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Penetration of Third-Stage Larvae of *Angiostrongylus cantonensis In Vitro*
I. Penetration Apparatus and Its Application

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Introduction

When the infective larvae of *Angiostrongylus cantonensis* are ingested by a rat, they quickly enter the stomach or intestinal walls and soon afterwards, the majority of them enter the venules and find their way to the liver by way of the portal vein (Alicata, 1965; Otsuru, 1977). Jindrák (1968) also indicated in his study on early migration of *A. cantonensis* in rats that following oral infection, at the end of the 1st hr, most third-stage larvae were in the walls of the stomach and intestine, and at the 2nd hr, in the wall of the stomach and the liver. Furthermore, the third-stage larvae could penetrate the host skin only when its surface had been abraded (Alicata and Brown, 1962). However, quantitative studies on the penetration of the third-stage larvae through the walls of the stomach and intestine are limited. Studies on the penetration of the third-stage larvae of *A. cantonensis* through the walls of the stomach and intestine are very important for better understanding of the early phases of infection of the nematode. Somewhat quantitative experiments were, therefore, conducted on the penetration of the third-stage larvae of *A. cantonensis* through the walls of intestine of a permissive host (rat) *in vitro.*

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Materials and Methods

1. Preparation of third-stage larvae

A. cantonensis was maintained in our laboratory by using aquatic snails, *Biomphalaria glabrata* and albino rats. The third-stage larvae of *A. cantonensis* used in these experiments were recovered from *B. glabrata* infected with the first-stage larvae 3 to 4 weeks previously. The snails were minced and digested in mechanically stirred digestive fluid (H_2O, 100 ml; granular pepsin, 0.1 g; conc. HCl, 1 ml) at room temperature (26°C) for 3 hr.

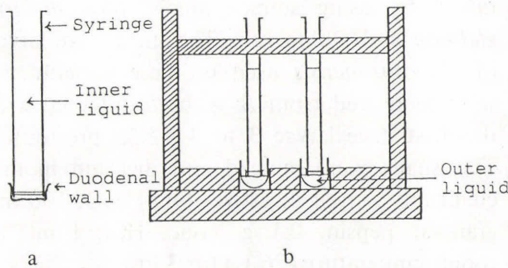
2. Preparation of tissue

The female Sprague Dawley rats (200–300 g in weight) were killed with ether. Immediately after death, the duodenum was carefully dissected. The duodenum was cut into 1 cm length, becoming about 1 cm^2 pieces.

3. Penetration apparatus

The tip of a 1 ml disposable syringe (4.5 mm in inside diameter, Terumo Co.) was cut horizontally. Then, the luminal surface of the duodenum was put downward over the cut tip and fixed with thread (Fig. 1a). The syringe with the tissue was then placed in a rack as shown in Fig. 1b and 100 third-stage larvae in 1 ml of the digestive fluid above-mentioned were placed in each syringe (inner liquid). In other experiment, the medium, NCTC 109 with 50% horse serum (abbreviated to NCTC-

50HS) was used instead of the digestive fluid as a inner liquid. Then, the tissue on the tip of the syringe was brought into contact with the surface of the outer liquid, NCTC-50HS in a glass tube (16 mm in diameter, 16 mm in height). The rack with the apparatus was placed in an incubator with a gas phase of 5% CO_2 and 95% air at 37°C for an appropriate incubation period. The number of third-stage larvae remaining in the inner liquid and of those emerging into the outer liquid were counted at the end of the incubation period under a dissecting microscope. The number of larvae penetrating into the duodenum was calculated by subtracting the total number of larvae in the inner and outer liquids from the initial number of larvae applied (100 larvae). Penetration rate used in this study is expressed as the number of larvae deducted by the number of remaining larvae in the inner liquid at the end of incubation period from 100 larvae initially placed in the inner liquid.



Figs. 1a, b Apparatus used for assessing the penetration of *A. cantonensis* third-stage larvae (syringe: 4.5 mm in inside diameter, 70 mm in length).

