A New Subculture and Nematocidal Assay Using a Species of Diplogastridae

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A new subculture method and a novel microplate assay for nematocidal activity using a species of Diplogastridae have been developed. The assay gives results rapidly, with high sensitivity in 4 h, and indicated good correlation between action mechanism and the nematode shape when examining 15 known compounds, including the antiparasitic avermectin, antimalarial quinine, and the γ-amino-*n*-butyric acid$_A$ (GABA$_A$) activated Cl$^-$ channel antagonist picrotoxinin. Thus new assay could be used as a primary screening method for new nematocidal compounds.

Key words nematode; Diplogastridae; nematocidal activity, microplate assay; subculture; avermectin

Nematodes are the most ubiquitous species on earth.[1] It is estimated that at least 40000 species of nematode exist, of which about 16000 have been described. About 30% of the described species are parasites to vertebrates and the remainder are either parasites of plants, invertebrates or are free-living. Even though almost all that is known about the neurobiology of nematodes comes from work done on *Caenorhabditis elegans*, which is a free-living nematode, it is likely that these studies can readily be extrapolated to many other nematodes.

We have developed a new subculture and a new microplate assay with a species of Diplogastridae, for the purpose of searching for nematocidal compounds.

Experimental

Collection and Identification Nematodes were obtained from soil on the campus of Toho University by means of the improved Baermann funnel technique,[2] and centrifuged at 2000 rpm for 10 min. The 3rd instar larvae of the nematode, which had the most vigorous movement, were collected from the sediment and passed through a 5 cm-column filled with glass beads (0.177—0.250 mm in diameter).[3] The free-living nematode was identified as class: Secernentea, order: Rhabditida, family: Diplogastridae[4] by Dr. Masaaki Araki, National Institute of Agro-Environmental Sciences.

Establishment of the Subculture Method Concentration of Bean Curd Lees Extract: In this study, we attempted to develop a new medium that would be able to subculture nematodes. Bean curd lees extracts were chosen as the main nutrient to add to the medium since glycinoeclepin A in soy bean extract is known to induce the hatching of parasite eggs.[5] Bean curd lees were extracted with hot water in a proportion of 1 : 10, and the extracts were freeze-dried. The obtained bean curd lees extracts were prepared at various concentrations and placed in the plate for incubation. Optimum concentration of the bean curd lees extract was determined by the survival rates of the nematodes, which were calculated under an inversion microscope.

Concentrations of Agar Powder and Nutrient Agar: Next, nematode survival rates were examined on agar powder (Wako Pure Pharmaceutical) and nutrient agar (Eiken) on plates at various concentrations. It is known that simple application of the nematode-suspended solution onto an agar powder plate results in poor survival because of the change in the environment and desiccation. In the present experiment, a 5 mm diameter hole was bored in the center of the medium, which was 90 mm in diameter and 10 mm in height. Furthermore, 4 holes of the same size were bored to form a cross an equal distance from the center. The central hole was filled with 20 worms/20 μl nematode-suspended water and the other 4 holes were filled with water. On the basis of standard concentrations of both agar powder and nutrient agar, medium was prepared and the nematode survival rates were calculated for each concentration.

Positive Control Nematocidal assay was performed employing the following compounds, kainic acid, GABA, piperazine, bicuculline, picrotoxinin, avermectin, santonin, diethylcarbamazine citrate, pyrantel pamoate, levamizole, metrifonate, thiabendazole, albendazole, chloroquine diphosphate, and quinine hydrochloride. These compounds are well known antiparasitic agents and most have clearly understood mechanisms (Table 1).[6,7] Water was employed as the main solvent. A small amount of dimethyl sulfoxide (DMSO) was utilized in order to increase the solubility of the samples. An appropriate concentration of DMSO that had no influence on the larvae was determined to be 2%.

Results and Discussion

Establishment of the Subculture Method Concentration of Bean Curd Lees Extract: The number of 3rd instar larvae were counted at the beginning. The larvae in water die completely within 5 d. At concentrations greater than 10%, the larvae could not be observed under the microscope, thus we tried to examine the number of living larvae at extract concentrations from 0.005 to 10%. The larvae died within 3—24 h at 0.5—10%, and within 3—5 d at 0.3—0.45%. At 0.3—10% the number of living larvae were less than that in water only. At 0.008—0.02%, the number of living larvae increased temporarily and survived after the 15th day, and life cycles of 5—6 d were observed with egg laying, hatching, and larvae growing. At less than 0.008%, although the larvae survived after the 15th day, the life cycles were not observed. Further investigation, as shown in Fig. 1, indicated that the larvae increased temporarily at each concentration from 0.008 to 0.02%, but decreased gradually after the 9th day. A concentration of 0.01% maintained a higher survival rate than the other conditions.

