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Some factors which influence the *in vitro* maintenance of *Anisakis simplex* (Nematoda)

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Abstract. Several culture media as well as some factors that may affect the *in vitro* development of the nematode *Anisakis simplex* Rudolphi, 1809 have been studied. After testing six media and four temperatures, the conditions for the *in vitro* culture selected were as follows: RPMI-1640 + 20% (v/v) heat-inactived fetal bovine serum or Meyer's M3 (without agar) media, at 37°C, under 5% CO_2 in air atmosphere, and renewal of the medium twice a week. The average survival rates of the larvae were significantly increased when the pH of the culture medium was increased (from 4.0 to 7.2) or decreased (from 7.2 to 4.0) after L_3 to L_4 moulting. The length of the larvae at the onset of culture affected the survival and moulting of themselves, but these were culture medium-dependent. On the other hand, we have observed that several L_3 and L_4 were attached, by means of a brown unknown substance apparently secreted by themselves, to the bottom of the substratum. Frequently, when a larva was spontaneously detached, a "cap" of the brown substance blocked, apparently, its mouth. The possible absorption of nutrients through the L_3 larvae cuticle of *A. simplex* is discussed.

Human anisakidosis is a parasitic disease caused by nematode larvae of the family Anisakidae Skrjabin et Karokhin, 1945. It affects particularly human gastrointestinal tract and may cause *visceral larva migrans* syndrome in some cases. This parasitic disease usually results from consumption of raw or semi-raw fish carrying larvae of the genera *Anisakis*, *Pseudoterranova* or *Contracaecum*. Over a short space of time several anisakidosis cases have been described in Spain (Arenal-Vera et al. 1991, López-Vélez et al. 1991, Barros et al. 1992, Valero et al. 1992, Clavel et al. 1993) and parasitation of commercial fish by anisakids has been reported (Ruiz-Valero et al. 1992, Adroher et al. 1996).

There have been several attempts to culture marine anisakids *in vitro* to study the development, maturation and production of eggs from the L_3 larvae. Up to now, the complex media have given the most satisfactory results in the *in vitro* culture of anisakids (Van Banning 1971, Grabda 1976, Likely and Burt 1989, 1992).

Several semi-defined culture media as well as several factors, such as temperature, CO_2, pH, and initial length of larvae, that may affect the *in vitro* development of this parasite were tested in our laboratory. The aim was to obtain cultures in increasingly defined media to facilitate the physiological, biochemical and immunological studies of the anisakid nematodes.

MATERIALS AND METHODS

The worms selected for our study were L_3 larvae of *Anisakis simplex* Rudolphi, 1809 isolated from the host *Micromesistius poutassou* Risso, 1826 (blue whiting), family Gadidae, from the fishmarket of Granada (Southern Spain). The nematodes were collected as described by Adroher et al. (1996) and washed in a 0.9% NaCl solution several times. Then they were identified according to morphological characteristics (Hartwich 1974, Petter and Maillard 1988).

Before proceeding to the culture itself, each larva was individually placed in an antibiotic-antimycotic solution (80 mg gentamicin sulfate, 0.625 mg amphotericin B, 10,000 IU penicillin G, 10 mg streptomycin sulfate and 4.5 ml Hanks' solution for a final volume of 10 ml of solution) for 30 minutes. The culture was grown on a sterile polystyrene 24-well tissue culture plate. Once 1 ml of culture medium was dropped into each well, one parasite per well was introduced. The cultures were observed daily for mobility, moulting, and survival of larvae.

Six media were tested at four different incubation temperatures (23°, 28°, 33° and 37°C): RPMI-1640 supplemented with heat-inactivated foetal bovine serum (IFBS) at 20% v/v (RPMI), MTL without hemoglobin (Baena et al. 1987); LIT + R9 (Sadigursky and Brodskyn 1986); M3 (Meyer 1970) without agar (M3M); Eagle's MEM + 20% IFBS; and Grace's medium + 10% IFBS.

