



Title	CIRCUMOVAL AND CIRCUMLARVAL PRECIPITATE REACTIONS OF ANGIOSTRONGYLUS CANTONENSIS
Author(s)	OKU, Yuzaburo; KAMIYA, Masao
Citation	Japanese Journal of Veterinary Research, 32(1), 23-39
Issue Date	1984-01-31
DOI	<a href="https://doi.org/10.14943/jjvr.32.1.23">https://doi.org/10.14943/jjvr.32.1.23</a>
Doc URL	<a href="https://hdl.handle.net/2115/2306">https://hdl.handle.net/2115/2306</a>
Type	departmental bulletin paper
File Information	KJ00002374149.pdf



## CIRCUMOVAL AND CIRCUMLARVAL PRECIPITATE REACTIONS OF *ANGIOSTRONGYLUS CANTONENSIS*<sup>1</sup>

Yuzaburo OKU<sup>2</sup> and Masao KAMIYA<sup>2</sup>

(Received for publication November 29, 1983)

Using sera obtained from *Angiostrongylus cantonensis*-infected or -transferred rats and immunized guinea pigs with *A. cantonensis* homogenate, positive circumoval and circumlarval precipitate reactions on living *A. cantonensis* at various developmental stages were recognized after incubation at 34°C for 24 hr. Characteristic precipitates were mainly formed on the excretory pore and cuticle of the 3rd-stage larvae; the oral opening, excretory pore and cuticle of the 4th-stage larvae; and the vulva, anus, cloacal and oral openings, excretory pore and cuticle of immature adults. However, precipitates were not formed on the living 1st-stage larvae. Precipitates began to appear 1 to 4 weeks after infection. These reactions were recognized irrespective of the sex and developmental stage of the worm. Positive cross-reactions were observed in the sera from *A. costaricensis*-infected rats. Applying a technique of immunofluorescence, it was confirmed that immunoglobulins were specifically incorporated into the precipitates. The results of these reactions were compared with those of the indirect hemagglutination and double diffusion tests. For the immunodiagnosis of angiostrongyliasis, the excellency of the circumoval and circumlarval precipitate reactions was noted.

Key words: *Angiostrongylus cantonensis*, *Angiostrongylus costaricensis*, circumoval precipitation, circumlarval precipitation, immunodiagnosis

### INTRODUCTION

SARLES & TALIAFERRO (1936) reported that masses of precipitates were found around the anterior end and in the intestine of larvae of *Nippostrongylus brasiliensis* retained in the skin and lungs of immune rats. A similar *in vitro* reaction in the serum of rats reinfected with *N. brasiliensis* was demonstrated by SARLES (1938). This reaction has been demonstrated in other nematodes by various authors. These

<sup>1</sup> This study was supported by an Overseas Scientific Research Grant, No. 58043005, from the Ministry of Education, Science and Culture, Japan

<sup>2</sup> Department of Parasitology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

precipitate formations on the larvae of nematodes were proved to be due to antigen-antibody reactions.<sup>6,8,14,24,25)</sup> Moreover, the circumlarval precipitate (CLP) reaction has been used for a practicable method for diagnosis of *Trichinella spiralis* and *Toxocara canis* infections.<sup>15,19)</sup>

The circumoval precipitate (COP) reaction is a diagnostic method used in schistosomiasis<sup>7,17)</sup> and it has also been experimentally demonstrated around the eggs of *Trichosomoides crassicauda* and *N. brasiliensis* incubated in sera from rats infected with the respective nematodes.<sup>9,23)</sup>

*Angiostrongylus cantonensis* is one of the rat lung worms. The third-stage larvae reach the central nervous system in a variety of mammals, including man, where they provoke eosinophilic meningoencephalitis.<sup>18)</sup> *Angiostrongylus costaricensis* is a causative agent of abdominal granulomatous inflammation.<sup>13)</sup>

The present investigation examined the precipitate formation on eggs, the 1st-, 3rd- and 4th-stages larvae and immature adults of *A. cantonensis* in sera from infected or immunized animals. In addition, the sera were tested by the double diffusion and indirect hemagglutination tests.

