages of *Necator americanus, in vitro,* to anthelmintics

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Abstract: The *in vitro* response of adults (males and females) and free-living stages of *Necator americanus* one of the human hookworms, to a wide variety of 20 broad and narrow spectrum anthelmintics was tested. Almost all the broad spectrum anthelmintics influenced males, females and free-living stages at different levels and showed good activity with EC_{50} values varying from about 0.0002 and 0.0007 mg/l for pyrantel pamoate and tricofenol piperazine respectively to about 8.47 and 7.6 mg/l for morantel tartrate and amoscanate respectively. Certain drugs (emetine, praziquantel and suramin) exerted their effect either on male or female or free-living stages at 10.0 mg/l level. It is concluded that either sex or life-cycle stage alone may not be an effective criteria for screening of anthelmintics which have been employed at large; and females of nematodes (in particular those of *N. americanus*) should be taken into account for proposing EC_{90} as they were found to require relatively highest EC_{50} level in almost all the instances studied presently.

During a survey of nematode parasites of Meerut region, *Necator americanus* was found to be one of the most prevalent human parasites. Various notable *in vitro* tests have been devised for screening of anthelmintics employing parasitic adults (*Cooperia punctata,* Leland *et al.*, 1975; *Nippostrongylus brasiliensis,* Jenkins *et al.*, 1980, Jenkins and Carrington, 1982; *Trichinella spiralis,* Jenkins and Carrington, 1981), free-living juveniles (horse strongyle larvae, Levine, 1950; *Obeliscoides cuniculi* and *Trichostrongylus calcaratus,* Tiner, 1958; eggs to third stage larvae of *N. brasiliensis,* Phillipson, 1966 and *N. brasilensis, Nematodirus dubius. Haemonchus contortus, Trichostrongylus colubriformis* and *Ostertagia ostertagi,* Ibarra and Jenkins, 1984 and the free-living nematode *Caenorhabditis elegans* (Simpkin and Coles, 1981). In addition, literature (Steward, 1955; Copp *et al.*, 1958; Sheffield *et al.*, 1959; Broome and Greenhalgh, 1961; Standen, 1963; Stone *et al.*, 1965; Katiyar and Sen, 1969; Coles and Mc Neillie, 1977; Jenkins, 1982) reveals that no study is available employing different sexes and life-cycle stages altogether of a single nematode to investigate anthelmintic response. The criteria of screening of anthelmintics has remained restricted chiefly to either free-living or adult stages of the nematode parasites. This may sometime lead to false results as different anthelmintic concentrations (EC_{50}) may be required to affect different sexes or life-cycle stages, which has been explored during present investigations which were undertaken chiefly depending upon this view. The *in vitro* response of male, female and third stage infective larvae (L_3) of *N. americanus* to twenty widely used anthelmintics is now reported.

MATERIALS AND METHODS

Motile *N. americanus* adults were obtained from the intestines during post-mortem examination in local hospitals or from pathological laboratories. The worms were washed thoroughly in physiological saline and the two sexes were separated. To obtain eggs the females were incubated in physiological saline at 37°C. The eggs laid were washed at least thrice in distilled water and adjusted to a known density in water using the McMaster technique.

Male and female adults and eggs were axenized for 30 min. in warm (37°C) saline (0.15 M-NaCl) containing antibiotics (penicillin, 124 µg; streptomycin, 70 µg; mycostatin, 100 units per ml). 150 adult worms (males and females separately) per 200 ml of culture medium in stoppered Erlenmeyer flasks or 5,000 eggs per ml of culture medium in stoppered glass containers on a roller drum, were incubated for 16 h (adult) or for 5 days (eggs) in predetermined (unpublished observations) optimal conditions (5% CO_2, 10% O_2 and 85% N_2; 38 ± 1°C). The incubation medium consisted of RPMI–1640 medium (Grand Island Biological Company, NY, USA) supplemented with 0.0225 mg glucose per ml and antibiotic mixture as described above plus drug solution/ suspension. Water insoluble drugs were dissolved in a suitable volatile solvent (acetone or diethyl ether) and a known volume was added to the container. After the solvent evaporated completely leaving a thin film of drug deposit over the basal surface of each container, the culture medium was added. Culture medium controls were prepared and treated identically with the exception that the drugs were not included. Males, females and eggs were incubated in the control culture media exactly as described before.

Each drug was tested initially at concentrations of 30, 20 and 10 mg/l whole compound. Subsequently drugs showing activity at 10.0 mg/l were titrated in a retest using decrements either 1: 2, 1: 3 or 1: 4 and so on in order to determine the concentrations required to affect approximately 50% of the worm (EC_{50}) according to earlier practices (Ibarra and Jenkins, 1984). The media were not changed during the experiments. For each anthelmintic, observations were carried out either macroscopically (for adults) or under a binocular microscope at x 40 magnification (for eggs and larvae). The number and physical condition of males, females, eggs and larvae present in each test and control containers were recorded on 0 h. The adults were examined again after 16 h and the number of dead, paralysed and abnormal worms was recorded. Eggs were examined after 5 days and the number and condition of eggs and larvae present were recorded. Activity of the compounds was assessed by comparing the test worms with the controls.

The drugs that killed, paralysed or made the worms greatly abnormal relative to controls or that elicited ovicidal or larvicidal effects or arrested or slowed the development of the larvae relative to the controls, were regarded as active. EC_{50} values were determined by subjecting the data obtained for the effects observed at each serial concentration of drug where between 10 to 90% of the test worms were affected relative to the controls, to linear regression analysis.