After first trials, two of these culture media (RPMI and M3M) were selected and culture of larvae was carried out in air atmosphere with or without 5% CO_2 at 37°C. Next, both media were modified as follows: a) RPMI supplemented with 1% v/v BME vitamin solution (100 ×) added (RPMIVT); 'b) M3M without yeast extract, with 1% v/v BME vitamin solution (100 ×) added (M3VT); c) Mixture with equal volumes of RPMI and M3VT media (M3VT + RPMI). These media were used for culturing in air with 5% CO_2 at 37°C.

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Table 1. Percentage of L3 to L_4 moulting of *Anisakis simplex* in different media throughout culture time.

Culture medium	n[a]	2nd day	3rd day	4th day	5th day
RPMI	22	0	37	90	100
RPMIVT	14	0	21	93	100
M3M	24	0	71	100	–
M3VT	20	0	0	55	100
M3VT+RPMI	20	0	0	75	100

[a] – Initial number of parasites in the culture medium

Table 2. Survival rates (days) of *Anisakis simplex* larvae in different culture media.

Culture medium	n[a]	S_{50}	$S_{av} \pm SD^b$	S_{max}
RPMI	22	25	24.9 ± 3.7	37
RPMIVT	14	21	23.3 ± 4.3[ns]	30
M3M	24	26	27.7 ± 6.2[ns]	39
M3VT	20	28	29.4 ± 4.7*	37
M3VT+RPMI	20	29	30.4 ± 6.9*	47

[a] – Initial number of parasites in the culture medium
[b] – Average survival ± standard deviation
Statistical comparison with the RPMI medium using Student's *t* distribution: * $P < 0.005$; [ns] – not significant

Table 3. Influence of change of culture medium in the survival rates (days) during maintenance of *Anisakis simplex* larvae.

Initial culture medium	Change of culture medium	n[a]	S_{50}	$S_{av} \pm SD^b$	S_{max}
RPMI	M3VT – 5th day	17	20	20.6 ± 5.1	29
	M3VT – 15th day	16	24	24.1 ± 5.0	35
M3VT	RPMI – 5th day	19	21	22.5 ± 4.0	29
	RPMI – 15th day	20	27	25.5 ± 4.5	35

[a] – Initial number of parasites in the culture medium
[b] – Average survival ± standard deviation

Table 4. Influence of initial length of L3 in the survival rates (days) during maintenance of *Anisakis simplex* larvae.

Change of medium[a]	Initial length	n[b]	S_{50}	$S_{av} \pm SD^c$	S_{max}
RPMI to M3VT	<12 mm	7	15	14.8 ± 6.8	21
	>20 mm	7	28	26.8 ± 4.3	35
M3VT to RPMI	<12 mm	10	24	24.9 ± 3.8	31
	>20 mm	9	26	26.7 ± 2.8	32

[a] – The culture medium was changed on 7th day
[b] – Initial number of parasites in the culture medium
[c] – Average survival ± standard deviation

The pH of every medium was adjusted to 7.2, except when indicated. The culture medium was renewed twice a week.

Two batches of larvae were used to observe the influence of pH on parasite development. First, a culture of a batch of parasites was started at pH 7.2 and then changed to pH 4.0 on the 5th day, when all the parasites had moulted into L_4 larvae. Simultaneously, another batch of parasites was maintained in the same medium starting at pH 4.0. This was increased to 7.2 on the 5th day, when all the parasites had moulted to L_4 larvae.

The average length of the larvae used in the previous test was 20 mm. Several experiments with parasites of different length were carried out in order to examine the influence of the L_3's initial length on its subsequent *in vitro* development. Some of these parasites were initially over 20 mm in length, while others were less than 12 mm. At first, the batches were maintained in the RPMI or M3VT media, and were changed to M3VT and RPMI, respectively on the 7th day.

The terms maximum survival (S_{max}), survival 50 (S_{50}), and average survival (S_{av}) have been defined by us as follows:

S_{max} expresses the day of culture in which the death of the last living nematode of the experiment occurs.

