Acta Veterinaria Hungarica 45 (4), pp. 439–445 (1997)

ATTEMPTS TO ANALYSE *ANGUILLICOLA CRASSUS* INFECTION AND THE HUMORAL HOST RESPONSE IN EELS (*ANGUILLA ANGUILLA*) OF LAKE BALATON, HUNGARY

L. BÉKÉSI[1], S. HORNOK[1] and CS. SZÉKELY[2]

[1]Department of Parasitology and Zoology, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary; [2]Veterinary Medical Research Institute, Hungarian Academy of Sciences, H–1581 Budapest, P.O. Box 18, Hungary

(Received May 13, 1997; accepted July 10, 1997)

Since the introduction of *Anguillicola crassus* into Europe, anguillicolosis has been a considerable problem in several countries. From 1991, periodical eel mortality occurred in Lake Balaton, Hungary. However, eels with a worm burden of 20 to 50 parasites did not show severe swimbladder lesions, which observation cast doubts on the primary aetiological role of the parasite in the eel kill. In order to study the pathology of the infection, from the spring of 1996 until October of the same year, 51 eels were collected from two regions of Lake Balaton and examined for swimbladder changes. To detect humoral antibodies, an enzyme-linked immunosorbent assay (ELISA) was performed, using cuticular-oesophageal worm antigen. The results of the test show the applicability of the method. However, no direct correlation was found between antibody levels or the intensity of infection and the swimbladder lesions. The low level of specific antibodies and the increasing severity of swimbladder changes in the autumn suggest that parasite-induced immunity is insufficient to prevent reinfection.

Key words: *Anguillicola crassus*, host response, immunity

Anguillicola nematodes (Camallanata, Dracunculoidea, Anguillicolidae) parasitise the swimbladder of various eel species in different geographical regions of the world (Moravec and Taraschewski, 1988). Adult worms are located in the lumen of the swimbladder, while 3rd and 4th stage larvae inhabit the wall of that organ. Adult worms feed on host blood.

The East Asian species *Anguillicola crassus* Kuwahara, Niimi et Itagaki, 1974 was brought into Europe by the introduction of the Pacific eel (*Anguilla japonica*) at the end of the 1970s. The appearance of this parasite in Hungary was demonstrated in 1990 by Székely et al. (1991). Highly intensive infection caused by the new parasite coincided with a massive eel kill in Lake Balaton (Hungary) during the summer of 1991 (Molnár et al., 1991). Since the introduction of *A. crassus* into Europe, a wide range of research has been carried out on

0236–6290/97/$ 5.00

its prevalence and biology. However, only few data concerned the pathology of, and humoral host response to this nematodosis.

The purpose of the present study was to compare the intensity of *A. crassus* infection with the pathological changes and the humoral immune response elicited by this parasite.

Materials and methods

Eels were collected in two periods from Lake Balaton. The spring sampling (5 April) was performed from outlet-trapping catch at Siófok, while the summer-autumn sampling (25 June, 25 July, 1 October) from electro-fishery catch at Keszthely.

The live fish were immediately transported to the laboratory and kept in aquaria until used for analysis. Prior to dissection, fish were measured and blood was taken from the caudal vein. The sera were stored at –20 °C until used. After dissection, swimbladders were checked for external lesions and size. In the summer-autumn sampling period, air content was measured using a syringe attached to a hypodermic needle. During parasitological examination the swimbladders were opened, the wall thickness and lumen content were recorded, and the worms were counted. Based on the severity of swimbladder lesions, scoring was performed as described by Csaba et al. (1993). Briefly, the most serious lesions (score 7) were established when the lumen of swimbladder was full of detritus or exudate, wall thickness reached as much as 3 mm and the organ itself was distended or shrunken.

ELISA. Fifty adult worms were collected for antigen preparation. They were washed in PBS three times, their head-part with the oesophagus was detached under binocular stereo-microscope. Separated head-parts were homogenised on ice for 1 h in PBS containing 4 mM phenyl-methyl-sulphonyl-fluoride (PMSF) and 2 mM ethylenediamine tetra-acetic acid (EDTA). The mixture was centrifuged for 1 h at 4 °C on 45,000 *g*. The protein content of the supernatant was determined by the method of Bradford (1976). Antigen was stored at –20 °C until used. Pooled negative eel sera were used for the production of polyvalent anti-eel rabbit serum (Human Biological Co., Gödöllő, Hungary). ELISA plates (Grainer Labortechnik, Frickenhausen, Germany) were coated at 4 °C overnight with 100 µl of antigen solution per well containing 1 µg protein in 0.06 M carbonate buffer (pH 9.6). Rabbit anti-eel serum was diluted in 5% skimmed milk powder in PBS to 1:400. To detect rabbit immunoglobulins, polyvalent swine anti-rabbit immunoglobulins conjugated to horseradish peroxidase (DAKO, Glostrup, Denmark) were applied at a dilution of 1:1000 in SM-PBS at 37 °C for 4 h. As substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma Chemicals Co., St. Louis, Missouri) was used. The optical density (OD)