#### MATERIALS AND METHODS

##### *Preparation of various developmental stages of A. cantonensis*

Eggs were obtained from the uterus of mature female worms. They were washed and suspended with saline. The suspension was adjusted to 5,000 eggs per ml. The first-stage larvae were harvested from feces of rats which had been infected for at least 8 weeks. The third-stage larvae were obtained from laboratory reared *Biomphalaria glabrata* infected with the first-stage larvae for at least 5 weeks. The fourth-stage larvae were obtained from the brain of rats 6 to 8 days after infection. These larvae were washed and suspended in saline. The suspensions of the 1st-, 3rd- and 4th-stage larvae were adjusted to about 800 and 400 larvae per ml, respectively. Immature adults were obtained from the brain of rats and mice 14 days after infection.

##### *Serum*

*A. cantonensis*-infected rats; six-week-old female rats (Wistar) were divided into 5 groups of 5 rats each. The first group served as a control and was not infected. Groups II, III, IV and V were given orally 25, 50, 100 and 250 third-stage larvae of *A. cantonensis*, respectively.

*A. cantonensis*-transferred rats; immature adults of *A. cantonensis* were obtained from the brain of the rats 25–28 days after infection. Male and/or female immature adult worms were introduced into the pulmonary artery of 19 six-week-old female rats, through the cervical vein by a syringe with needle, gage No. 16. Subsequently, these recipients were sacrificed 5 or 10 weeks after transfer.

*A. costaricensis*-infected rats; three female wistar rats were inoculated perorally with 50 or 100 third-stage larvae of *A. costaricensis*. They were killed and necropsied 7 weeks after infection.

*A. cantonensis*-infected mice; ten female 6-week-old mice (dd) were infected with 50 larvae. Two mice each were killed and bled weekly from 1 to 5 weeks after infection.

Immunized guinea pigs with *A. cantonensis*; twelve Hartley strain guinea pigs were given footpad inoculations with emulsified adult female worms in Freund's complete adjuvant. Six guinea pigs were immunized similarly with adult male worms.

#### *COP and CLP reactions*

One drop, about 0.025 ml, of the suspension containing living larvae, eggs or 6–8 specimens of immature adults was put into the well of a slide, and 3 drops, about 0.075 ml, of serum were added. A cover slip was placed over the well. The slide was incubated at 34°C. After 24 hr, the slide was examined by microscope for the appearance of precipitates attached to the worms or eggs.

#### *Fluorescence antibody method*

Immature adults or eggs were incubated in phosphate-buffered saline (PBS), normal serum or *A. cantonensis*- or *A. costaricensis*-infected rat serum at 34°C for 24 hr, and washed gently 6 times with chilled PBS for 6 hr. Then they were placed in FITC labeled anti-rat IgG serum (MILES) diluted with PBS at 0°C for 36 hr and washed as above. They were examined by fluorescent microscope (OLYMPUS BH-RFL) using an UV exciting filter system.

#### *Indirect Hemagglutination test (IHA)*

The IHA method of KAMIYA & TANAKA (1969) was used. The antigen for sensitization was the buffered saline extract (1:4,000 w/v) of female adult *A. cantonensis*. The antigen sensitized cells were frozen in liquid nitrogen and stored at -80°C. Sera with a IHA titer of 1:16 or more were regarded as positive.

#### *Double diffusion (DD)*

The method of DD was similar to that of KAMIYA et al. (1977) and the antigen was the buffered saline extract (1:10 w/v) of female adult *A. cantonensis*.

## RESULTS

Eggs of *A. cantonensis* were incubated in sera from the rats infected with 50–250 third-stage larvae of *A. cantonensis*, the rats in which male and/or female worms were transferred, the guinea pigs immunized with *A. cantonensis* or the rats infected with *A. costaricensis*. Precipitates were formed in and around the degenerating eggs (table 1,

photos 1-8) but not around the intact eggs. In the sera of normal rats, normal guinea pigs and the rats infected with 25 third-stage larvae of *A. cantonensis*, no reactions were observed. The precipitates were hyalin-like bodies which varied in size and formed a thick membrane, band, globule, etc. They appeared as early as 1 hr of incubation and enlarged gradually by 2 days. The COP-positive egg ratio of the *A. cantonensis*-infected rat sera was lower than that of the immunized guinea pig and *A. costaricensis*-infected rat sera (table 2). Moreover, the precipitates in the former were smaller than those in the latter. In the former, small precipitates were attached to a part of the egg; however, in the latter, some eggs were covered entirely with precipitates. Positive COP reaction was observed as early as 1 week after infection with *A. cantonensis*.