S_{50} expresses the day of culture in which the deaths of 50% of nematodes of the experiment occur.

S_{av} expresses the arithmetic mean of the day in which the death of each nematode of the experiment occurs. The data are expressed as the mean ± standard deviation.

Similarly, M_{av} expresses the arithmetic mean of the day in which the moulting of each nematode of the experiment occurs. The data are expressed as the mean ± standard deviation.

All media, sera and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Bio-Whittaker (Walkersville, MD, USA).

Statistical comparison of the culture data was done using the Student's *t* distribution.

RESULTS

The longest survival time and the largest number of moultings occurred in the 37°C media. Highest scores (mobility, moulting, and survival) were observed in the RPMI and M3M media (results not shown).

The influence of CO_2 on *Anisakis simplex* survival and its moult from L_3 to L_4 is very clear. As shown in Fig. 1, the presence of 5% CO_2 in the atmosphere stimulates moulting, and prolongs the survival.

Once it was shown that the most suitable conditions correspond to RPMI and M3M media at a temperature of 37°C in an air 5% CO_2 atmosphere, different modifications of these media were studied (see Materials and methods section). In Table 1 it is shown that moultings into L_4 occurred in all individuals and the media[1]. The larvae survival rates were higher in the media derived from M3M, but were not significantly affected by the vitamins added to any of the tested media (Table 2). The replacement of yeast extract by the concentrated

[1]In some cases, a few L_3 (<10%) did not moult during their routine maintenance in the laboratory.

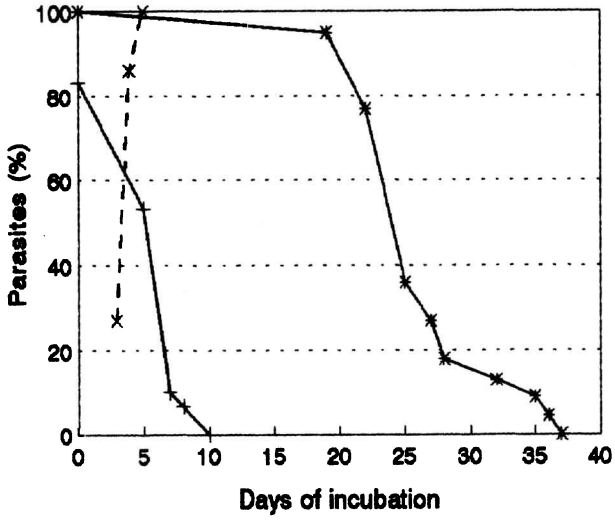

Fig. 1. Influence of 5% CO_2 in the L_3 into L_4 moulting and in the survival of *Anisakis simplex* larvae incubated in RPMI–1640 + 20% (v/v) IFBS, pH 7.2, at 37°C. Symbols: Larvae survival under air (+), and under 5% CO_2 in air (∗). Moulting to L_4 (×) only occurred under 5% CO_2 in air.

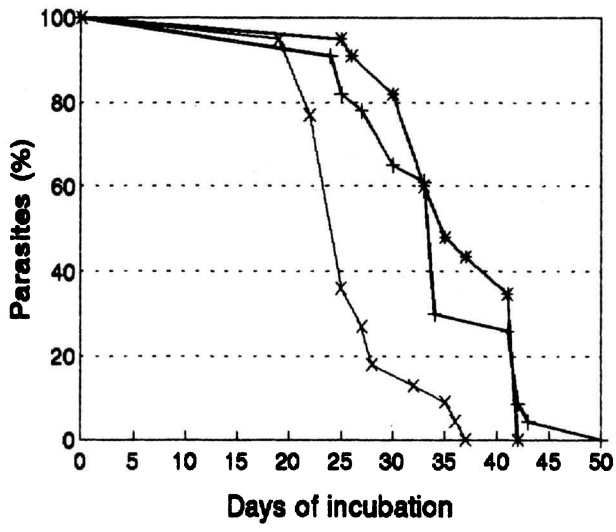

Fig. 2. Influence of pH change of culture medium in the survival of *Anisakis simplex* after moulting to L_4. Symbols: (×) RPMI, pH 7.2; (+) RPMI pH 7.2 changed to pH 4.0 on 5th day; (∗) RPMI pH 4.0 changed to pH 7.2 on 5th day.

vitamin solution did not lead to significant changes of larvae survival at M3M. However, the mixture with equal volumes of RPMI and M3VT improved considerably the S_{max} (10 days more) in both media, as well as the S_{av} in RPMI ($P < 0.004$) (Table 2).

