

The Occurrence of *Angiostrongylus cantonensis* in Toads and Frogs in Okinawa Prefecture, Japan

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Introduction

Since Rosen *et al.* (1967) reported *Angiostrongylus cantonensis*, a metastrongylid lung-worm of rodents, as the causative agent of human eosinophilic meningitis or meningoencephalitis in the Pacific Islands, a number of reports have been published concerning the distribution and incidence of the parasite in its definitive and intermediate hosts. In these publications, a wide variety of molluscan species were reported as the intermediate hosts of the parasite. In addition to these high incidences of the parasite in molluscan species, the infective-stage larvae of *A. cantonensis* have been reported in nature from such paratenic hosts as land planarians (Alicata, 1962), freshwater shrimp (Alicata and Brown, 1962; Rosen *et al.*, 1967) land crabs (Alicata, 1964a; 1965) and frogs (Ash, 1968).

In our country, 12 suspected cases of human angiostrongyliasis have been known in Okinawa prefecture since 1969 (reviewed by Otsuru, 1977; Asato *et al.*, 1977) and two of them were quite interesting cases because the fresh liver of toads orally administered as a medicine was suspected of the source of the infections (Kinjo *et al.*, 1975).

The authors report the occurrence of *A. cantonensis* larvae in some species of toads and frogs collected in Okinawa prefecture and the results from the experimental infection with infective *A. cantonensis* larvae in frogs collected in Niigata prefecture where no *A. cantonensis* has been reported.

Materials and Methods

1) Natural infection with *A. cantonensis* larvae in toads and frogs:

A total of 203 toads and frogs consisting of eight species (107 *Bufo asiaticus*, 26 *Bufo marinus*, 44 *Rana catesbeiana*, 8 *Rana limn-charis*, 4 *Rhacophorus leucomystax*, 8 *Rhacophorus japonicus*, 5 *Rhacophorus viridis* and 1 *Microhyla ornata*) were collected in five areas of Okinawa prefecture and examined for natural infections with *A. cantonensis* larvae. The organs examined were as follows: (1) stomach wall, (2) intestinal wall with mesentery, (3) liver, (4) muscle and (5) heart and lung. After peptic digestion of tissue for 2 to 3 hours at 37 C using an aqueous solution of 0.6% of 1:10,000 pepsin and 0.8% HCl, the digested material was filtered through a stainless-steel sieve, the filtrates were allowed to sediment and the sediments thus formed were examined microscopically for the 3rd-stage larvae of *A. cantonensis*.

2) Experimental infection with 3rd-stage

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larvae of *A. cantonensis* in frogs :

Seven of adult *R. catesbeiana*, collected in Niigata prefecture where on *A. cantonensis* has been reported, were used. The strain of *A. cantonensis* from Taiwan has been maintained in our laboratory since 1972 by passage in aquatic snails, *Biomphalaria glabrata*, and albino rats, *Rattus norvegicus*. The 3rd-stage larvae were recovered from the experimentally infected snails by the peptic digestion and 700 of these larvae were given orally to each frog. The frogs were maintained at 25 ± 2 C throughout the experiment, and examined for 3rd-stage larvae in the organs stated previously at 3, 7, 14, 20, 45, 58 and 70 days after the administration in the same manner as mentioned above.

3) Morphological and histological examinations :

The morphological features of the larvae recovered from naturally or experimentally infected toads and frogs were observed microscopically and their measurements were compared with those of the 3rd-stage larvae from *Biomphalaria glabrata*. The microscopic measurement was done with 20 living larvae after fixation with 70% methanol.

Five toads, *B. asiaticus*, collected in Miyako-Island were sacrificed and the organs, such as liver, intestine and muscle of leg, were fixed in a 10% formalin solution. After dehydration in ethanol series, small pieces of the organs were embedded in para-

ffin, sectioned and stained with hematoxylin and eosin. *A. cantonensis* larvae in the tissues were observed microscopically.

4) Infection of rats with the larvae recovered from toads and frogs :

In order to determine whether the larvae recovered from toads and frogs possess their infectivity to rat, the 78, 52 and 59 larvae from *B. asiaticus* in Miyako-Island and 50 larvae from each of experimentally infected *R. catesbeiana* were fed to 10 albino rats respectively. The pulmonary arteries of these rats were examined for adult worms 50 days after the infection. The adult worms obtained from the rats were also used for further identification on their morphological features.