Concentrations of Agar Powder and Nutrient Agar The number of living larvae was observed at agar powder concentrations from 0.3 to 1.5%. The life span was longer than that in water only. At 0.7—1.5% the life cycles (3—4 d) were observed, but at less than 0.7% the life cycle was longer and the number of eggs laid decreased. Thus, in view of the larvae number, concentrations from 0.7 to 1.5% were evaluated again. As a result, the life cycle was observed at each concentration. In Fig. 2, the living larvae increased temporarily except at 1.5%, but decreased after

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Table 1. Specific Shapes of the Larvae in Response to Each Compound with a Different Action Mechanism

Drugs	Mode of nematocidal action	Variations in activities	Shapes of larvae
Kainic acid	Agonist at Glu receptors	→	Arc
GABA Piperazine	Agonist at GABA receptors	→	Arc
Bicuculline	Competitive antagonist at $GABA_A$ receptors	—	Wave
Picrotoxinin	Noncompetitive antagonist at $GABA_A$ receptors (Cl^- channel blocker at $GABA_A$ receptors) Noncompetitive antagonist at glycine receptors	→	Coil, S
Avermectin	Opening of $GluCl^-$ channels Inhibiting GABA activated Cl^- channels	→	Needle
Santonin	Inhibitory and stimulatory actions through stimulating gabergic and cholinergic mechanisms	↘	Coil, S
Diethylcarbamazine citrate	Inhibitor of arachidonic acid metabolism and stimulation of innate immunity	→	Needle, arc
Pyrantel pamoate Levamizole	Agonist at nicotinic ACh receptors	→	Wave, arc
Metrifonate	ChE Inhibitor	↗	Needle, black tissue
Thiabendazole Albendazole	Bind selectively to β-tubulin, and inhibit microtubule formation	↗	Coil, S
Chloroquine diphosphate	The exact mechanism of action is not known	→	Arc
Quinine hydrochloride	The exact mechanism of action is not known	↗	Hook, black tissue

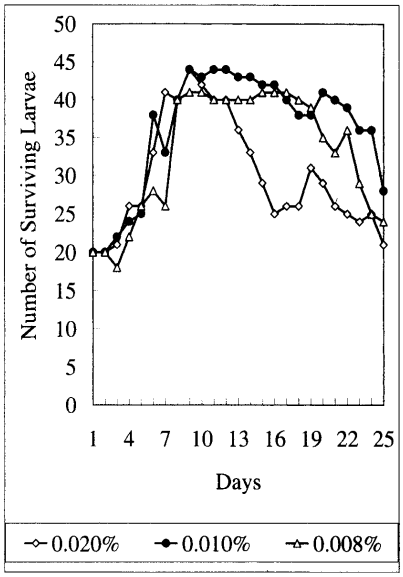

Fig. 1. Concentrations of Bean Curd Lees Extracts and Number of Surviving Larvae ($n=10$)

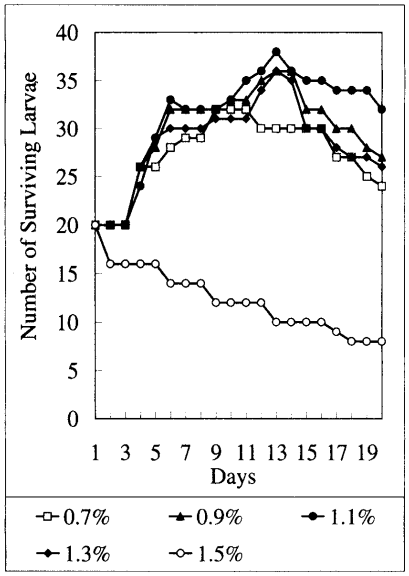

Fig. 2. Concentrations of Agar Powder and Number of Surviving Larvae ($n=10$)

the 13th day. At 1.5% agar powder, the number of dead larvae increased and the number of eggs laid decreased more than that at other concentrations, presumably due to the restricted movement. At 0.9—1.3%, more stable life cycles were observed, but they were not observed at 0.7%. Consequently, agar powder concentrations of 0.9 to 1.3% were regarded as suitable.

At each suitable concentration of agar powder (0.9 to 1.3%) and bean curd lees extract (0.01%), the number of living larvae was examined. Figure 3 shows a gently sloping increase, compared to that in Fig. 2. Larvae were observed to have life cycles of 3 d, but began to die after about 20 d.

Therefore, 1.1% agar powder and 0.01% bean curd lees extract was the most suitable combination.

At concentrations of nutrient agar from 0.5 to 4.5%, larvae were short-lived: they began to die from the 7th day. It was thought that eggs did not hatch and ovulatory cycles were not constant on account of the restricted movement due to the hard medium. Thus, nutrient agar appeared to be unsuitable as the main nutrient to add the medium.