Results

1. Preliminary experiment

The effects of incubation time on the survival rates of the third-stage larvae in the digestive fluid, distilled water and NCTC-50HS were investigated for determining the observation period. The third-stage larvae used were obtained by 3-hr peptic digestion of minced snail tissues at room temperature (Fig. 2, PI). Harvested larvae were counted and larval sus-

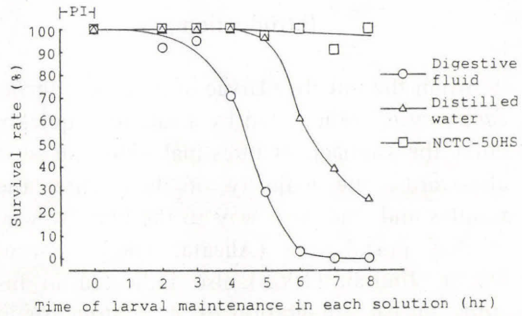
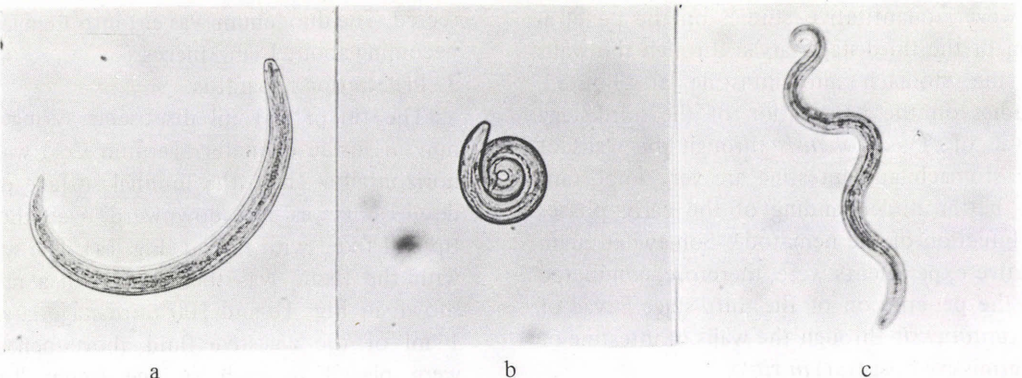


Fig. 2 Effect of incubation period on survival rates of *A. cantonensis* third-stage larvae treated with digestive fluid (H_2O, 100 ml; granular pepsin, 0.1 g; conc. HCl, 1 ml), distilled water and NCTC-50HS (NCTC 109 with 50% horse serum) in a incubator with a gas phase of 5% CO_2 and 95% air at 37°C (PI: Pre-incubation in digestive fluid for 3 hr at room temperature).



Figs. 3a–c Three types of *A. cantonensis* third-stage larvae observed under experimental conditions. a, b and c indicate the crescent-shaped larva as the dead, coil-shaped as the uncertain, and other-shaped as the surviving, respectively.

pension was adjusted to contain 50 third-stage larvae in 1 ml of each solution in a glass tube (16 mm in diameter, 16 mm in height). Glass tubes were incubated in an incubator with a gas phase of 5% CO_2 and 95% air at 37°C for an appropriate incubation period. On the basis of motility and shape, the third-stage larvae observed at the completion of the incubations were classified into 3 types as follows: (1) crescent-shaped larvae as the dead, (2) coil-shaped larvae as the uncertain, and (3) other-shaped and moving larvae as the surviving as shown in Figs. 3a–c. The survival rate (%) was expressed as the number of type 3 larvae against the initial number of living larvae.

As shown in Fig. 2, the survival rate of third-stage larvae during the first 3 hr was more than 90% in all solutions tested. In case of distilled water, it began to decrease after 6th hr of incubation. The survival rate decreased with the increasing incubation period in case of digestive fluid although it was nearly maintained the initial value in case of NCTC-50HS for subsequent 5 hr. From the results obtained, it seemed that 3-hr observation period was enough to know the ability of larval penetration. We decided to adopt 5-hr observation period by way of caution.