Results

1) Incidence of *A. cantonensis* 3rd-stage larvae in toads and frogs in Okinawa prefecture :

The results obtained from a total of 203 toads and frogs collected in Okinawa prefecture were summarized in Table 1. The larvae were found in 35 of 83 toads (*B. asiaticus*) collected in Miyako-Island and one of 10 in Kitadaito-Island. Seven of 44 *R. catesbeiana* in Kume-Island, 1 of 8 *R. limnocharis* in Okinawa-Island and 1 of 4 *R. leucomystax* in Okinawa-Island were also positive. No larva, however, was detected in all toads (14 *B. asiaticus* and 26 *B. marinus*)

Table 1 Incidence of *A. cantonensis* larvae in toads and frogs collected in Okinawa prefecture

Species	Locality	No. examined	No. positive	Positive rate(%)
<i>Bufo asiaticus</i>	Miyako-Island	83	35	42.2
	Kitadaito-Island	10	1	10.0
	Minamidaito-Island	14	0	0
<i>Bufo marinus</i>	Minamidaito-Island	26	0	0
<i>Rana catesbeiana</i>	Kume-Island	44	7	15.9
<i>Rana limnocharis</i>	Okinawa-Island	8	1	12.5
<i>Rhacophorus leucomystax</i>	Okinawa-Island	4	1	25.0
<i>Rhacophorus japonicus</i>	Okinawa-Island	8	0	0
<i>Rhacophorus viridis</i>	Okinawa-Island	5	0	0
<i>Microhyla ornata</i>	Okinawa-Island	1	0	0

Table 2 Numbers of *A. cantonensis* 3rd-stage larvae found in the organs of toads and frogs collected in Okinawa prefecture

Species	Locality	Stomach	Intestine with mesentery	Liver	Muscle	Heart & lung	Total		
<i>Bufo asiaticus</i>	Miyako-Island	126	79	0	N.D.	0	205		
		288	248	125	N.D.	0	661		
		340	158	290	N.D.	0	788		
		30	47	0	N.D.	0	77		
		30	40	0	N.D.	0	70		
		0	58	0	N.D.	0	58		
		40	30	0	N.D.	0	70		
		0	0	59	N.D.	0	59		
		30	0	0	N.D.	0	30		
		40	44	0	N.D.	0	84		
		250	155	170	N.D.	0	422		
		650	504	380	N.D.	0	1,534		
		20	43	0	N.D.	0	64		
		0	48	0	N.D.	0	48		
		1,728	0	0	N.D.	0	1,728		
		250	0	0	N.D.	0	250		
		264	0	0	N.D.	0	264		
		313	0	14	N.D.	0	327		
		84	0	0	N.D.	0	84		
		113	0	0	N.D.	0	113		
		10	0	0	N.D.	0	10		
		15	0	0	N.D.	0	15		
		292	66	13	N.D.	0	371		
		181	65	0	N.D.	0	246		
		1,802	690	764	N.D.	0	3,256		
			(90)			78	0	N.D.	168
			(70)			83	63	N.D.	215
			(150)			98	0	N.D.	248
			(407)			127	466	N.D.	1,000
			(34)			94	162	N.D.	264
			(76)			0	250	N.D.	326
			(217)			0	0	N.D.	217
	(0)			0	47	N.D.	47		
	(14)			0	32	N.D.	46		
	(25)			11	0	N.D.	36		
<i>Rana catesbeiana</i>	Kitadaito-Island	0	0	15	N.D.	0	15		
	Kume-Island	25	0	0	N.D.	0	25		
		18	0	0	N.D.	0	18		
		30	0	0	N.D.	0	30		
		(76)			0	0	N.D.	76	
		(43)			0	0	N.D.	43	
		(24)			0	0	N.D.	24	
	(0)			0	15	N.D.	15		
<i>Rana limnocharis</i>	Okinawa-Island		(562)*		110	N.D.	672		
<i>Rhacophorus leucomystax</i>	Okinawa-Island	0	83	0	N.D.	0	83		

Figures in parentheses and those in parentheses with asterisk indicate a total number of larvae from stomach, intestine with mesentery and that from stomach, intestine and liver respectively. N.D.: not done

Table 3 Numbers of *A. cantonensis* 3rd-stage larvae found in the organs of frogs (*R. catesbeiana*) experimentally infected with the 3rd-stage larvae of *A. cantonensis*

Days after infection	No. of the larvae recovered from :					Total (Recovery rate)
	stomach wall	intestinal wall with mesentery	liver	kidney	heart & lung	
3	172	111	13	75	13	384(54.9)
7	50	23	15	40	0	128(18.3)
14	73	42	73	26	0	214(30.6)
20	86	29	54	14	0	183(26.1)
45	31	32	28	15	0	106(15.1)
58	38	46	51	21	0	156(22.3)
70	27	33	28	7	0	95(13.6)

collected in Minamidaito-Island, and in 5 *R. viridis*, 8 *R. japonicus* and 1 *M. ornata* in Okinawa-Island.

