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Research Note

Failure of Antihelmintic Treatment to Control *Anisakis simplex* in Trout (*Oncorhynchus mykiss*)

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The life cycles of *Anisakis* species are marine: adults infect mammals and euphasiid crustaceans are the intermediate hosts, while numerous species of marine fish may act as paratenic hosts (Smith and Wootten, 1978). One exception to this rule is constituted by the presence of *Anisakis* in euryhaline fish, such as migratory salmonids (Hoffman, 1967). There have also been occasional reports of *Anisakis* sp. larvae from wholly fresh-water hosts such as trout, and trout have been reported to act as paratenic hosts (Kane, 1966; Wootten and Smith, 1975). In previous work (Santamarina *et al.*, 1994), we obtained a 74% recovery rate when rainbow trout (*Oncorhynchus mykiss*) were intraperitoneally injected with third-stage larvae of *Anisakis simplex*, indicating that this is an effective method for experimental infection. Larvae were located in the viscera largely on piloric caeca. Encapsulation began in the second week of infection and was completed within 41 days of infection. Larvae obtained from experimentally infected trout infection and transferred to an *in vitro* culture showed a similar capacity for ecdysis as larvae obtained from blue whiting (*Micromesistius poutassou*), a natural host. These results indicate that the trout is a useful experimental model for studies of *Anisakis simplex*. In the work reported here, we used this model in experiments designed to screen various antihelmintic drugs for anti-*Anisakis* activity.

Tests were carried out on rainbow trout *Oncorhynchus mykiss* (100–200 g) from Piscifactorías Coruñesas (Carballo, La Coruña, Spain). They had been fed only on dried pelleted food. Prior to tests the fish were acclimatized for at least 36 h in 250 l tanks with a constant flow of water (15±3°C, pH 6.5±0.5) from a spring close to the laboratory. Oxygen was bubbled through the water. Trout were anaesthetized with tricaine methane sulphonate (MS222, Sandoz) at 50 mg/l.

Third-stage larvae (L_3) of *Anisakis simplex* (identified as per Smith, 1983) were extracted manually from the viscera and body cavity of commercially caught blue whiting (*Micromesistius poutassou* (Risso, 1826)). 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 to or alteration of the cuticle were discarded, and only apparently healthy larvae were used (following storage in PSS at 4°C). The culture medium was 10% Earle's balanced salt solution (EBBS; Gibco) in glycine-HCl buffer (pH 2.5). Assays were carried out in 5 ml wells. Five larvae were placed in each well. The cultures were maintained at 37°C for 7 days (Tojo *et al.*, 1992).

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±5°C; 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

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Table 1 Antihelmintics assayed against *Anisakis simplex*

Antihelmintic	Commercial name	Presentation	Manufacturer
Mebendazole	P.c.	powder	Esteve
Flubendazole	P.c.	powder	Esteve
Parbendazole	P.c.	powder	Smith Kline
Triclabendazole	Fasinex	suspension	Ciba-Geigy
Piperazine dihydrochloride	P.c.	granulated	Syva
Netobimin	Hapasil	powder	Bayer
Trichlorfon	Neguvón	powder	Bayer
Nitroscanate	Lopatol	pills	Ciba-Geigy

P.c. Pure compounds.

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 (Santamarina *et al.*, 1994).

Experimentally infected trout were weighed and assigned to groups of five fish of similar weight in 10 l tanks (Letica, Barcelona). The drug (Table 1) was administered via an orogastric tube, following anaesthesia, on three consecutive days (31, 32 and 33 days postinfection) at 24-hour intervals. On each day, every fish received 500 mg of drug per kg body weight, homogenized in a mortar with a 4% aqueous solution of carboximethylcellulose (CMC) to a volume of 0.3 ml. Fish in the control group were sham-treated (i.e. administration of CMC alone). Six days post-treatment (= 39 days p.i.), all fish were killed. The abdominal cavity was opened and the viscera removed and examined. The musculature was not examined in detail. All larvae found were examined under a stereomicroscope for assessment of viability (dead, damaged or apparently healthy; criteria as per Tojo *et al.*, 1992). Two larvae per trout were selected for subsequent *in vitro* culture. Viability was assessed, and presence of ecdysis recorded, on day 7 of culture.

Our results are displayed in Figure 1, showing jointly; the mean intensity of parasitation (=A) (calculated on the number of recovered larvae in the dissection of every fish), and the *in vitro* survival capacity of these larvae (=C) (obtained on the percentage of survival *in vitro*).