2. Penetration experiments

The time course of the distribution of third-stage larvae in the inner liquid (digestive fluid), the duodenum and the outer liquid (NCTC-50HS) was investigated during the first 5 hr (Exp. 1). The larvae in the inner and outer liquids were counted at the appropriate incubation period during 5 hr.

As shown in Table 1, the penetration rates were 58% and 86% at 0.5th hr and 5th hr, respectively. Among the larvae penetrating, most of them were observed in the tissue at the first 0.5 hr. Five percent of larvae were observed in the outer liquid at 1st hr. Fourteen percent and 63% of larvae were found in the inner and outer liquids respectively when the observation was terminated.

Similar experiment was performed in the use of NCTC-50HS as the inner liquid. Thoroughly washed with NCTC-50HS 100 larvae in 1 ml of NCTC-50HS as an inner liquid were placed in each syringe. Then, the time course of the distribution of third-stage larvae was investigated (Exp. 2).

As shown in Table 2, the penetration rates were 91% and 95% at 0.5th hr and 5th hr, respectively. Ninety-one percent of larvae were observed in the tissue at the first 0.5 hr. This value is significantly higher than that of Exp. 1. In spite of such a higher penetration rate, 5% of larvae remained in the inner liquid after 5 hr. Besides, though many larvae penetrated into the tissue, little larvae emerged into the outer liquid in the Exp. 2.

Table 1 Distribution of *A. cantonensis* third-stage larvae in the inner liquid, rat duodenal wall and outer liquid, and penetration rate after appropriate incubation periods

Site	Incubation period (hr)			
	0.5	1.0	2.0	5.0
Inner liquid*	42.0±7.4***	34.8±18.0	19.8±13.7	13.8±5.6
Duodenal wall	57.3±8.7	60.5±17.3	57.0±17.7	23.0±9.6
Outer liquid**	0.8±1.5	4.8± 5.0	23.3± 8.1	63.3±9.1
Penetration rate	58.0±7.4	65.3±18.0	85.3± 4.1	86.3±5.6

*H_2O, 100 ml; granular pepsin, 0.1 g; conc. HCl, 1 ml

**NCTC 109 with 50% horse serum

***Figures indicate mean percent of larvae recovered from four replicates and those following ± sign, standard deviation.

Table 2 Distribution of *A. cantonensis* third-stage larvae in the inner liquid, rat duodenal wall and outer liquid, and penetration rate after appropriate incubation periods

Site	Incubation period (hr)			
	0.5	1.0	2.0	5.0
Inner liquid*	9.5±3.5**	9.5±3.5	6.5±0.7	5.0±7.1
Duodenal wall	90.5±3.5	90.5±3.5	93.0±1.4	94.5±6.4
Outer liquid*	0	0	0.5±0.7	0.5±0.7
Penetration rate	90.5±3.5	90.5±3.5	93.5±0.7	95.0±7.1

*NCTC 109 with 50% horse serum
**Figures indicate mean percent of larvae recovered from two replicates and those following ± sign, standard deviation.

Discussion

An *in vitro* method for studying the penetration of third-stage larvae of *A. cantonensis* was established. It was ascertained that third-stage larvae could penetrate into the rat duodenum *in vitro* showing more than 80% penetration rate after 5 hr. In case of the digestive fluid as an inner liquid (Exp. 1), 57% of the larvae on an average were observed in the rat duodenum at 0.5th hr, and the larvae began to appear in the outer liquid at the 1st hr (Table 1). On the contrary, in case of NCTC-50HS as an inner liquid (Exp. 2), though 91% of the larvae were observed in the tissue at 0.5th hr, little larvae appeared in the outer liquid even after 5 hr. It is uncertain that the reason why the penetration rate in Exp. 2 at 0.5th hr was higher than that of Exp. 1 since almost all the larvae were actively moving (Fig. 2). It was considered that as the blood vessels in the duodenum were tightly fixed with thread to the syringe in the penetration apparatus, the larvae appeared in the outer liquid. However, although many larvae penetrated into the tissue, the reason that most larvae did not emerge into the outer liquid in the Exp. 2 is not clarified yet (Table 2). Further studies on this phenomenon are now in progress. In both experiments, some larvae did not penetrate and remained in the inner liquid even after 5 hr (Tables 1, 2). It is presumed that this penetration process may be one of the first barrier to the ingested third-stage larvae for their further development *in vivo*.