Numbers of the larvae including dead larvae in the digests recovered from above 45 naturally infected toads and frogs were summarized in Table 2. The larvae were most frequently found in the stomach wall and intestinal wall with mesentery, and some were in liver and muscle. However, no larva was detected in heart and lung in all animals. The number of the larvae recovered from one toad, *B. asiaticus*, in Miyako-Island was maximal among examined animals, so many as 3,256 in total.

2) Experimental infection of frogs with *A. cantonensis* 3rd-stage larvae :

As a control, 12 *R. catesbeiana* collected in Niigata prefecture were examined for natural infection with *A. cantonensis* and no larva regarded as *A. cantonensis* was detected in all frogs. From other 7 frogs experimentally infected with 3rd-stage larvae of *A. cantonensis*, the larvae were found in all organs except heart and lung during the period from 3 to 70 days after the administration. The recoveries of the larvae from these frogs were shown in Table 3. More than half of the larvae were found in stomach and intestinal walls with mesentery. The total recovery showed a tendency to decrease as increasing time after the administration. Among these larvae, the rates of living larvae were 90.0% at 3 days, 92.1% at 7 days, 69.1% at 14 days, 73.7% at 20

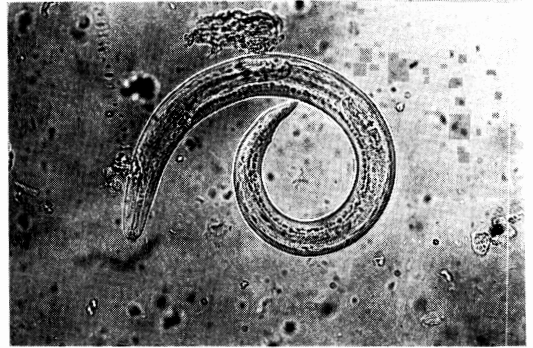


Fig. 1 A live *A. cantonensis* 3rd-stage larva from liver of a toad (*B. asiaticus*) collected in Miyako-Island.

days, 69.8% at 45 days, 65.3% at 58 days and 70.5% at 70 days.

3) Morphological and histological observations :

The morphological features of the larvae obtained from the naturally or experimentally infected toads and frogs, as seen in Fig. 1, were consistent with those of *A. cantonensis* 3rd-stage larvae with cone-shaped tail. It was also determined from the microscopic measurements that the larvae did not differ from the 3rd-stage larvae in *Biomphalaria glabrata*. This result was shown in Table 4.

In histological observations, many oval capsules surrounded with fibrous cells were observed in the walls of stomach and intestine, and each capsule usually contained one or two larvae (Fig. 2, A). Infiltration of cells around these capsules were not so re-

Table 4 A comparison of the size of the larvae recovered from snails, toads and frogs

Larvae recovered from :	<i>Biomphalaria glabrata</i>	<i>Bufo asiaticus</i>	<i>Rana catesbeiana</i>	
			(20 days after infection)	(70 days after infection)
Body length	0.467 (0.438~0.485)	0.462 (0.440~0.497)	0.459 (0.447~0.485)	0.473 (0.453~0.487)
Body width	0.028 (0.023~0.031)	0.028 (0.025~0.030)	0.028 (0.024~0.034)	0.029 (0.025~0.036)
Esophagus length	0.183 (0.176~0.187)	0.185 (0.175~0.195)	0.183 (0.176~0.194)	0.188 (0.179~0.191)
Length from tail tip to anus	0.038 (0.034~0.040)	0.039 (0.035~0.042)	0.038 (0.036~0.040)	0.040 (0.037~0.043)

Biomphalaria glabrata : experimentally infected with 1st-stage larvae, *Bufo asiaticus* : naturally infected in Miyako-Island, *Rana catesbeiana* : experimentally infected with the 3rd-stage larvae. The measurements in millimeter are means of 20 larvae and the ranges are in parentheses.