For moulting to L_4 under natural conditions, L_3 must pass from a physiological pH medium (in the tissues of the intermediate host) to a gastric pH environment (in the stomach of the definitive host). Therefore, we set up an experiment in which the larvae were subjected to a change of pH or medium. The results of the first experiment (Fig. 2) showed that either kind of pH change (pH 7.2 to 4.0, $S_{av} = 34.2 \pm 6.9$ for 23 larvae; or pH 4.0 to 7.2, $S_{av} = 35.5 \pm 6.8$ for 23 larvae) produced a significant increase of 9–12 days in S_{av} compared with RPMI medium at pH 7.2 ($P < 1.10^{-4}$). Likewise, an increase of up to 7 days in the S_{max} was observed (Fig. 2).

When the M3VT medium was replaced by RPMI medium or *vice versa* on the 5th or 15th day of the cultivation, no significant differences were seen, although those cultures which underwent a change of medium on the 15th day showed higher survival rates (3 days or more) than the others (Table 3).

Finally the effect of L_3's length on the *in vitro* development was studied, using $L_3 > 20$ mm and $L_3 < 12$ mm in M3VT and RPMI media, which were substituted by RPMI and M3VT, respectively, on the 7th day. The results (see Table 4) show that the survival rates are lower in larvae < 12 mm when cultivation commenced with RPMI. Moulting was delayed (by the 11th day, M_{av} is 6.8 ± 3.1 for five larvae < 12 mm, and 4.4 ± 0.5 for 7 larvae > 20 mm) and in two cases, moulting did not occur in the smaller larvae. In an initial M3VT culture, although moulting of the L_3 < 12 mm was delayed in one case (by the 11th day, M_{av} is 4.4 ± 2.5 for ten larvae < 12 mm, and 4.2 ± 1.2 for nine larvae > 20 mm), they all moulted, and the survival rates were similar to those for larvae of a larger length.

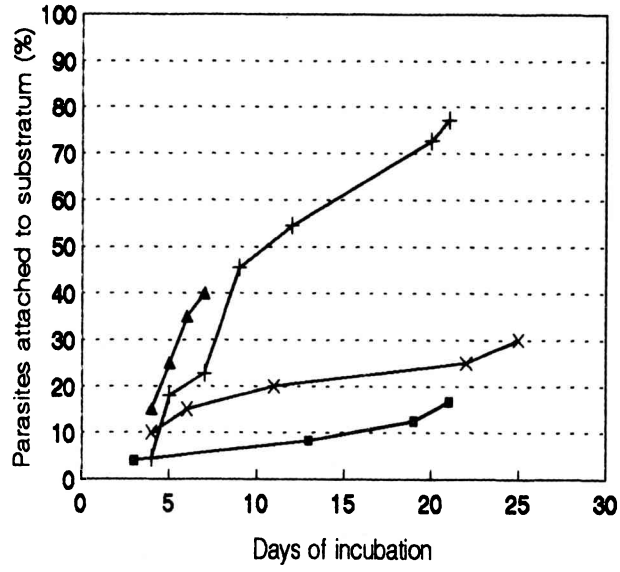

Fig. 3. Accumulative percentage of *Anisakis simplex* larvae maintained in an *in vitro* culture attached to substratum incubated in RPMI (+), M3M (■), M3VT (×), and M3VT + RPMI (▲) media. Larvae maintained in RPMIVT medium remained non-attached to substratum throughout culture time.

