

INFLUENCE OF THE PH OF THE CULTIVATION MEDIUM ON SURVIVAL AND  
DEVELOPMENT OF STAGE III LARVAE OF *ANISAKIS SIMPLEX*

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**A b s t r a c t.** The cultivation was done on Eagle's media as well as Eagle's media enriched with the supplement of 10 and 20% of bovine serum. In the media without serum the larvae lived significantly shorter and did not get through molting. They lived the longest and came through molting the fastest in the media with pH 2.0 supplemented with 20% SB. In the media with the pH 4.0 and 7.3 the larvae lived significantly shorter and only a small proportion reached stage IV.

### INTRODUCTION

*In vitro* breeding of parasites offers a very useful research tool in studies on their development and control of infestations. According to many authors (SILVERMANN 1963; SMYTH 1969, JABŁONOWSKI and PIECHOCKI 1988) the major problems to be solved in *in vitro* cultivation are obtaining parasites of uniform physiological status, maintaining aseptic conditions and selection of the appropriate cultivation medium. The basis for that last decision are the standard, chemically determined media used in culturing tissues, which should be additionally supplemented to adjust them to the physiological needs of the parasites. A detailed review of the media used in *in vitro* cultivation of nematode parasites enriched with various additives and their influence upon the success of cultivation operation were given in the descriptive paper by JABŁONOWSKI and PIECHOCKI (1988).

The majority of so far published papers on *in vitro* cultivation of parasites concerned cultivation of *Ascaris suum* larvae, significantly fewer publications can be found on *in vitro* cultivation of stage III larvae of *Anisakis simplex* (BANNING 1971, GRABDA 1976, SOMMERVILLE and DAVEY 1976, IGLESIAS et al. 1997). Marine mammals are the final hosts of that parasite. Free living stage II larvae (L<sub>2</sub>) hatching from eggs reach the bodies of shellfish with

water, get through another molting and as stage III larvae form the invasive form for the end hosts. In case of *A. simplex* fish are the parateneal hosts. Together with the food of marine mammals, i.e. fish and shellfish, the larvae reach the stomach of the final host where they actively penetrate the mucous membrane and sub-mucosa. As a result of the penetration by the larvae, numerous crater-like ulcerations are observed. In the wall of the stomach of their final host the larvae undergo another molting. Adult parasites live in the lumen of the stomach causing no mechanical damages but only irritation. Stage II larvae (L<sub>3</sub>) may also cause infestation of accidental hosts, e.g. humans or other land mammals. In case of small laboratory animals such as rats or guinea pigs, the larvae penetrate the walls of the alimentary tract and wander to other organs. In the majority of mammals, as well as humans, the larvae anchor in the mucous membrane of the stomach or intestine causing vast damages, hemorrhages, eosinophilic infiltrations, swelling and ulcerations. The disease is generally known as anisakiosis or eosinophilic granuloma and it manifests through stomachaches, nausea, diarrhea and high body temperature.

This study aimed at verifying the suitability of Eagle's medium for cultivation L<sub>3</sub> larvae of *A. simplex* in vitro, the medium that is quite frequently used for cultivation other nematodes. The medium was additionally supplemented with bovine serum. As the majority of L<sub>3</sub> larvae in the body of the host live in the stomach contents with acid pH, it was decided to check how the development and survival of the larvae in vitro are influenced by the pH of the medium.

#### MATERIALS AND METHODOLOGY

L<sub>3</sub> larvae of *A. simplex* were obtained from herrings caught in the Baltic Sea. They were stored in the sterile Thyrod's liquid at 4°C. Before establishing the experiment the larvae were flushed with the sterile solution of physiological salt with antibiotics and mikocides added (penicillin – 100U/ml, streptomycin – 100µl/ml and nystatin 100 j. m/ml). Next, 5 larvae were placed in test tubes containing 5 ml of Eagle's medium each. One series of test tubes was supplemented with 10% and another with 20% of bovine serum (SB). Prior to placement of the larvae, some of the tubes were brought to pH 2.0 or pH 4.0 using 1N solution of HCl. The remaining larvae were cultivated in the pH 7.3 medium. All tubes were maintained in a heater at 37°C. The survival (%) and mobility of the larvae (highly mobile +++; mobile ++; poorly mobile +) and the percentage of larvae that completed molting reaching stage L<sub>4</sub> were determined every second day by stereoscopic microscope observation.

## RESULTS

It was observed that during the initial 4 days of the experiment the larvae stayed alive in all tubes (tab.).

TABLE  
Influence of medium pH upon survival and development of stage III larvae of  
*A. simplex*.

pH	Day	Medium					
		MEM		MEM+10% SB		MEM+20% SB	
		% live larvae	% L <sub>4</sub>	% live larvae	% L <sub>4</sub>	% live larvae	% L <sub>4</sub>
2,0	2	100 ++	0	100 +++	0	100 +++	0
	4	100 ++	0	100 +++	40	100 +++	80
	6	80 +	0	100 ++	80	100 +++	100
	8	40 +	0	100 ++	80	100 +++	100
4,0	2	100 ++	0	100 ++	0	100 +++	0
	4	100 +	0	100 +	0	100 ++	0
	6	0	0	60 +	0	80 +	30
	8	0	0	40 +	0	40 +	40
7,3	2	100 +	0	100 +	0	100 ++	0
	4	100 +	0	100 +	0	100 +	0
	6	0	0	60 +	0	80 +	0
	8	0	0	20 +	0	40	0