No precipitates were formed on the living first-stage larvae in any sera. The degenerated first-stage larvae very rarely formed precipitates on their surface in the sera from the immunized guinea pigs (photo 9).

Precipitates were formed on the third- and fourth-stage larvae and immature adults in the sera of the *A. cantonensis*-infected or -transferred rats, immunized guinea pigs with *A. cantonensis* homogenate and *A. costaricensis*-infected rats, but not in control

TABLE 1 *Results of the circumoval precipitate reaction of Angiostrongylus cantonensis in various sera*

SOURCE OF SERUM	NO. OF POSITIVE SERA / NO. OF SERA EXAMINED
I group (normal) rats	0 / 5
II group (25 larvae-infected) rats*	0 / 3
III group (50 larvae-infected) rats*	3 / 5
IV group (100 larvae-infected) rats*	2 / 3
Rats transferred with male worms**	2 / 7
Rats transferred with female worms**	3 / 6
Rats transferred with male and female worms**	5 / 6
<i>Angiostrongylus costaricensis</i> -infected rats***	3 / 3
Normal guinea pigs	0 / 2
Guinea pigs immunized with male worm	6 / 6
Guinea pigs immunized with female worm	12 / 12

\* 12 weeks after infection

\*\* 10 weeks after transfer

\*\*\* 7 weeks after infection

TABLE 2 Percentages of COP-positive eggs in various sera

SOURCE OF SERUM*		% OF POSITIVE EGGS
	PBS	0
Control	Normal rat	0
	Normal guinea pig	0
	1 week	1.5
	2 weeks	2.6
250 larvae-infected rats	3 weeks	3.1
	5 weeks	1.0
100 larvae-infected rat	10 weeks	3.2
	A	12.9
<i>A. costaricensis</i> -infected rats	B	17.7
	C	9.3
Guinea pigs immunized with male worm		17.1
Guinea pigs immunized with female worm		25.0

\* Each examined serum was randomly selected.

sera of the normal rats or guinea pigs.

On the third-stage larvae, precipitates were formed frequently at the excretory pore of the living worm and at the cuticle of the degenerated worms, but rarely at the oral opening of the living worms or at the anus of the degenerated worm (photo 10).

On the fourth-stage larvae, precipitates were formed frequently at the excretory pore and oral opening of the living worm and at the cuticle of the degenerated worm, but rarely at the oral opening of the living worms or at the anus of the degenerated worm (photo 11).

On the immature adults, the precipitates which were formed at the anus, vulva, oral and excretory and cloacal opening were frequent and larger (photos 12–15). Precipitate formation of the worms obtained from mice and rats were identical.

Even in the sera obtained from rats one week after infection, precipitates were formed on the immature adults. And in the sera from immature adult-transferred rats, precipitates were formed on the third-stage larvae. Furthermore, in the sera obtained from single sex worm transferred rats, precipitates were formed at the opening of the genital organs of the opposing sex worm. Generally, the size of the

precipitates increased from 1 to 3 weeks after infection and persisted thereafter.

In the infected mice, positive CLP reaction on the immature adults was observed from 3 weeks after infection. And the sera from 19 transferred rats, 18 immunized guinea pigs with *A. cantonensis* and 3 rats infected with *A. costaricensis* were all positive for CLP reaction on the immature adults.

Results of groups I–IV in CLP reaction on immature adult worms are shown in table 3. Group I controls were all negative. The sera from the infected rats of groups II, III and IV began to turn to positive 1 or 3 weeks after infection, and were all positive 4 weeks after infection. Positive conversion of group II occurred 2 week later than that in groups III and IV. No rat converted from positive to negative throughout the experimental period.

COP and CLP reactions were observed in the sera heated at 56°C for 30 min as well as in the unheated sera from *A. cantonensis*-infected rats and immunized guinea pigs with *A. cantonensis* homogenate.

Fluorescence were observed on the precipitates around eggs and immature adults which had been incubated in sera obtained from *A. cantonensis*- or *A. costaricensis*-infected rats (photos 16–19).