Medium for Subculture In Fig. 3, the living larvae began to decrease after about 3 weeks due to shortage of nutrients. Therefore, nutrient agar was examined as an additional nutritive source. A media with 1.1% agar powder and 0.01% bean curd lees extract was tested with various concentrations of nutrient agar, ranging from 0.05 to 0.2%. Survival d was greatly extended at every concentration of the media, as shown in Fig. 4. In particular 0.10% nutrient agar revealed remarkably high survival rates, and ovulatory cycles of 3 d became stable as well. Based upon the above experiment, the basic

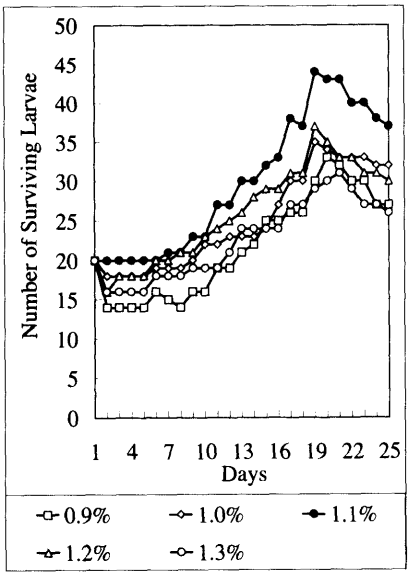

Fig. 3. Concentrations of Agar Powder for the Larvae at 0.01% Bean Curd Lees Extract ($n=10$)

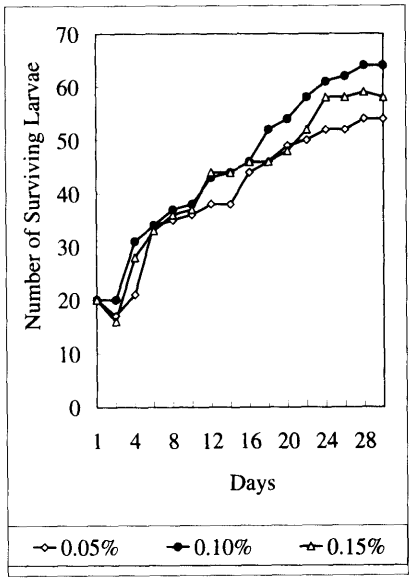

Fig. 4. Concentrations of Nutrient Agar for the Larvae at 0.01% Bean Curd Lees Extract and 1.1% Agar Powder ($n=10$)

medium for subculture of the larvae was established, and contained 0.01% bean curd lees extract, 1.1% agar powder, and 0.10% nutrient agar. A subculture at these concentrations gave the larvae in stable numbers.

Establishment of the Nematocidal Assay Judgement of Dead Larva: Accurate judgement of living and dead larvae is crucial in evaluating nematocidal activity of tested compounds. We have set up a simple method using a microscope to judge the state of the larvae with respect to movement, shape, and staining with alkaline methylene blue. We attempted to be as consistent as possible in our judgement of a larva's death by setting the following five conditions (Fig. 5). 1) The larva is immobile. 2) The larva adopts a needle or arc form. 3) The larva cells become vitreous. 4) The larva develops segmentation. 5) The larva is stained with alkaline methylene blue.

Our criteria for judging larva death were: the larva was immobile and its body was in the form of needle or arc.

Fig. 5. Photograph of the Dead Larva Taken under Microscope ($\times 200$)

The judgement of dead larva was based on the following conditions: fixed body, vitreous cells, segmentation, and stained body with alkaline methylene blue.

Another indication was its keratoderma and subkeratodermal-layer being stained with alkaline methylene blue. If the esophagus alone was stained, we regarded this to be the result of intake of the stain and the larva was still alive.

Procedure for Nematocidal Assay: Preparation of Samples 1) Samples were dissolved in water with 2% DMSO. 2) Samples were diluted to yield a series of concentrations and placed in microplate wells.

Collection of the 3rd Instar Larvae 1) Nematodes were collected from 5—7 d-old media by pouring a small amount of water into the holes already existing in the media. 2) The water suspension of nematodes was centrifuged at 2000 rpm for 10 min. 3) The 3rd instar larvae were obtained by passing the nematodes through a 5 cm-column filled with glass beads (0.177—0.250 mm in diameter).

Assay Method 1) Blank and control experiments were carried out at the same time. 2) The 3rd instar larvae (20 worms/20 μl) were added to each well and the contents incubated at 28 °C for 4 h. 3) Incubation at 28 °C for 80 min is resumed after 4% alkaline methylene blue was added to each well, in order to confirm the dead larvae. 4) Minimum lethal concentration (MLC) is calculated according to the foregoing definition of the dead larvae.

