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Experimental Infection of Rainbow Trout (*Oncorhynchus mykiss*) by *Anisakis simplex* (Nematoda: Anisakidae)

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Abstract

Experimental intraperitoneal infection of rainbow trout (*Oncorhynchus mykiss*) with third-stage larvae of *Anisakis simplex* was more successful (74% recovery rate) than oral infection (9% recovery rate). In both cases, most larvae were located on the pyloric caeca by 55 days postinfection. Larval encapsulation was completed within about 40 days. Encapsulated larvae obtained from experimentally infected fish and cultured *in vitro* displayed a similar capacity for ecdysis to those obtained from a natural host (blue whiting, *Micromesistius poutassou*). These results indicate that intraperitoneal infection of rainbow trout with *A. simplex* may be a useful experimental model for studies of this nematode.

Key words: *Anisakis simplex*, rainbow trout, intraperitoneal infection

Introduction

Much has been written about *Anisakis* in recent years following recognition of this nematode as pathogenic in man (Ishikura and Namiki, 1989; Ishikura and Kikuchi, 1990). The life-cycle of *Anisakis* is entirely marine: adults infect marine mammals, white crustaceans (euphausiids) are the intermediate hosts and a large number of marine fish species act as paratenic hosts (Smith and Wootten, 1978). Despite numerous works, there are no reliable animal models for long-term maintenance of this parasite in the laboratory. Like man, the common laboratory animals (mice, rats, rabbits, etc.) are merely accidental hosts of this parasite: in such animals, the normal pattern of events is for the larva to die a few days postinfection, with subsequent granuloma formation around it. In mice, for example, most larvae are viable at day 14 postinfection; by day 21, however, the larvae die and the infected tissue is invaded by inflammatory cells (Jones *et al.*, 1990; Iglesias *et al.*, 1993).

Although *Anisakis* is usually restricted to the marine environment, there have been reports of infection of euryhaline fish such as migratory salmonids, which spend part of their adult life in the sea and presumably acquire the larvae during this time (Hoffman, 1967). In addition, *Anisakis* larvae have also been reported from the brown and rainbow trouts, a strictly freshwater species (Kane, 1966; Wootten and Smith, 1975) and previous studies have suggested that the trout may be a useful laboratory model of paratenic hosts of *Anisakis*. Rainbow trout (*Oncorhynchus mykiss*), because of its importance as a source of human food, is one of the most intensively studied fish species. In the work reported here, we infected rainbow trout with third-stage larvae of *Anisakis simplex*, with the aim of developing a new experimental model for studies of this nematode.

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Materials and Methods

Parasite. Third-stage larvae (L_3) of *Anisakis simplex* (identified as per Smith, 1983) were extracted manually from the viscera and body cavity of locally purchased blue whiting (*Micromesistius poutassou*). Capsules were removed by dissection with fine needles. Following extraction, larvae were kept in physiological saline solution (PSS). Prior to assay, all larvae were carefully examined under a stereomicroscope; all individuals showing any form

of damage or alteration of the cuticle were discarded, and only apparently healthy larvae were used for experiments (following storage in PSS at 4C).

Trout. Tests were carried out on rainbow trout *Oncorhynchus mykiss* (15–20 cm body length) from a local fish farm (Piscifactorías Coruñesas, Carballo, La Coruña, Spain). Prior to the experiment, the fish were acclimatized for at least 36 h in 10 l plastic tanks (Letica, Barcelona, Spain) with a constant flow of water (15±3C, pH 6.5±0.5) from a spring close to the laboratory. Oxygen was bubbled through the water. In the fish farm, the fish had been fed only on dried pelleted food; the same food was supplied daily in the laboratory.

Anaesthesia. Trout were anaesthetized with tricaine methane sulphonate (MS222, Sandoz) at 50 mg/l.

Experimental infection. Three methods of infection were tested: orogastric, oral and intraperitoneal.

