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A Tetrazorium Dye (MTT) Assay for Testing Larval Viability Using Second-stage Larvae of *Toxocara canis*

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Abstract

A tetrazorium-based (MTT) assay was developed to assess killing effects of anthelmintic compounds on second-stage larvae of *Toxocara canis*. A linear correlation exists between the number of living larvae (0 to 3,000) and MTT-formazan crystal production. More than 1,000 larvae are required for sufficient absorbance; however, the number can be decreased to half when the alkaline buffer is added to dissolve the MTT-formazan crystals. Hexyl 2,4-dihydroxybenzoate, a strong larvicidal synthetic analogue and the active ingredient of *Usnea purnustri*, and six common anthelmintics showed a good correlation between the number of living larvae and MTT-formazan crystal production. These results suggest that the MTT assay is available for estimating larvicidal activity of drugs.

Key words: *Toxocara canis*, MTT, second-stage larvae, *in vitro* assay, anthelmintics

Introduction

Visceral larva migrans, caused by the larval stage of *Toxocara canis*, is difficult to cure, because few anthelmintics are available. We have been investigating the effective plant-derived drugs that have been used in tropical countries as folk medicine. To assess larval impairment *in vitro* we initially used the relative mobility (RM) index of the larvae (Kiuchi *et al.*, 1987). The RM index showed a good correlation with larval impairment. However, the index could not easily determine whether the larvae were alive or dead. In a previous report, therefore, we introduced a dye exclusion (DE) index to estimate larval morbidity (Akao *et al.*, 1992). With some drugs tested, both indices showed a striking discrepancy that suggested the low mobility or high stainability of the larvae does not indicate low activity. Therefore, we concluded that more than two assays must be conducted to ascertain larval impairment.

A tetrazorium-based (MTT) assay has been used to evaluate the *in vitro* viability of *Onchocerca gutturosa*, *O. volvulus* (Townson *et al.*, 1989), *Schistosoma mansoni* (Nare *et al.*, 1991), and the *in vitro* effects of anthelmintics (Townson *et al.*, 1990, 1991). However, no information is available about the MTT-assay/*Toxocara* system. In this study, we describe an MTT assay using second-stage *T. canis* larvae, and compare the results with the DE assay that estimates larval morbidity (Akao *et al.*, 1992). This comparison provides more information about the precise mechanisms of action of larvicidal drugs.

Materials and Methods

Second-stage T. canis *larvae*

We obtained newly hatched *T. canis* larvae by modifying the method of de Savigny (1975) described elsehwere by Kondo *et al.* (1981), and maintained the larvae in Eagle's minimum essential medium (pH 7.2, Nissui, Tokyo, Japan) supplemented with antibiotics (100 U/ml of penicillin G, 250 mg/ml of streptomycin and 100 mg/ml of kanamycin).

Larvae that had been cultured for less than 20

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weeks were used for the assay because their chemosusceptibility changed with long-term incubation *in vitro* (Akao *et al.*, 1993).

Chemicals

3,[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazorium bromide (MTT, Sigma Chemical Company, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS, pH 7.2) and stored in 4°C until used.

Hexyl 2,4-dihydroxybenzoate (HDB) (0.1 mg/ml), a larvicidal ingredient of *Usnea purnustri* (Ahad *et al.*, 1991), was synthesized in our laboratory. We also tested the following common anthelmintics for the assay: diethylcarbamadine citrate (Tanabe Seiyaku, Osaka, Japan), mebendazole (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan), bithionol (Tanabe Seiyaku, Osaka, Japan), thiabendazole (Dainippon Seiyaku, Osaka, Japan), santonin (Nippon Shinyaku Co., Ltd., Kyoto, Japan), and pyrantel pamoate (Taito Pfizer Co. Ltd., Tokyo, Japan). These drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Company, USA) and assayed at the concentration of 1 mg/ml in sterilized PBS to keep the DMSO concentration at 2%.

Assay

The larvae were plated out in 100 μl of sterilized PBS in the volumes of from 0 to approximately 3000 larvae in 1.5 ml-polypropylene microcentrifuge test tubes (Treff Lab, Degersheim, Switzerland). The final volume of PBS was adjusted to 100 μl by adding PBS. The number of larvae in each tube was counted under a microscope after following completion of the assay.

To assess the MTT formazan produced by the larvae, various numbers of the larvae were incubated with 10 μl of MTT (5 mg/ml) for 24 hours at 37°C in 5% CO_2 in air. To evaluate chemosensitivity, the larvae were incubated with drug dissolved in PBS for 3 or 24 hours at 37°C in 5% CO_2 in air. After washing three times with sterilized PBS at 1500 rpm for 3 minutes, the volume was adjusted to 100 μl by sterilized PBS. Ten microliters of MTT was then added and incubated for an additional 24 hours under the same conditions.