Nematocidal Activities of Positive Controls MLC: The MLC values of each compound are indicated in Fig. 6. Levamisole and metrifonate showed the highest potency, with MLC values of 0.8×10^{-2} and 1.6×10^{-2} μmol/ml, respectively.

As expected the well-known active compounds for strongyloidiasis, avermectin and thiabendazole, exhibited strong nematocidal activity. Therefore, this new assay was thought to be effective for primary screening of nematocidal compounds against *Strongyloides stercolaris*.[8]

Variations in Activity: During the 4 h course of this assay, the time-course of activity of the test compounds was examined. The tested compounds possess a different mode of action as shown in Table 1. In the case of santonin,

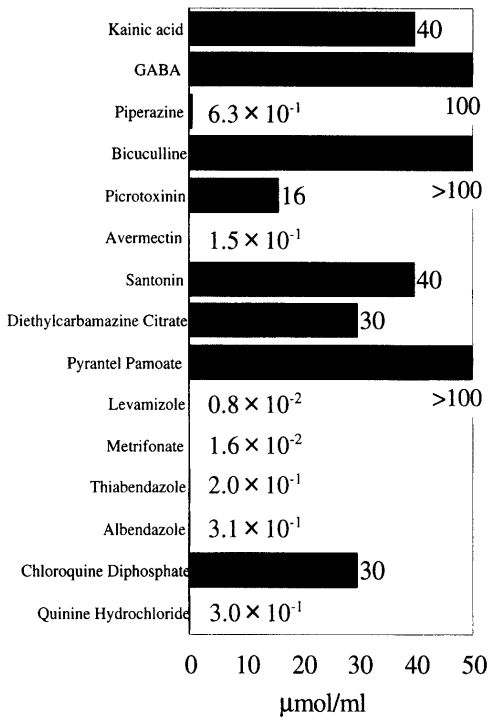

Fig. 6. MLC Values of Well-known Antiparasitic Agents, Most Have Clearly Understood Mechanisms

activity was the highest in the beginning, but became progressively weak with time. On the contrary, metrifonate, thiabendazole, albendazole and quinine hydrochloride increased their nematocidal activity gradually and reached maxima at 2—3 h and this effect lasted until 4 h later. Other compounds maintained constant activity from beginning to end. Thus, activities against 3rd instar larvae were variable and differences in the larvae shapes were observed.

Shapes of Larvae: The shapes of larvae can be classified into several types, which are roughly correlated with the different mechanisms of action of these compounds (Table 1).[6-7] GABA and piperazine, agonists of $GABA_A$ receptors, caused the bodies to adopt arc forms immediately. With pyrantel pamoate and levamizole, the larvae died instantly and the bodies were observed in wave or arc forms. Similarly, thiabendazole and albendazole, which act on β-tubulin binding, caused the bodies to twist into S or coil forms immediately. Avermectin caused the bodies to be fixed in needle or loose wave forms, different from the case of thiabendazole although both compounds exhibited very high activity. The effect of quinine hydrochloride was peculiar. At higher concentration, the larvae died immediately with its keratoderma peeled off, and its tissues discolored black with the body in the form of an irregular curve resulting from shrinkage of the tissue. An increase in the diameter of the body was also noticed. Furthermore, the living larvae in quinine hydrochloride tested at low concentrations began to die in the needle form gradually, but the shapes were clearly different from those of immediate death and the tissues did not discolor. For metrifonate, the dead larvae displayed the same black tissues as in the case with quinine hydrochloride, however, the larva shape upon gradual death was the arc or wave form, with no black tissues observed. Picrotoxinin, santonin and thiabendazole induced coil or S forms, but the variation in activities were different from each other. The activities were constant for picrotoxinin, decreasing with santonin and increasing for thiabendazole over time. The body shapes, and the variation in the activities were the same in the case of kainic acid, GABA, and chloroquine, in spite of their different mechanisms (Table 1). For kainic acid and GABA, a similar partial structure may be a contributing factor to the similar results.

As can be seen from Table 1, compounds possessing the same nematocidal mechanism result in the same larva shape and have a similar trend in terms of the variation of activity. Thus, in other words, the mechanism of action can be roughly estimated based upon the shapes of the larva and the variation of activity.

Conclusion

A new subculture method for the free-living nematode (family of Diplogastridae) has been set up. The best medium was found to contain 0.01% bean curd lees extract as the main nutrient, 1.1% agar powder and 0.10% nutrient agar. The subculture has given stable life cycles for the nematode and enabled us to collect enough nematode for subsequent nematocidal assay. A simple method for nematocidal assay was established and its effectiveness has been proven by using known compounds. The rapid microplate assay lasted for just 4 h and consumed only small amounts of sample. Therefore, the assay should be suitable as a primary screening method for new nematocidal compounds. The respective mode of action of the tested compounds can be roughly estimated from the shapes of the larvae, as depicted in Table 1.

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