The IHA titers of I–IV groups were shown in figure 1. The controls, group I, were all negative. *A. cantonensis*-infected rats, group II–IV, were negative until 6 weeks after infection. Five of the 13 infected rats increased in the IHA titer 8 weeks after infection. Group V rats (1–5 weeks after infection) and normal guinea pigs were all negative. Immunized guinea pigs and *A. costaricensis*-infected rats were all positive. The majority of transferred rats (11/19) were negative.

Results of DD groups I–IV are shown in table 4. In group I, a false positive reaction was present. Most of the sera from the infected rats, groups II–IV, were

TABLE 3 Results of the circumlarval precipitate reaction on immature adults of control rats (I) and rats infected with 25 (II), 50 (III) and 100 (IV) third-stage larvae of *A. cantonensis*

GROUP	WEEKS AFTER INFECTION									
	0	1	2	3	4	5	6	8	10	12
I	0/5*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
II	0/5	0/5	0/4	3/4	4/4	4/4	4/4	4/4	4/4	3/3
III	0/5	3/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
IV	0/5	3/5	4/5	5/5	5/5	4/4	4/4	4/4	4/4	3/3

\* No. of positive sera/No. of sera examined

positive 1 week after infection. But 4 false negative reactions were found 4 weeks after infection. Eighteen immunized guinea pigs and 3 *A. costaricensis*-infected rats were all strongly positive. Positive reaction was observed in 4 of the 7 rats transferred with male and female worms.

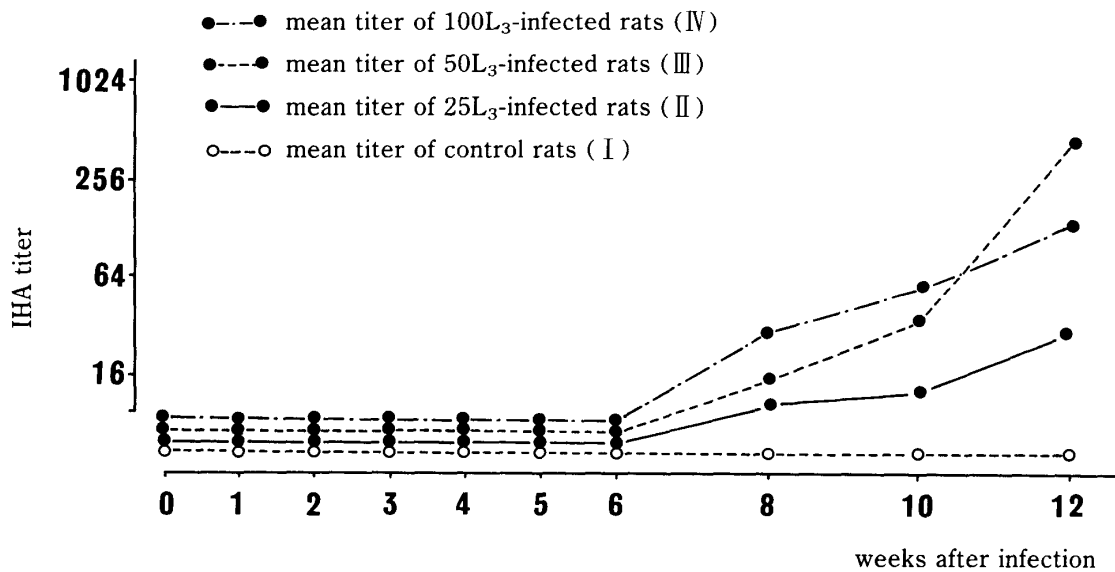


FIGURE 1 Time course of mean IHA titer of control rats and rats infected with 25, 50 and 100 third-stage larvae of *A. cantonensis*

TABLE 4 Results of the double diffusion of control rats (I) and rats infected with 25 (II), 50 (III) and 100 (IV) third-stage larvae of *A. cantonensis*

GROUP	WEEKS AFTER INFECTION										
	0	1	2	3	4	5	6	8	10	12	
I	0/0*	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	
II	0/5	3/5	2/4	2/4	1/4	2/4	2/4	3/4	2/4	3/3	
III	0/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	
IV	0/5	4/5	4/5	4/5	4/5	4/4	4/4	4/4	4/4	3/3	

\* No. of positive sera/No. of sera examined

## DISCUSSION

In the present investigation, the precipitate formation around the eggs and the worms was observed in the sera from the infected or the hyperimmunized animals. These reactions were found both in the unheated sera and in the sera heated at 56°C for 30 min. By fluorescent antibody, it was confirmed that the precipitates incorporated with immunoglobulins. Thus, the precipitate formation is considered to be an antigen-antibody reaction, which does not need the complement.