The supernatant was suctioned carefully, and the MTT-formazan crystals produced by the larvae were dissolved in 100 μl of DMSO and mixed well for 1 hour with a plate mixer (Tomy Seiko Co., Ltd., Tokyo, Japan). In some experiments, 12.5 μl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) was added in DMSO to enhance absorbance (Plumb *et al.*, 1989).

The colored supernatant was plated out in 96-well microtiter plates (#25960, Corning, NY, U.S.A.). The absorbance was measured with a microplate reader (Model 450, Bio-Rad Laboratories, Ltd., Richmond, VA, U.S.A.) at a wavelength of 540 nm. Wells containing only PBS and MTT were used as controls for the plate reader, and appropriate numbers of larvae in PBS with MTT were used in each experiment to obtain a simple linear regression line. The larvae were counted at least three times, and their absorbance was measured. The expected absorbance levels of the intact larvae at the same number of drug-treated larvae was calculated using a regression line. The drug-induced larval morbidity was expressed by the cytotoxicity index (CI) as follows:

$$CI = \left(1 - \frac{\text{Average absorbance of drug-treated larvae}}{\text{Expected absorbance of intact larvae}}\right) \times 100$$

Two or three tubes were used during the performance of the assay for each drug. An elevated CI indicates a high degree of larval impairment.

Results

Relationship between larvae number and MTT-formazan production

Formazan crystals formed around the esophagus and nerve ring of living larvae (Fig. 1) and in the incubation medium. These products in the larva were entirely dissolved after 1 hour incubation with DMSO. Figure 2 shows the amount of MTT formazan produced by the larvae when the various numbers of larvae were incubated with MTT (5 mg/ml). A linear relationship is seen up to approximately 3000 larvae. More than 1000 larvae are needed to obtain sufficient absorbance.

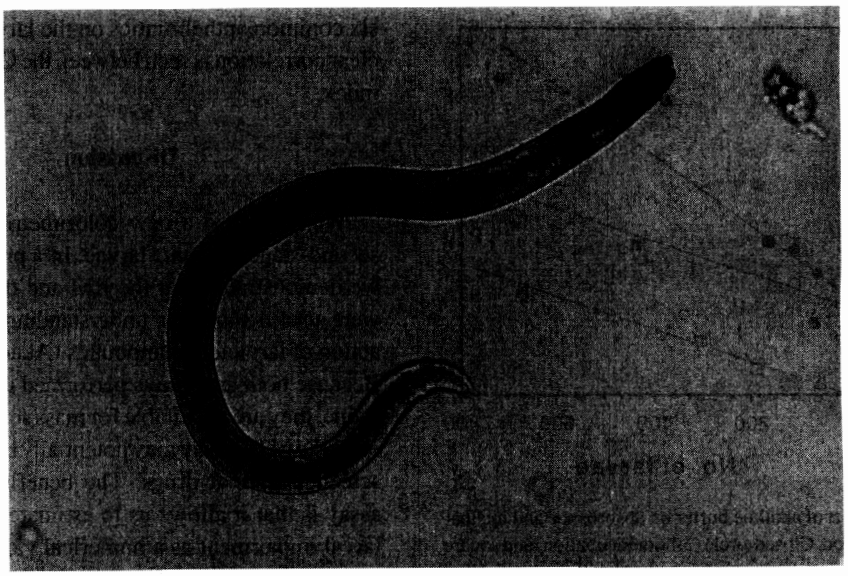

Fig. 1 MTT-formazan crystals in second-stage *T. canis* larva.

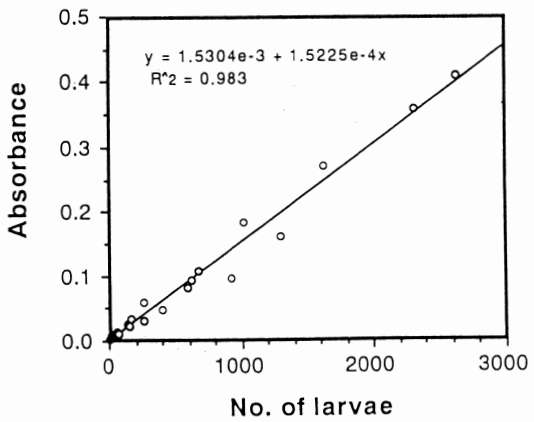

Fig. 2 Relationship between living larvae number and MTT-formazan crystal production.

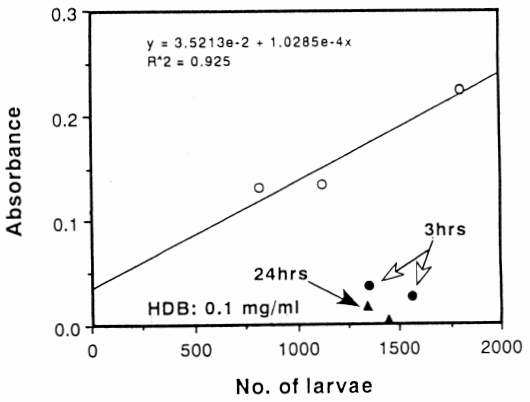

Fig. 3 Larvicidal effect of HDB on *T. canis* larvae. Closed circle and triangle indicate the absorbance after 3-hour and 24-hour incubation, respectively.