Orogastric infection. Ten trout were maintained without food for 48 h; following anaesthesia, five larvae were then introduced into the stomach of each fish using a polythene tube attached to a pipette. The trout were allowed to recover from the anaesthesia in fresh water and observed for 15 min.

Oral infection. After the trout had fasted for 48 h, 30 larvae per fish were added to the tanks. The trout were observed for 30 min, during which all larvae were ingested.

Intraperitoneal infection. Larvae were first sorted into groups of five in Petri dishes containing PSS, then disinfected by placing in 2% glacial acetic acid for 30 sec (Poggensee *et al.*, 1989), then rinsed in PSS and transferred manually to a 2 ml syringe containing 1 ml of PSS. Throughout this procedure all media were maintained at 30±5C; at this temperature the larvae are active and do not tend to aggregate, thus facilitating handling and subsequent injection. Following anaesthesia, each trout received 10 larvae (two injections, each of five larvae) by injection into the abdominal region just anterior to the pelvic fins, inserting the needle to a depth of about 1 cm in posteroanterior direction, parallel to the longitudinal axis of the body so as not to affect internal organs. Note that the syringe must be visually inspected just prior to injection, to ensure that the larvae are not aggregated; otherwise the needle may become blocked.

Evaluation of the infection methods. Ten orally and ten intraperitoneally infected trout were killed 55 days postinfection (p.i.). The viscera or each fish were removed and examined immediately, and the number and location of all parasites were recorded. In paratenic hosts, larvae only infrequently locate in muscle tissues, and the trout musculature was therefore not examined in detail.

Following extraction, larvae were placed in PSS and examined under a stereomicroscope in order to select ten individuals not showing any internal damage or alteration of the cuticle. These larvae were then washed several times in PSS, briefly immersed in 2% acetic acid to prevent bacterial contamination and cultured for determination of viability. Viability was also determined in a control group of larvae obtained from a natural infection of blue whiting (*Micromesistius poutassou*).

Time-course for encapsulation. An additional test was carried out in which trout which had been intraperitoneally infected with five larvae per fish were killed at intervals between 1 and 50 days p.i. The viscera were removed and all larvae present were examined and the stage of encapsulation noted.

In vitro culture. The culture medium was 10% EBBS (Gibco) in 0.1 M glycine HCl buffer (pH 2.5). Assays were carried out in a culture plate with 5 ml wells, with five larvae per well. The cultures were maintained at 37C for seven days. Larvae were examined under a stereomicroscope on days 4, 5, 6 and 7 of culture, and developmental stage (third-stage, ecdysial or fourth-stage) and viability (dead, damaged or apparently healthy) was determined as previously described (Tojo *et al.*, 1992).

Results

The effectiveness of the infection methods tested varied. Orogastric infection proved ineffective, since large numbers of larvae were regurgitated within a few minutes of infection.

Following both oral and intraperitoneal infection, all larvae recovered 55 days p.i. were alive and fully encapsulated. Following oral infection, however, only half of the trout were infected by day 55, and the overall percentage recovery was only 9%: infection intensity was minimal in three trout (1

larva/trout) but high in two trout (13 and 10 larvae/trout). Intraperitoneal infection was more successful: as expected, all trout were infected 55 days p.i. The recovery rate was 74% and infection intensity was relatively uniform (Table 1).