Alicata and Brown (1962) confirmed experimentally that the third-stage larvae of *A. cantonensis* could penetrate rat skin only when the surface of the skin had been abraded. Ubelaker *et al.* (1981) also established the infection of *Angiostrongylus costaricensis* in a permissive host, *Sigmodon hispidus,* by cutaneous penetration of its third-stage larvae only when the skin had been abraded. Furthermore, Ubelaker *et al.* (1981) indicated significant differences in the recovery of adult worms from *S. hispidus* between oral or intraperitoneal routes and subcutaneous or abraded skin routes. The penetration apparatus used in this study may be useful as a tool for understanding these interesting topics which have been shown by above-mentioned investigators.

Summary

A method for studying the penetration of third-stage larvae of *A. cantonensis* was established. Namely, 100 larvae in 1 ml of the inner liquid were placed in each 1 ml disposable syringe with rat duodenum fixed on the tip. Subsequently, the tissue on the tip of the syringe was brought into contact with the surface of the outer liquid in a glass tube and placed in an incubator with a gas phase of 5%

CO_2 and 95% air at 37°C for the appropriate period. The number of third-stage larvae remaining in the inner liquid and of those emerging into the outer liquid were counted at the end of the incubation period under a dissecting microscope. As a result, in case that the inner liquid was digestive fluid (H_2O, 100 ml; granular pepsin, 0.1 g; conc. HCl, 1 ml) and the outer liquid was NCTC 109 with 50% horse serum (NCTC-50HS), penetration rate was 58% after 0.5 hr and 86% after 5 hr. In case that both inner and outer liquids were NCTC-50HS, the penetration rate was 91% after 0.5 hr and 95% after 5 hr. However, some larvae (5–14%) did not penetrate and remained in the inner liquid even after 5 hr. Furthermore, in case that the inner liquid was NCTC-50HS, most larvae did not appear in the outer liquid, although 63% of larvae appeared in the outer liquid after 5 hr when the inner liquid was digestive fluid.

The availability of penetration apparatus used in this study for clarifying the tissue invasiveness of other parasitic nematode larvae was discussed.

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In vitro における広東住血線虫３期幼虫の穿通について
Ⅰ．実験装置およびその応用

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広東住血線虫３期幼虫の組織侵入性について，*in-vitro* で検討した．実験方法は以下のとおりである．すなわち，１ ml のディスポーザブル注射筒の先端を切断し，新鮮なラットの十二指腸壁を糸で固定する．*Biomphalaria glabrata*から人工消化液(0.1%ペプシン／１％塩酸液）処理により得た３期幼虫 100 匹を含む１ ml の内液（人工消化液または培地：ＮＣＴＣ 109 +50%馬血清）を注射筒内に入れ，はりつけた十二指腸壁が外液（培地）に接触するように垂直に固定する．37℃・湿度飽和・５％炭酸ガス培養器に入れ，一定時間後，内液・外液の幼虫数を解剖実体顕微鏡下で調べた．

内液を人工消化液，外液を培地にした場合（実験１），0.5および５時間後の穿通率はそれぞれ58%，86%であった．内液・外液共に培地の場合(実験２）にはそれぞれ91%，95%であった．いずれの場合にも少数の幼虫が穿通しないで内液に残った．さらに実験１の場合には，５時間後に63%の幼虫が外液に出現したのに対し，実験２の場合にはほとんどの幼虫が外液に出現しなかった．

本実験で用いた実験装置は，他の線虫の組織侵入性を解明するうえでも有用である．