RESULTS

Results are summarised in Table 1. Out of 20 compounds studied 15 showed significant activity ($P < 0.01$) relative to the controls at concentrations below 10 mg/l against both adult (males and females) and juvenile stages. Out of 15 active compounds the most potent were pyrantel pamoate and tricofenol piperazine which were fully active at the lowest concentrations used and the least potent were amoscanate and morantel tartrate. Ivermectin, pyrvinium pamoate and thiabendazole were moderately potent. Females and juveniles required relatively maximum and minimum drug concentrations respectively to show significant response. 100% adult worms survived in all control incubation media and 96% worms were fully active after 16 h. All the 15 active drugs exerted lethal effects on the adult worms at different concentrations. Females, however, showed maximum resistance. After 16 h the worms that were alive became greatly lethargic in all the 20 drugs. In ivermectin, pyrantel pamoate and tricofenol piperazine live worms were pale and highly coiled. Piperazine, diethylcarbamazine and praziquantel, however, exerted no lethal effects on either male or female. Praziquantel and suramin were slightly lethal to L_3 and female respectively. Emetine was a bit lethal to both female as well as L_3. Both of them developed grossly abnormal appearance. 99 - 100% control eggs attained full development into third infective larval stage within 5 days. Compounds were regarded to exert ovicidal effects where the eggs did not show development and hatching, larvicidal effects when the larvae died during development and hatching or were inhibitory when development of the larvae was either slowed down significantly or arrested completely. The drugs studied showed a variety of such activities. Though thiabendazole, levamisole and thiophanate showed no ovicidal activity even at concentrations as high as 30 mg/l, all the three

TABLE 1

Response of adult (males and females) and free-living stages of Necator americanus *to 20 broad and narrow spectrum anthelmintics* in vitro. *Approximate concentration mg/l required to affect 50% of the worms (EC_{50})*

Anthelmintic	Life-cycle stage		
	Parasitic adult		Free-living (L_3) juvenile
	Male	Female	
Amoscanate	4.4	7.6*	3.8
Bephenium hydroxynaphthoate	0.47	±	0.4
Diethylcarbamazine citrate	–	–	–
Dithiazanine iodide	0.027	0.11*	0.02
Emetine	–	±	±
Febantel	0.76	0.91*	0.84
Ivermectin	0.004	0.005*	0.001
Levamisole HCl	0.021	0.074*	0.011
Morantel tartrate	5.81	8.47*	3.07
Nitroscanate	0.53	0.74*	0.43
Phenothiazine	0.024	0.14*	0.09
Piperazine hexahydrate	–	–	–
Praziquantel	–	–	+
Pyrantel pamoate	0.0002	0.0005*	0.0003
Pyrvinium pamoate	0.006	0.009*	0.003
Stilbazium iodide	0.021	0.03*	0.017
Suramin	–	+	–
Thiabendazole	0.003	0.004*	0.0038
Thiophanate	0.84*	0.77	0.8
Tricofenol piperazine	0.008*	0.0007	0.006

* = Highest (true) EC_{50} for *N. americanus* species as a whole
\+ = Good activity, > 50 % test worms affected relative to controls at 10.0 mg/l whole compound
± = Weak activity, between 20 and 50 % worms affected relative to controls at 10.0 mg/l whole compound
– = No activity, < 20 % test worms affected relative to controls at 10 mg/l whole compound

showed larvicidal activity. Ivermectin and phenothiazine markedly inhibited the development of larvae and their motility was greatly reduced. At high concentrations ivermectin and thiabendazole produced small blister like patches on the larval body wall. In rest of the drugs larvae became lethargic.

DISCUSSION

Most of the available anthelmintic screening tests carried out *in vitro* are based on free-living life-cycle stages alone, Jenkins (1982) has, however, pointed out and discussed disadvantages and limitations of such screens and has stated that there is a possibility of getting "false positive or negative" results. Present investigations provide experimental evidence that the EC_{50} values obtained for free-living stages may not be reliably taken over to the adults and also provide a new criteria of screen. Even the two sexes of *N. americanus* required different drug concentrations to show significant ($P < 0.01$) response. The relatively low EC_{50} values of drugs for free-living stages if are taken into account as criteria for selection of drug of choice for preparation of doses, it will result to insignificant effects and the probability of entire worm expulsion by intake of drug selected in this way by the host, is extremely low. It is therefore, considered that EC_{50} values obtained for females may be more reliably regarded to influence the species effectively. Since adult parasitic stages inhabit the host and particularly females are responsible for the spread of infection, it is more appropriate to screen and select the drugs taking their effect on females as basic criteria. In few drugs, however, EC_{50} values were higher for males or L_3. In such cases the relatively highest EC_{50} value

among male, female or L_3 is advisable.

The screen undertaken presently was highly specific as it was intended to detect the activity against *N. americanus* only.

No ovicidal effect of the drugs was probably due to the egg shell which serves as an effective barrier for the penetration of the drug. During anti-trichostrongyle screens, levamisole and morantel tartrate have been reported (Ibarra and Jenkins, 1984) to show no ovicidal effects even at concentrations as high as 50 mg/l but both were markedly larvicidal and ivermectin to markedly inhibit both development and motility of the larvae.

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RESUMEN

Se probó la respuesta *in vitro* de *Necator americanus* a 20 antihelmínticos. Con valores de EC_{50} desde 0.0002 hasta 0.0007 mg/L, los antihelmínticos de espectro amplio afectaron a machos, hembras y estadíos libres. Ciertas drogas (emetine, praziquantel y suramin) afectaron selectivamente a los grupos mencionados al nivel de 10.0 mg/L. Las hembras requirieron mayores niveles de tratamiento, lo que tiene importantes implicaciones en la aplicación de antihelmínticos.

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