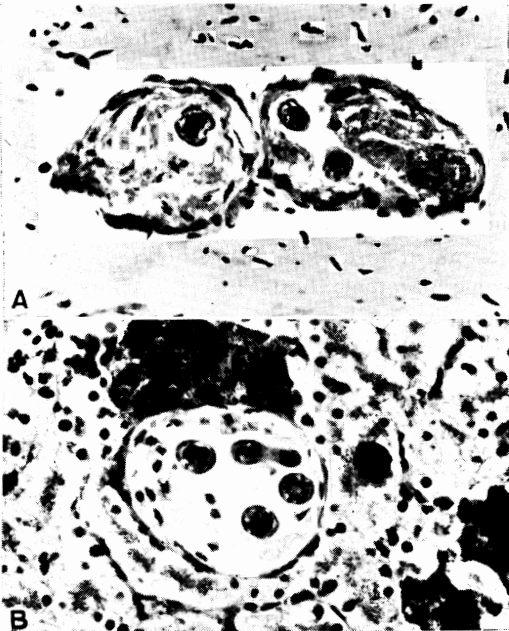


Fig. 2 The larvae of *A. cantonensis* surrounded with fibrous cells in the wall of the stomach (A) and liver (B) of an infected toad (*B. asiaticus*).

markable. The capsules identical to those above mentioned were also randomly distributed in the liver parenchyma (Fig. 2, B) and muscle tissues of legs. In liver parenchyma, the capsules were often surrounded with many pigment cells.

4) Infection of rats with the larvae recovered from toads and frogs :

When three rats infected with 78, 52 and 59 larvae from naturally infected toads (*B. asiaticus*) were examined, 36, 23 and 28 adult worms were found in their pulmonary arteries and the recovery rates were 46.1%, 44.2% and 47.4% respectively. On the other hand, the recoveries from seven rats infected with 50 larvae recovered from experimentally infected frogs at 3, 7, 14, 20, 45, 58 and 70 days after the infection were 42 (84%), 38 (76%), 34 (68%), 12 (24%), 27 (54%), 22 (44%) and 18 (36%) respectively.

These adult worms showed morphological features of *A. cantonensis* adult worms. For example, their blood-filled intestine was straight and ran parallel with the genital tube in male adult, and it was spirally wound with milky-white uterus in a "barber's pole" pattern in female adult.

Discussion

A. cantonensis larvae have been found in some vertebrate and invertebrate paratenic hosts in nature (Alicata, 1962; Alicata and Brown, 1962; Alicata, 1964a; Rosen *et al.*, 1967; Ash, 1968), some of which have been shown to be epidemiologically significant as a source of infection to man (Rosen *et al.*, 1967). In addition to these natural infections, it has been shown experimentally that the larvae can survive also in freshwater shrimps and crabs (Wallace and Rosen, 1966).

Although fishes have not been found infected in nature, it has been demonstrated experimentally that the larvae can migrate to the musculature of a freshwater cichlid fish, *Tilapia mossambica* and a carangid marine fish, *Trachuroops crumenophthalmus*, and live in the muscles at least 4 weeks following infection (Wallace and Rosen, 1967). Ash (1968) also showed that a sea snake, *Laticauda colugrina*, was a paratenic host. Alicata (1963) failed to recover *A. cantonensis* larvae from chicks given 1,000 larvae 15 days previously, but he could demonstrate that pigs and calves were poor paratenic hosts of the parasite (Alicata, 1964b).

In the present study, *A. cantonensis* 3rd-stage larvae infective to rat were found in 45 out of 203 toads and frogs collected in Okinawa prefecture. These larvae were most frequently found in stomach and intestinal walls with mesentery and some were in liver and muscle. In histological examinations on five toads, *B. asiaticus*, the larvae were observed in small capsules surrounded with fibrous cells in the tissues. These results were similar to those obtained by Ash (1968) as a result of the study on frogs (*Hyla aurea*) in New Caledonia.