A number of intestinal helminthiases of fish have been successfully treated with drugs originally developed for use in man or domestic animals. For example, niclosamide (Molnár, 1970), praziquantel (Molnár, 1977) and mebendazole (Alarcón and Castro, 1988; Sanmartín *et al.*, 1989) have all been reported to show activity against *Botriocephalus*. However, very little is known about the effectiveness of orally administered drugs for combatting extraintestinal endoparasites of fish, though Schmahl *et al.* (1989) has shown amprolium and fumagillin to be effective against extraintestinal infections by certain microsporeans and myxosporeans.

There have likewise been few *in vivo* studies of the effectiveness of drugs for combatting *Anisakis* species. Sweney and Ridway (1975) reported piperazine to be effective in marine mammals, in which *Anisakis* locates in the stomach. A number of other studies have evaluated the activity of various drugs against third-stage larvae of *Anisakis simplex*, largely on the basis of *in vitro* trials; some drugs have been shown to have larvicidal activity (triclabendazole and nitroscanate – Tojo *et al.*, 1992) while others simply prevent transition to the fourth stage (mebendazole and flubendazole – Woo, 1987; Oshima *et al.*, 1990; Tojo *et al.*, 1992; pirantel-Kasuya *et al.*, 1990; Goto *et al.*, 1990; Tojo *et al.*, 1992).

The activity of some drugs thus appears to depend on the life-cycle stage of the parasite. In some cases, this may be attributable to *between-stage* differences in location. This may explain why drugs which are effective against third-stage *Anisakis* larvae *in vitro*, or against adult *Anisakis* in the stomach of marine mammals, are not effective (when admin-

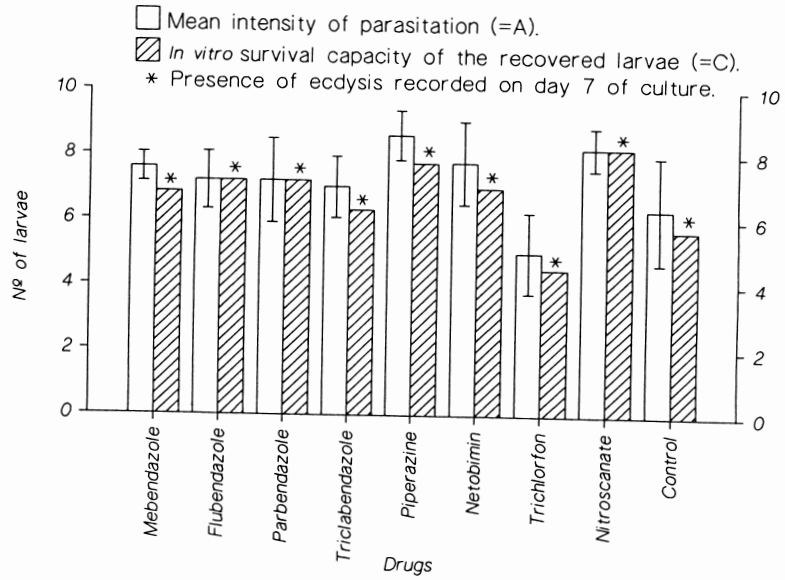

Fig. 1 Summarized results of tests to screen for the anti-*Anisakis* activity of various drugs.

istered orogastrically) against third-stage larvae in trout. Since in fish the larvae encyst in the peritoneal cavity, successful orogastric treatment would require intestinal absorption of the drug and its subsequent transfer (in sufficient amount) to the infected tissues.

As displayed in Fig. 1, none of the drugs tested in the present study displayed larvicidal activity, since all larvae recovered 6 days post-treatment were alive. Although 100% recovery of larvae was rarely achieved, this is probably either because the experimental infection procedure was not 100% efficient; it is unlikely that some of larvae had died and been absorbed by the host, since – as we have demonstrated in previous study (Santamarina *et al.*, 1994) – such absorption takes more than 20 days.

Similarly, none of the drugs tested affected the capacity of third-stage larvae to undergo ecdysis to the fourth stage.

In conclusion, a number of drugs which have proven anti-*Anisakis* activity *in vitro*, in the posology tested are ineffective against third-stage *Anisakis* larvae in trout. For each drug, however, it is not possible to state whether the negative results of this study are due to a genuine lack of pharmacological activity, to insufficient absorption/transfer, or to a combination of both factors. Further studies are necessary to clarify these questions.

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