Enhancement of MTT-formazan absorbance by alkaline buffer

The use of fewer larvae is preferable when performing a mass screening. Therefore, MTT-formazan crystals were dissolved in DMSO with alkaline buffer (12.5 μl per tube, pH 10.5) to enhance absorbance (Plumb *et al.*, 1989). Figure 3 shows the effect of buffer on absorbance and the incubation period. The absorbance increased with the addition of the buffer and with increasing incubation time. This result indicated that 500 larvae are sufficient to perform the MTT assay according to this method.

Larvicidal effect of HDB on T. canis *larvae*

HDB is a strong larvicidal synthetic analogue of the active ingredient of *Usnea purnustri* (Ahad *et al.*, 1991). Figure 4 shows the results of MTT assay

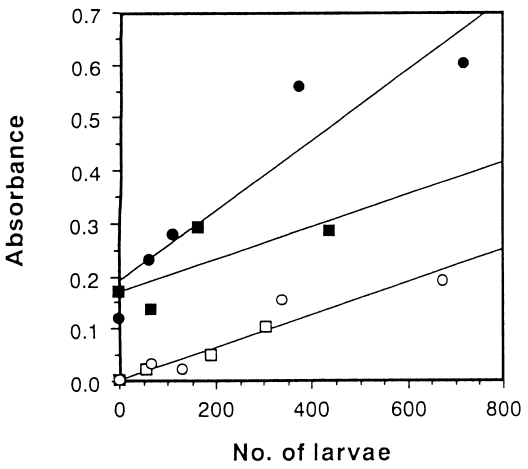

Fig. 4 Effect of alkaline buffer on absorbance and incubation period. Closed circle (3-hour incubation) and square (24-hour incubation) indicate the absorbance with alkaline buffer, and open circles and square indicate without the buffer.

Table 1 Comparative results between CI and RM of six common anthelmintics at 10^{-3} g/ml on second-stage *T. canis* larvae

Anthelmintics	CI*	RM†
Diethylcarbamadine citrate	6	97
Mebendazole	11	33
Bithionol	84	0
Thiabendazole	16	33
Santonin	40	42
Pyrantel pamoate	63	0

*CI; cytotoxicity index at 3 hours after incubation
†RM; relative mobility at 3 hours after incubation

without adding alkaline buffer. When the larvae were incubated with 0.1 mg/ml of HDB, the larval viability decreased markedly during the first 3-hour incubation (CI=80), and completely disappeared at 24 hours of incubation (CI=97). These results agreed with our previous results obtained with the RM assay (Akao *et al.*, 1992).

Larvicidal activities of six common anthelmintics in vitro

The MTT assay was conducted with the addition of the alkaline buffer. Table 1 shows the effects of six common anthelmintics on the larvae *in vitro*. A clear correlation is seen between the CIs and the RM index.

Discussion

We described a new colorimetric assay using second-stage *T. canis* larvae. In a previous report, we demonstrated that the RM and the DE indices were useful tools for understanding the mode of action of larvicidal compounds (Akao *et al.*, 1992). Because both are assays performed under a microscope, they are unsuitable for mass screening. However, the MTT assay may potentially be used to mass screen larvicidal drugs. The benefit of the MTT assay is that it allows us to estimate the degree of larval impairment as a numerical value because of the linear correlation between the number of living larvae and the MTT-formazan products.

MTT is reduced by the enzyme, cytochrome C_1, that exists in mitochondria of live but not dead cells. Consequently, a water-soluble yellowish MTT dye becomes a water-insoluble blue formazan product. Therefore, *in situ* formazan crystals indicate the location of mitochondria in a living larva. In this study, these crystals formed around esophagus and nerve ring, suggesting that many mitochondria are crowded at both regions. These results agreed with a recent study that a large number of mitochondria are aggregated in the somatic cells around the esophagus (Konishi, 1992; Bowman *et al.*, 1993).

HDB was used in a practical application of the MTT assay. We found a direct relationship between the incubation period and the MTT-formazan products in HDB-treated larvae. The results of a previous report revealed that the RM index rapidly decreased within 3 hours after incubation with HDB, while the DE index maintained a high score during the same incubation period. After 24 hours of incubation, HDB killed the larvae, but they were judged to be alive by the DE assay (Akao *et al.*, 1992). In this study, however, the larvae treated with HDB for 24 hours died using the MTT assay. There was also a good correlation between the RM and MTT-assays with six common anthelmintics, which suggested that the MTT assay is useful for estimating larval viability.

The drawback to this method is that the MTT

assay requires at least 500 larvae for a screening despite addition of the alkaline buffer. We are now investigating ways to improve the sensitivity of this assay.

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