We have also observed that several L_3 and L_4 were attached, by means of an unknown brown substance apparently secreted by themselves, to the bottom of the wells throughout the culture time irrespective of ecdysis, at least in RPMI media (Fig. 3). Frequently, when a larva was spontaneously detached, a "cap" of the brown substance apparently blocked its mouth, and lips-print of larva on the bottom of culture well was observed.

DISCUSSION

Several authors (Sommerville and Davey 1976, Mercer et al. 1986), who worked with the 199 medium, observed the stimulatory and, particularly, synchronizing effects of the CO_2 on the *Anisakis simplex* moult from L_3 to L_4. We have confirmed these results with other culture media, such as RPMI (Fig. 1) and M3M. The latter is a reducing medium (due to presence of cysteine and glutathion) used for the culture of such anaerobic intestinal parasitic protozoans as *Giardia* (Meyer 1970). Recently, Likely and Burt (1992) reported that cysteine is required in the API-1 medium for *in vitro* cultivation of anisakids. While in the 199 medium all the larvae moult in a period of 3–5 days (Sommerville and Davey 1976, Mercer et al. 1986), in the RPMI medium they do so in 3 days, and in the M3M in two days, bearing in mind that the moulting always commences on the 3rd day of the culture (Table 1). M3M medium is a partially defined medium including yeast extract and serum as non-defined components. When the yeast extract was replaced by a concentrated BME vitamin solution, the onset of moulting was delayed one day, although it was completed in only two days (Table 1).

This could be due either to the absence of the extract yeast or to the presence of the vitamins that somehow influence the moulting, which probably means that some of them are used by the L_3 of the parasite. This may appear to disagree with the assertions of Yasuraoka et al. (1967) and Sommerville and Davey (1976) who concluded that feeding does not commence until ecdysis is completed. Perhaps these products are absorbed through the larvae cuticle. Hence it could explain the fact that the size of L_3 increases in their development even if they do not feed through the mouth, and low molecular weight nutrients are probably absorbed through their cuticle. However, the added vitamins do not seem to affect significantly the larvae survival (Tables 2 and 3).

When the recently moulted L_4 larvae in the RPMI medium were transferred to the same medium with a pH of 3.2 higher units (pH 4.0 to 7.2) or 3.2 lower units (pH 7.2 to 4.0), a high and significant increase of survival rates was observed (Fig. 2). These results suggest that this parasite is perfectly adapted to the drastic change to which it is exposed in the stomach of its final host.

Apart from the medium, when the length of the L_3 at the onset of the culture was considered (Table 4), it was observed that, by starting the culture with M3VT medium and replacing it by RPMI on the 7th day, there was no difference in the moulting or in the survival in relation to the initial length of L_3. However, when we used RPMI to start the culture and it was then replaced by M3VT on the 7th day, a substantial delay was observed in the moulting of the smallest larvae (<12 mm) as well as a noteworthy decrease of their survival rates. It appears that the vitamins added to the medium at the onset of the culture (M3VT), and not subsequently to the moulting to L_4, are absorbed by L_3 larvae, particularly the smallest ones. This enables them to behave (moult and survival) in a similar way to that of the largest ones (Table 4).

Finally, Sommerville and Davey (1976) observed in *in vitro* culture of *Anisakis* sp. larvae that the heads of some L_4 were encrusted with a "brown deposit", and Mercer et al. (1986) reported that L_3 of *A. simplex* were often immobile during the period of cuticle formation and ecdysis being attached to the substratum by an "oral plug". Likely and Burt (1992) also observed the attaching of L_3 of *Contracaeum osculatum* to the wall of the culture vessel by means of a "cephalic cap". We have also observed that some L_3 and L_4 were capable of attaching to the bottom of the wells throughout the culture time irrespective of ecdysis, at least in RPMI media (Fig. 3). The mean of this attachment and the nature of this "brown deposit" are unknown. The survival of these larvae in the culture media also indicates the absorption of nutrients through the worm cuticle. More studies are necessary to elucidate these questions.

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