Table 1 Number of *Anisakis simplex* L_3 recovered from rainbow trout 55 days after oral infection or intraperitoneal infection

Fish No.	Infection method	
	Oral (30 larvae/trout)	Intraperitoneal (10 larvae/trout)
1	13	10
2	10	10
3	1	10
4	1	9
5	1	8
6	0	8
7	0	7
8	0	5
9	0	4
10	0	3
Total	26	74
Mean	2.6	7.4
Percent recovery rate	9%	74%

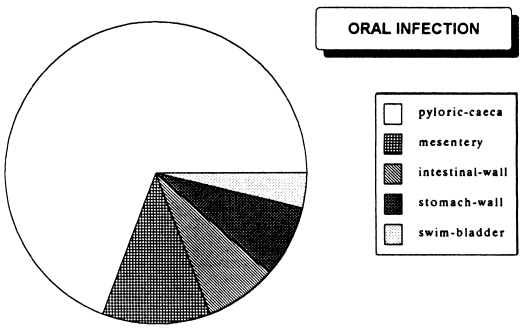

Fig. 1 Location of *Anisakis simplex* L_3 in rainbow trout (*Oncorhynchus mykiss*) 55 days after oral infection.

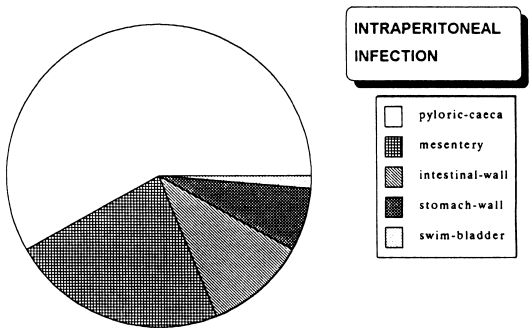

Fig. 2 Location of *Anisakis simplex* L_3 in rainbow trout (*Oncorhynchus mykiss*) 55 days after intraperitoneal infection.

Table 2 Development *in vitro* of *Anisakis simplex* L_3 obtained from an orally infected trout, showing the number of individuals in each category on days 4, 5, 6 and 7 of culture (L_3 = third-stage larvae, L_3/L_4 = larvae in ecdysis, L_4 = fourth-stage larvae, D = dead, A = apparently damaged, H = apparently healthy)

Culture day	Orally infected rainbow trout (*Oncorhynchus mykiss*)								
	L_3			L_3/L_4			L_4		
	D	A	H	D	A	H	D	A	H
4	1	1	7	0	1	0	0	0	0
5	1	1	3	0	0	0	2	0	3
6	1	1	2	0	0	0	2	0	4
7	2	0	1	0	0	0	2	0	5

The location of encapsulated larvae 55 days p.i. was similar following infection by both methods, with larvae being found at various sites within the body cavity (Figs. 1 and 2). The largest number of *Anisakis* larvae was found among the pyloric caeca, both after oral (18 larvae, 69%) and intraperitoneal infection (43 larvae, 58%); larvae were also found in the mesentery (3 larvae, 11%, after oral infection; 17 larvae, 23%, after intraperitoneal infection), on the outer wall of the intestine (2 larvae, 8%, after oral infection; 8 larvae, 11%, after intraperitoneal infection) on the stomach wall (2 larvae, 8%, after oral infection; 5 larvae, 7%, after intraperitoneal infection) and in the swim bladder (1 larva, 4%, after oral infection; 1 larva, 1%, after intraperitoneal infection). Larvae were not observed in the musculature in any fish.

Larvae recovered 55 days p.i. from the body cavity of orally (Table 2) and intraperitoneally (Table 3) infected trout and cultured *in vitro* displayed similar viability and a similar capacity for transition to the fourth-stage larvae. These results are similar to those for encapsulated larvae obtained from a natural host of *Anisakis* (blue whiting; Table 4). In

Table 3 Development *in vitro* of *Anisakis simplex* L_3 obtained from an intraperitoneally infected trout, showing the number of individuals in each category on days 4, 5, 6 and 7 of culture (L_3 = third-stage larvae, L_3/L_4 = larvae in ecdysis, L_4 = fourth-stage larvae, D = dead, A = apparently damaged, H = apparently healthy)