Positive COP reaction was found in the rat sera 1 week after infection, although the parasite did not begin to oviposit. And the rats transferred with only male worms showed positive reaction. These results suggested that oviposition of the parasite is not responsible for COP reaction, and that common antigens are shared among eggs, the 3rd- and 4th-stage larvae and male and female worms of *A. cantonensis*. However, in schistosomiasis there are many reports which support that specific anti-egg antibody is greatly responsible for COP reaction.<sup>1,2,17)</sup>

The fact that the precipitate formation was observed only around the damaged eggs but not in the intact ones, seems to indicate that the antigens responsible for COP reaction of *A. cantonensis* are released through the defective part of the damaged egg shell. A similar feature was illustrated in the COP with *Schistosoma japonicum* egg by electron microscopy.<sup>20)</sup>

In COP reaction as well as DD, the *A. costaricensis*-infected rats reacted stronger than the *A. cantonensis*-infected rats. These results may be related to the observation that many eggs were found in the mesentery lymph node of mice infected with *A. costaricensis* (unpublished data).

CHEN & SUZUKI (1974) reported that adult and the 3rd-stage larvae gave higher titers in the fluorescence antibody test than the 1st-stage larvae. Similar results were obtained in the present study, i. e., there was precipitate formation on the 3rd-stage larvae and immature adult but no precipitate formation on the living 1st-stage larvae.

In the precipitates formed on immature adults in the sera from rats 1 week after infection, in which the parasite developed to the 4th-stage larvae. And even in the sera from the rats transferred with the immature adult worms, precipitates were formed on the 3rd- and 4th-stage larvae. Thus, the stage specificity was not observed in the CLP reaction of *A. cantonensis*. It was also regarded that this reaction was not sex and organ specific, because even in the sera from single sex worm transferred rats and the rats 1 week after infection, precipitates were formed at both of the cloacal opening and vulva of immature adult.

OLIVER-GONZALES (1945) reported that rabbits produced two types of stage specific antibodies to *Trichinella spiralis* infection. However, CHUTE (1956) did not obtain similar results with *T. spiralis* infected rats and rabbits, and thus suggested that the

two types of antibodies reflected not qualitative, but quantitative differences of antigens in different stages.

Precipitate formation at the oral opening and excretory pore of the 3rd- and 4th-stage larvae suggested that antibodies were produced against antigens secreted and excreted from these portions of the parasites during a period of larval migration.

In IHA, *A. cantonensis*-infected rats were negative until 6 weeks after infection. KAMIYA & TANAKA (1969) and YOSHIMURA et al. (1976) indicated that an increase in the IHA titre was seen from 4–5 weeks after infection. This disparity may be due to the antigen concentration applied to red blood cells for sensitization and to the freezing of antigen-sensitized cells.

In DD, positive reaction was seen from 1 week after infection. Similar results were reported by KAMIYA & KANDA (1974) and YOSHIMURA et al. (1976). On the other hand, CHEN (1974) reported that counterelectrophoresis and DD gave positive reactions from 2 and 3 weeks after infection, respectively. This disparity may be derived from the difference in the amount of added antigen. In this investigation, false positive reaction was seen in normal rat serum. Similar observations on guinea pigs were reported.<sup>26)</sup>

Compared with results of the IHA and DD, the feasibility of the COP and CLP reactions was noted for the immunodiagnosis of angiostrongyliasis.