In experimental infection to the frogs, *R. catesbeiana*, the larvae persisted in stomach and intestinal walls with mesentery, liver and kidney of the administered frogs for at least 70 days. The morphological features and microscopic measurements of the larvae did not differ from those of the 3rd-stage larvae of *A. cantonensis* and these larvae also possessed infectivity to rat. The recovery of living larvae and their infectivities, however, showed a tendency to decrease as the time after the infection to the frogs. It was considered from the results that the toads and frogs were paratenic hosts for the parasites. These toads and frogs became probably contaminated to the parasites when they ingested slugs and snails as a food, because some indigested slugs and shell of snails were often observed in their stomach and it is well known that the mollusks are highly infected with the parasites in Okinawa prefecture.

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The finding of larvae in wild-caught toads and frogs has a significance since in two cases of eosinophilic meningoencephalitis in Okinawa prefecture the swallowing of fresh liver of toads (*B. asiaticus*) as a medicine appeared to be a possible source of the infections (Kinjo *et al.*, 1975). Among a part of people in some Oriental regions, the swallowing of living mollusks and fresh liver of toads are believed to be effective for a fever and cough and also for general health. In Okinawa prefecture, the swallowing of living slugs or fresh liver of the toads as a medicine were suspected as a source of the infection in five out of 12 cases (reviewed by Otsuru, 1977). Such a action as wilful ingestion of living mollusks or liver of toads for a therapeutic purpose is an important mode of human infection in this area as well as the habit of eating mollusks as a food in Southeast Asia, and more animals, especially those which will be ingested by man for various purposes, should be widely examined to elucidate whether they can become paratenic hosts for *A. cantonensis* or not.

Conclusion

A total of 203 toads and frogs consisting of eight species collected in Okinawa prefecture were examined for natural infection with *Angiostrongylus cantonensis* larvae. Thirty-six of 93 *Bufo asiaticus* collected in Miyako- and Kitadaito-Island, 7 of 44 *Rana catesbeiana* in Kume-Island, 1 of 8 *Rana limnocharis* in Okinawa-Island and 1 of 4 *Rhacophorus leucomystax* in Okinawa-Island were found to harbor the larvae of *A. cantonensis* in their organs such as stomach and intestinal walls with mesentery, liver and muscle. However, no larva was detected in 14 *Bufo asiaticus* and 26 *Bufo marinus* in Minamidaito-Island, and 5 *Rhacophorus viridis*, 8 *Rhacophorus japonicus* and 1 *Microhyla ornata* in Okinawa-Island.

Frogs (*Rana catesbeiana*) in Niigata prefecture, the nonendemic area of *A. can-*

tonensis, were experimentally infected with the 3rd-stage larvae isolated from snails, and in the frogs, the larvae survived over 10 weeks after infection.

The larvae of *A. cantonensis* recovered from these naturally or experimentally infected amphibians were 3rd-stage and they had infectivity to rats.

From these results, the toads and frogs are paratenic hosts for *A. cantonensis* and it has an epidemiological significance since in two suspected cases of angiostrongyliasis in Okinawa prefecture the swallowing of fresh liver of the infected toads as a medicine appeared to be a possible source of the infections.

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沖縄県カエル類の広東住血線虫寄生状況

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沖縄県で採集した8種のカエル類、合計203個体について、広東住血線虫幼虫の寄生状況を調査した。その結果、宮古島と北大東島で採集したアジアヒキガエル (*Bufo asiaticus*) 93個体中36個体に本線虫第3期幼虫の寄生を認めたほか、久米島のウシガエル (*Rana catesbeiana*) 44個体中7個体、沖縄本島のヌマガエル (*Rana limnocharis*) 8個体中1個体、シロアゴガエル (*Rhacophorus leucomystax*) 4個体中1個体にも寄生がみられた。他方、南大東島のアジアヒキガエル14個体、オオヒキガエル (*Bufo marinus*) 26個体、沖縄本島のアオガエル (*Rhacophorus viridis*) 5個体、ニホンカジガエル (*Rhacophorus japonicus*) 8個体、ヒ

メアマガエル (*Microhyla ornata*) 1個体からは幼虫は検出されなかった。幼虫の寄生は消化管壁、肝臓、筋肉中にみられ、これらの幼虫およびこれをラットに感染させて得た成虫の形態から、本幼虫は広東住血線虫第3期幼虫であると同定した。

新潟県で採集したウシガエルに第3期幼虫を実験的に感染させたところ、幼虫は第3期のままで10週間にわたってカエル体内で生存していた。

以上の結果より、カエル類は本線虫の待機宿主であり、すでに沖縄県ではアジアヒキガエルの肝臓生食が原因と考えられる人体症例2例が発生していることから、疫学上重要視されなければならない。