+++ highly mobile ++ mobile + poorly mobile

The larvae in the pH 2.0 medium were the most mobile. It was established that in the medium supplemented with 10% of bovine serum 40% of larvae went through molting on that day reaching development stage IV while in the medium supplemented by 20% of bovine serum the proportion of such larvae was as many as 80%. In the other media no molting process was observed. After 6 days in the medium without the bovine serum supplement and with pH 7.3 and with pH 4.0 no live larvae were observed. In the media with pH 2.0 ca. 80% of the larvae were still alive. They showed very poor mobility. During the following days a decreasing number of live larvae was observed in this medium and no molting was observed. In the media with pH 2.0 supplemented with 10% or 20% of bovine serum a slightly decreasing number of live larvae was observed. All the larvae showed high mobility and a vast majority went through molting reaching L<sub>4</sub> stage. In the media with pH 4.0 and

pH 7.3 supplemented with bovine serum the percentage of live larvae decreased rapidly during the following days. The larvae showed low mobility and the vast majority of them did not reach the stage IV larvae form.

### DISCUSSION

The here presented study showed that the pH of the cultivation environment has a quite significant influence on survival, mobility and development of L<sub>3</sub> stage larvae of *A. simplex*. The acid reaction medium was also used by other authors for cultivation of *A. simplex* larvae (GRABDA 1976, BANNING 1971). GRABDA (1976) did not use the defined medium but a medium prepared in her own laboratory from bovine liver extract supplemented by bovine blood. j. L<sub>3</sub> larvae cultivated on that medium went through molting in 4-7 days reaching stage L<sub>4</sub> and next after 12-14 days went through another molting reaching the pre-adult stage. Adult individuals laid eggs and next the first stage larvae were obtained hatching from the eggs. Similarly BANNING (1971) used acid reaction environment for cultivating L<sub>3</sub> larvae applying the medium prepared from bovine liver extract supplemented with bovine blood and sodium citrate.

In the available literature no papers were found on cultivation of L<sub>3</sub> larvae in the defined media brought to acid pH. SOMMERVILLE and DAVEY (1976), studying the influence of temperature and CO<sub>2</sub> upon the development of stage III larvae used the defined medium M<sub>199</sub> or Krebs-Ringer medium, with the pH at 7.0. IGLESIAS et al. (1997) studied suitability of media such as RPMI-1640, MTL, Meyer's M3, Eagle's for in vitro cultivation of L<sub>3</sub> larvae. Some of the media were supplemented with thermally inactivated bovine serum, solution of vitamins or agar. Cultivation was carried out at 37°C in the atmosphere containing or not 5% CO<sub>2</sub>. The authors also studied the influence of a change of the medium during cultivation and the influence of a change in pH of the medium upon the development of larvae. Their experiments were initially conducted in the media with the pH at 7.2. After 5 days, when the majority of the larvae went through molting and reached stage IV, the medium was changed to a medium with the same composition but the pH at 4.0. Such an operation influenced increasing the survival rate of the larvae. According to those authors, this indicates high adaptation abilities to the changing environmental conditions as it takes place in vivo during a change of the host.

The main objective of the study presented in this paper was to determine the influence of pH upon the mobility, time of molting and survival of the stage III larvae. In the in vivo conditions L<sub>3</sub> larvae together with the food enter the stomach of the final hosts, i.e. marine mammals. The majority of those larvae settle in the stomach and only a small proportion of them, together with the food go to the intestines. It is common knowledge that the stomach content

pH in case of the majority of animals ranges from 2 to 4 while the contents of the intestines range from 7 to 8. Taking the above into consideration, the medium used was brought to the values similar to those in the alimentary tract of the host. The study clearly showed that the reaction of the medium is of significant influence on the mobility, survival and development of the larvae. In the media with the pH 2,0 the larvae lived the longest, showed a higher mobility and went through molting faster. Not so acid environment with the pH 4.0 and that with the base reaction where the pH was 7.3 was less favorable for the above-mentioned experimental parameters.

Eagle's medium is too poor a medium for cultivation of stage III larvae of *A. simplex*. In that medium without the supplement of bovine serum and at pH 4.0 and 7.3, the larvae lived 50% shorter, showed lower mobility and did not go through molting as compared to the media supplemented with bovine serum. In that medium with the pH 2.0 the larvae survived longer, but did not molt. The supplement of bovine serum to the medium had a positive influence upon the mobility and survival of the larvae and speeded molting. That was consistent with the results obtained by other authors (IGLESIAS et al. 1997) who, using Eagle's medium for cultivation of *A. simplex* larvae, supplemented it with 20% of thermally inactivated bovine serum. The bovine serum is quite generally used for supplementing the media in cultivation of the larvae of nematodes (CLEELAND and LAURENCE 1962, JABLONOWSKI et al. 1997). CLEELAND and LAURENCE (1962) believe that supplementing M<sub>199</sub> medium for cultivation of *A. suum* larvae with 20% of bovine serum was more beneficial than 10% or 30% supplement. JABLONOWSKI et al. (1997) while applying Eagle's medium for cultivation of *A. suum* larvae decided that the supplement of 20% calf serum had the most beneficial influence upon the development and survival of the larvae. The lower, 10%, and higher, 30% and 50%, addition of calf serum was less favorable for development of *A. suum* larvae.

Taking into consideration own studies and the authors quoted above, in our conditions Eagle's medium supplemented with 20% of bovine serum and brought to acid pH is the most favorable for cultivation of L<sub>3</sub> larvae of *A. simplex*.

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