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## EXPLANATION OF PHOTOGRAPHS

## PLATE I

Precipitate formation on degenerated eggs and 1st-stage larva of *A. cantonensis*

Photo 1 Normal intact egg. Scale : 20  $\mu$

Photos 2-4 Weak circumoval precipitation reaction ; Precipitate (arrow) on eggs which had been incubated in the serum from rat infected with *A. cantonensis*. Scale : 20  $\mu$

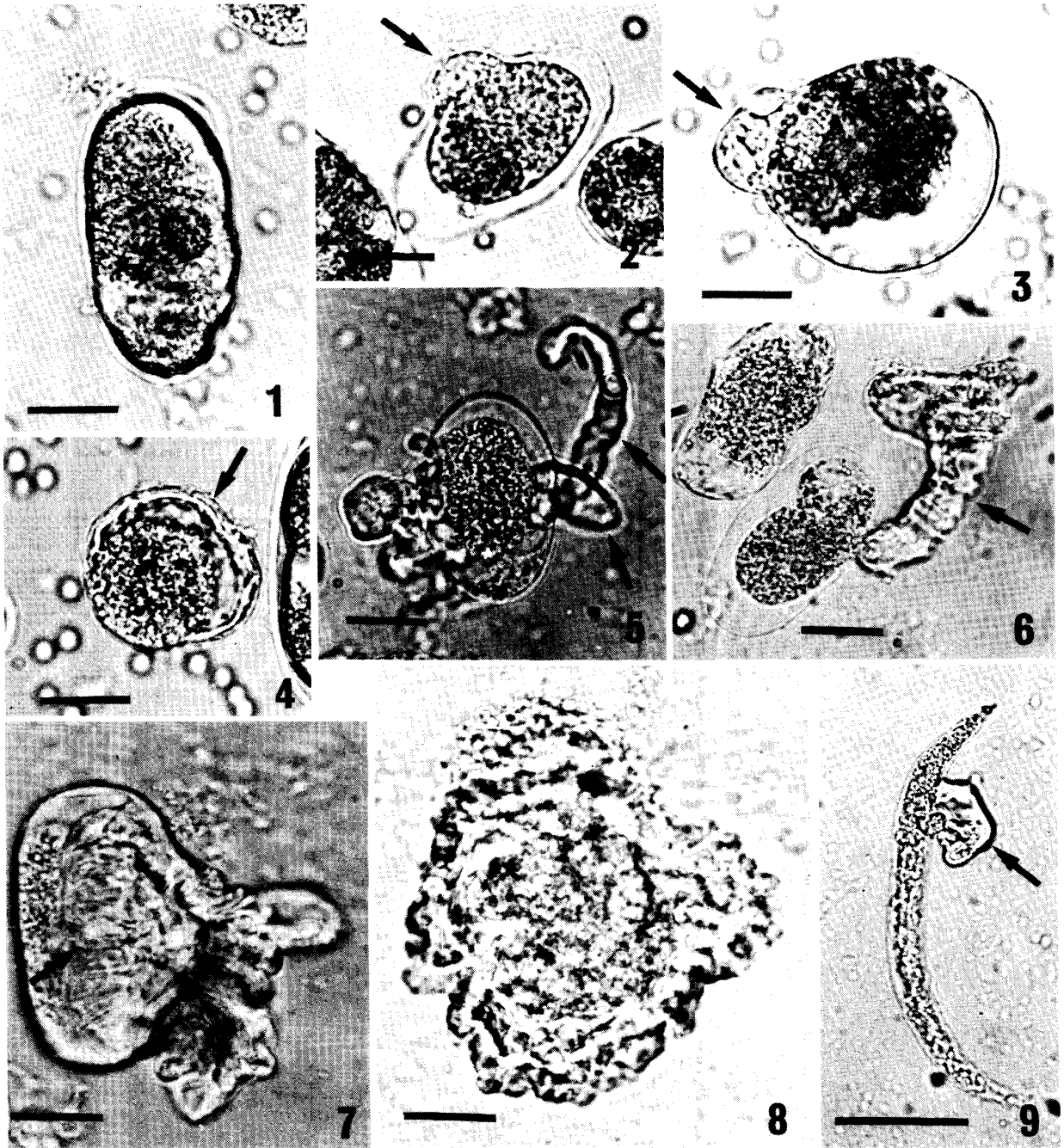
Photo 5 Filamentary precipitates (arrow) on an egg which had been incubated in the serum from guinea pig immunized with homogenate of *A. cantonensis*. Scale : 20  $\mu$

