IN VITRO CULTIVATION OF *ANISAKIS SIMPLEX*, CAUSAL AGENT OF THE HUMAN ANISAKIASIS.

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INTRODUCTION

Anisakis simplex is a parasite ascaridoid nematode, main etiological agent of the parasitic illness known as human anisakiasis, which affects the gastrointestinal tract. The natural final hosts are cetaceans. The intermediate/paratenic hosts are crustaceans, fishes, and cephalopods, which harbor the third larval stage (L3) of this nematode. When the human, who acts as an accidental host, consumes these raw, marinated or undercooked hosts harboring L3 of *Anisakis*, has a high risk of acquiring human anisakiasis.

OBJECTIVES

We are developing a cultivation method with the aim to achieve the complete lifecycle of *Anisakis* in the laboratory using only culture media. We study the development, ecdysis and survival of *A. simplex* to know the factors that are involved in the growth and survival of this parasitic nematode.

MATERIAL AND METHODS

A total of 48 *Anisakis* larvae from body cavity of *Micromesistius poutassou* Risso, 1826 (blue whiting), family Gadidae, were selected for cultivation. The fish were purchased from the fish market of Granada (Southern Spain) and came from Galician harbors (NW Spain). These larvae were morphologically identified as L3 of *Anisakis simplex* s.l. Rudolphi, 1809 according to Hartwich (1974) and Peter & Maillard (1988). The larvae

were axenized in an antibiotic-antifungal solution as indicated by Iglesias et al. (1997). Next, the culture medium (1 ml) was placed into each well of a sterile polystyrene 24-well tissue-culture plate. Worms were individually cultured (one parasite/well). These plates were then placed in an incubator at 36 ºC in wet air atmosphere with 5% CO_2. The culture medium was renewed twice a week. An inverted microscope was used to observe the worms for mobility, molting and survival every 48 h. The culture medium RPMI-1640 supplemented with heat-inactivated fetal calf serum and pepsin was adjusted at pH 4.0 (Iglesias et al., 2001) and used as control. This culture medium was also supplemented with tryptose (5 g/L) and/or calf's liver infusion (5% w/v) to test its effect on mobility, molting and survival of the worms. The media pH was also adjusted at 4.0. Next, the media were sterilized by filtration and kept cold.

RESULTS AND DISCUSSION

The development of the parasites in the assayed media was followed. The average and maximum survival, the day that the molting are completed (M3 and M4), and the number of larvae which complete the molting are recorded for every media. The M3 (L3 to L4 molting) is completed by all larvae at 3-5 days of culture in all media. The M4 (L4 to adult molting) is not completed by all larvae in culture (Table 1 and Fig. 1). The results show M4, when occurred, was slower in the control medium, but the percentage of larvae which molting to adult was much higher in this medium (75%) and in the medium supplemented with calf's liver infusion (66%) than in the other media (16,6% for medium with tryptose and 8,3% for medium with tryptose and liver infusion).

Table 1.- *In vitro* cultivation of *Anisakis simplex*: M4 and average survival (media ± SD) (n: number of larvae which molt to adult stage).

Culture media	L4-Adult molting (M4) days± SD (n)	Average survival days ± SD
Control	42,22 ± 11,37 (9)	66,86±20,05
+ tryptose	28,50 ± 2,12 (2)	38,5±16,81
+ calf's liver infusion	33,00 ± 8,40 (8)	71,75±32,78
+ tryptose +calf's liver infusion	30 (1)	35,25±14,62