	Intraperitoneally infected rainbow trout (*Oncorhynchus mykiss*)								
Culture day	L_3			L_3/L_4			L_4		
	D	A	H	D	A	H	D	A	H
4	0	0	5	0	0	0	0	0	5
5	0	0	4	0	0	1	0	0	5
6	0	0	4	0	0	0	0	0	6
7	0	0	2	0	0	2	0	0	6

Table 4 Development *in vitro* of *Anisakis simplex* L_3 obtained from a naturally infected blue whiting, showing the number of individuals in each category on days 4, 5, 6 and 7 of culture (L_3 = third-stage larvae, L_3/L_4 = larvae in ecdysis, L_4 = fourth-stage larvae, D = dead, A = apparently damaged, H = apparently healthy)

	Naturally infected blue whiting (*Micromesistius poutassou*)								
Culture day	L_3			L_3/L_4			L_4		
	D	A	H	D	A	H	D	A	H
4	1	0	7	0	0	1	0	0	1
5	1	0	5	0	0	2	0	0	2
6	1	0	3	0	0	0	0	0	6
7	1	0	1	0	0	0	0	0	8

Table 5 Developmental (encapsulation) stage of *Anisakis simplex* larvae recovered between 1 and 50 days after intraperitoneal infection with five larvae per fish. Stage of encapsulation: free = active and freely mobile, not confined to a single location; coiled = confined to a clearly defined location, adopting the characteristic pre-encapsulation position

Fish No.	Days p.i.	No. of larvae found	Stage of encapsulation
1	1	4	free
2	7	4	free
3	21	2	coiled
4	28	5	coiled
5	31	5	coiled
6	36	4	coiled and encapsulated
7		3	encapsulated
8	41	4	encapsulated
9		2	encapsulated
10		5	encapsulated
11	50	4	encapsulated
12		5	encapsulated

all three assays most larvae were still alive after 7 days of culture, and a large number reached the fourth stage.

The results of the test carried out to investigate the time-course of encapsulation indicated that larvae coil up between 7 and 21 days after infection. Some larvae were fully encapsulated within 31 days of infection, and all the larvae had encapsulated by day 41 (Table 5).

Discussion

There have been few reports of experimental infection of rainbow trout with *Anisakis* larvae. Natural infection by larvae of this nematode occurs as a result of ingestion by the host. Following experimental oral infection of rainbow trout, by introduction of larvae into the stomach (Wootten and Smith, 1975) or by feeding fish on larvae (present study), larvae successfully enter the body cavity as in natural paratenic hosts; however, the recovery rates obtained with these infection methods (27% and 9% respectively) are unacceptably low. While mean recovery rate after oral infection was low (9%), two of the ten fish in this group were successfully infected (in one case with recovery of 13 larvae). This raises the possibility that the infection technique is inadequate; in particular, it is possible that the number of larvae ingested varied greatly between fish, and/or that in some cases the larvae were killed or severely injured during ingestion. Intraperitoneal infection by injection of larvae into the body cavity, on the other hand, ensures a high recovery rate (74% in this study) and reasonably uniform infection intensity. Ours results show that after infection by this method, the location of larvae in the host is similar to that observed after oral infection, with larvae occurring principally on the pyloric caeca, but also in the mesentery, on the intestinal wall, on the stomach wall and on the swim bladder. Although the musculature was not examined in detail, no larvae were observed in muscle tissue during dissection of any of the fish; this suggests that, as in other paratenic hosts, the larvae

locate in the body cavity. As in Wootten and Smith's (1975) study, larvae were still uncapsulated 24 days after infection. By day 41, all larvae were encapsulated in a flat spiral, as occurs in natural paratenic hosts of this nematode. Furthermore, after both oral and intraperitoneal infection, the capacity for ecdysis and transition to the fourth stage was normal. In conclusion, the results of this study indicate that intraperitoneal infection of rainbow trout with *A. simplex* may be an experimental model of considerable value for studies of the biology of this nematode. We are currently investigating applications of this model in the evaluation of anthelmintic drugs for fish.

Acknowledgements

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