Photo 6 Band-form precipitate (arrow) on an egg which had been incubated in the immunized guinea pig serum. Scale : 20  $\mu$

Photo 7 Precipitate (arrow) in an egg which had been incubated in the immunized guinea pig serum. Scale : 20  $\mu$

Photo 8 Strong circumoval precipitation reaction : A large mass of precipitates surrounding an egg which had been incubated in the immunized guinea pig serum. Scale : 20  $\mu$

Photo 9 Precipitate (arrow) on a degenerated 1st-stage larva of *A. cantonensis* which had been incubated in the immunized guinea pig serum. Scale : 30  $\mu$



## PLATE II

Precipitate formation on various stage worms of *A. cantonensis* which had been incubated in the sera from rat infected with *A. cantonensis*

Photo 10 Precipitate (arrow) on the excretory pore of a living 3rd-stage larva. Scale : 30  $\mu$

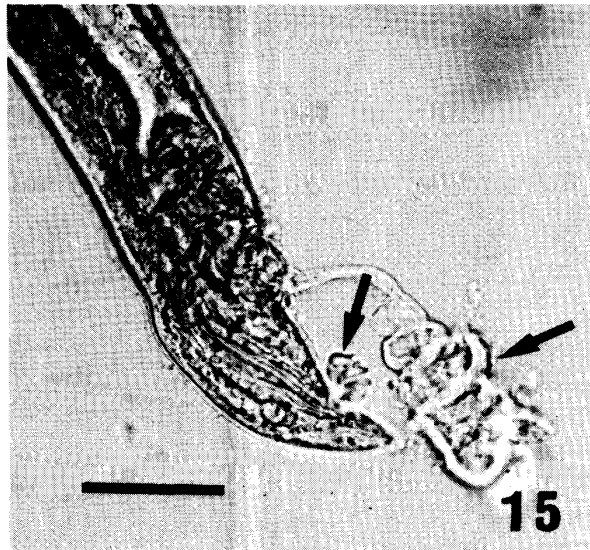
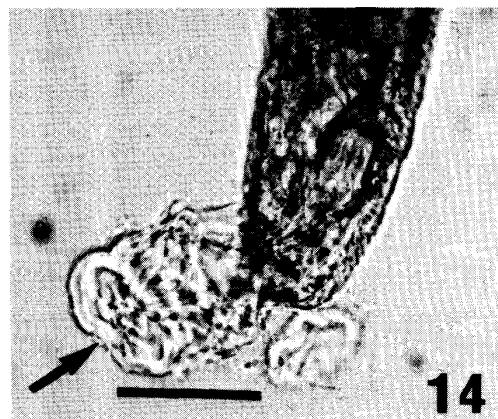
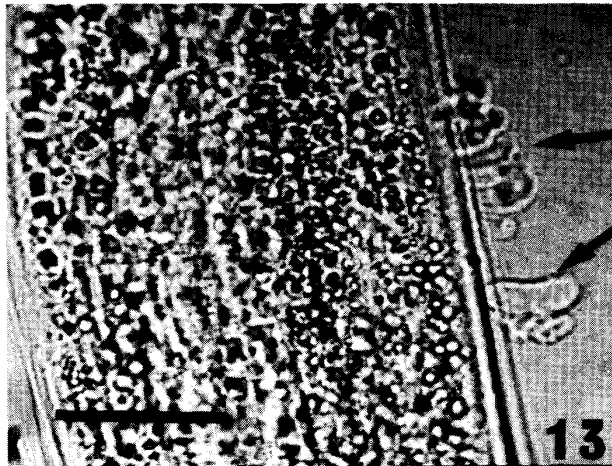
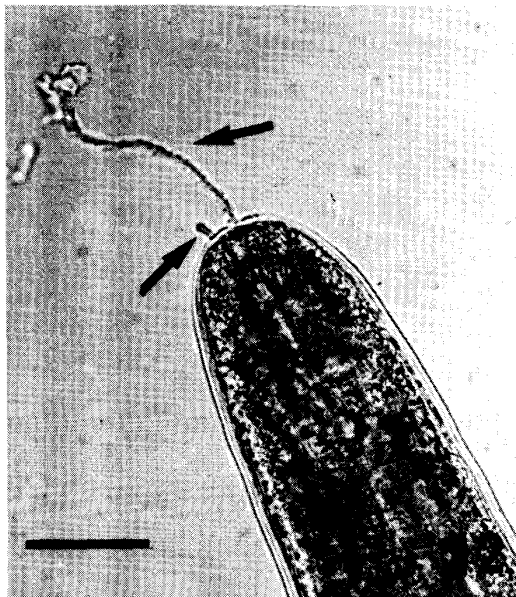
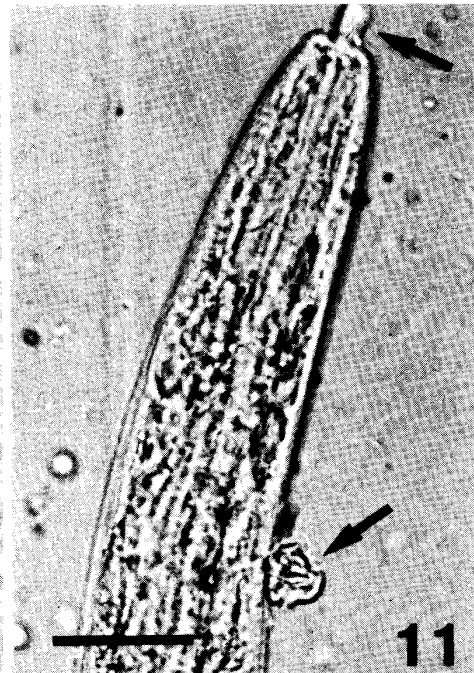
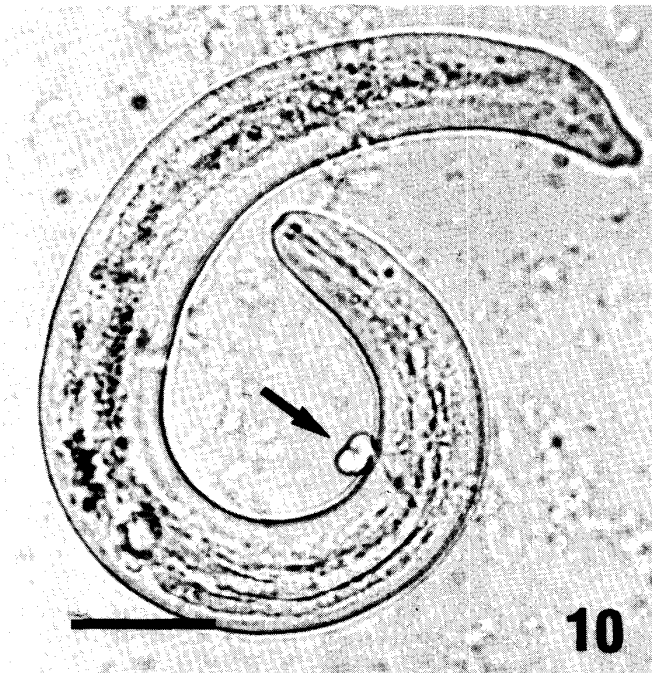
Photo 11 Precipitates (arrow) on the oral opening and excretory pore of a living 4th-stage larva. Scale : 30  $\mu$

Photo 12 Precipitates (arrow) on the amphid (?) and oral opening of a living immature adult. Scale : 60  $\mu$

Photo 13 Precipitates (arrow) on the surface of a living immature adult. Scale : 60  $\mu$

Photo 14 Clot of precipitate (arrow) on the cloacal opening of a male immature adult. Scale : 120  $\mu$

Photo 15 Precipitates (arrow) on the vulva and anus of a female immature adult. Scale : 120  $\mu$



## PLATE III

Photos 16 and 17 Fluorescent precipitates (arrow) on an egg which had been incubated in the serum from the rat infected with *A. costaricensis*, and then incubated with FITC labeled anti-rat IgG serum ; photographed in white light (16) and in near-ultraviolet light (17)  
Scale : 40  $\mu$

Photos 18 and 19 Fluorescent precipitate (arrow) on the cloacal opening of male immature worm which had been incubated in the serum from a rat infected with *A. cantonensis*, and then incubated with FITC labeled anti-rat IgG serum ; photographed in white light (18), and in near-ultraviolet light (19)  
Scale : 300  $\mu$