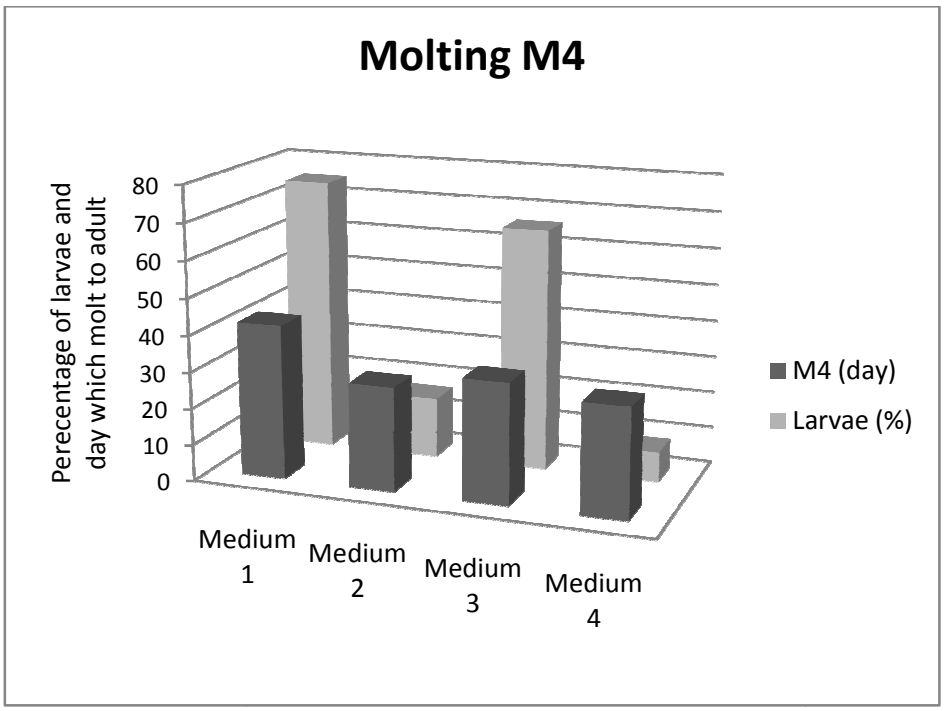

Fig. 1: Effect of the calf's liver infusion on the L4 to adult stage molting (M4) of *Anisakis simplex* in *in vitro* culture. Medium 1: control medium; Medium 2: control + tryptose; Medium 3: control + calf's liver infusion; Medium 4: control + tryptose + calf's liver infusion.

Other authors had previously shown that the use of calf's liver could be useful for the cultivation of *Anisakis*. Van Banning (1971), Grabda (1976) and Carvajal et al. (1981) obtained adults in a culture complex medium with calf's liver digested by pepsin and fresh blood. These authors obtained a yield of 50% or less of adults, while we have obtained more than 65% in this study with a simple culture medium.

Also, differences in the average survival of the worms in the culture media have been recorded (Fig. 2). As in the above case, the culture control medium and the medium supplemented with calf's liver infusion showed a higher survival (ca. 2-fold) than the other assayed media.

When comparing the evolution of the *A. simplex* in the culture media, it is observed that the medium which is only added the calf's liver infusion accelerates 9 days the development of this worm to adult stage regard to the control medium, as well it increases 5 days the average survival (see Table 1), and, moreover, the worms cultured in this medium were higher size than the cultured ones in the control medium. Worms grown in the other

tested culture media showed less development and survival, suggesting that the tryptose is not suitable for growing this helminth.

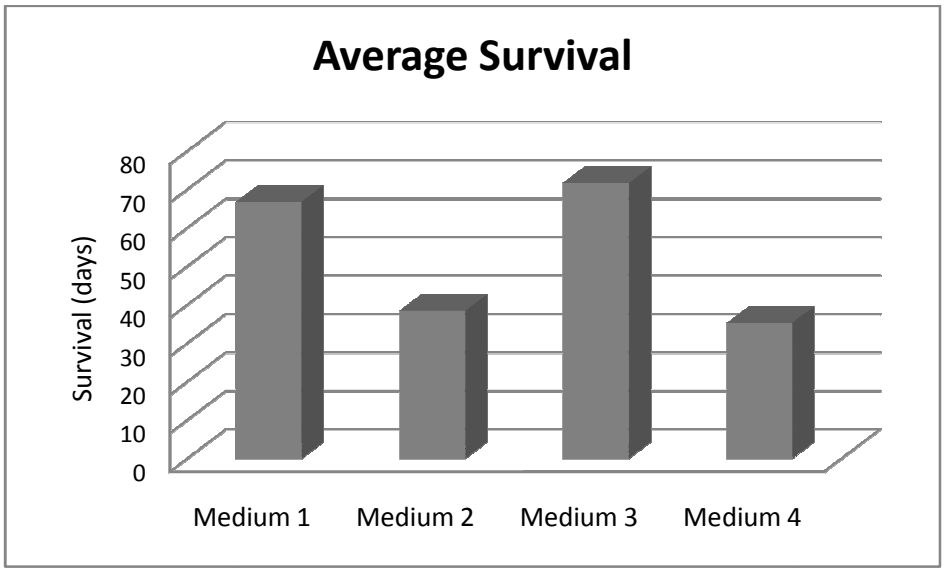

Fig. 2: Average survival of *Anisakis simplex* in the assayed culture media. Medium 1: control medium; Medium 2: control + tryptose; Medium 3: control + calf's liver infusion; Medium 4: control + tryptose + calf's liver infusion.

CONCLUSIONS

The use of calf's liver infusion in the media for cultivation of *Anisakis simplex* causes a positive effect since M4 molting occurs earlier, and it also increases the survival and the size of the worms.

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