values were measured at 405 nm after 1 h using a Multiscan Plus microplate reader model RS-232 C (Labsystem, Helsinki, Finland).

ELISA was performed on 21 sera from infected eels selected at random. Sera of two eels from an uninfected eel-farm were used as negative controls.

Results

The mean size of the fish examined was 63.82 cm (SD: ± 7.72). The controls measured 40.00 cm. As shown in Table 1, the eels represented different stages regarding to the severity of swimbladder changes (inflammation, haemorrhages, pigmentation, thickened wall, dead worms, accumulation of debris and exudate in the lumen) and the intensity of the worm burden. All stages on the 1–7 score-scale were represented in the sample. The mean severity of swimbladder changes was 3.9 (SD: ± 1.81). Swimbladder inflammation (haemorrhages, wall thickening) and degenerative changes (dead worms, debris and pigmentation in the lumen) were more severe in the summer and autumn months (5.08) than in the spring (2.77). The mean worm burden proved to be 7.37 (SD: ± 10.32), but the figures showed a higher intensity of infection (9.56 worms per eel) in the summer-autumn period. The gas content of the swimbladder, if measured, showed a mean of 8.09 cm^3 with a high SD value (± 5.84). OD values detected by ELISA at the dilution of 1:40 represented a mean of 0.365 (SD: ± 0.081), and two uninfected control sera showed a mean of 0.281 (Table 1).

Discussion

The first report on the pathology of a related species, *A. globiceps* in Japan revealed considerable thickening of the swimbladder wall (Yamaguti, 1935). Similarly, thickening of the swimbladder wall was found in Anguillicola-infected eels in Europe (Van Willingen and Dekker, 1989; Haenen et al., 1994). Mortality due to the infection has been reported by Sarti et al. (1985), Hartmann (1987), Boon et al. (1989), Molnár et al. (1991), Csaba et al. (1993). The swimbladder lesions caused by *A. crassus* infection were studied by Molnár et al. (1993) and further details were added to the histology of changes caused by abnormally located 3rd and 4th stage larvae (Molnár, 1994). Haenen et al. (1996) studied the swimbladder lesions developing after experimental infection with 3rd stage larvae and the effect of infection on the humoral immune response. Studies on the humoral responses show the applicability of somatic crude antigens or antigens prepared from different parts of the parasite's body (Buchmann et al., 1991; Höglund and Pilström, 1994, 1995).

Table 1

Comparison of the swimbladder lesions, worm burden, air content and ELISA optical density (OD) values in *A. crassus* infections of eels collected from Lake Balaton in two periods of 1996

	Date	Size (cm)	Severity of swimbladder lesions on a scale of 1–7	Number of worms	Air content (cm^3)	ELISA OD values
	05. 04		2,2,2,1,1,5,3,2,3,3,5,4,3,3,2,2,6,1,1,5,3,3,2,4,2 (n = 26)	0–30	*	(n = 9)
Mean SD ±		63.19 9.58	2.77 1.36	5.26 8.38		0.359 0.053
	25. 06 25. 07 01. 10		3,4,5,4,6,7,4,2,24,6,6,6,6,4,6,5,7,6,5,4,6,6,6,7 (n = 25)	0–36	1–22 (n = 22)	(n = 12)
Mean SD ±		64.48 5.14	5.08 1.44	9.56 11.69	8.09 5.84	0.371 0.064
Total mean SD ±		63.82 7.72	3.90 1.81	7.37 10.32	8.09 5.84	0.365 0.081
	15. 04		Negative controls 1, 1 (n = 2)	0.0	2–3	
Mean SD ±		40.00 2.82	1.0 0.0	0.0	2.5 0.70	0.281 0.086

* not measured

The differences found in this study between uninfected control and suspected positive OD values are too slight, suggesting the low specificity of this cuticular-oesophageal antigen. Similar results have been reported by Höglund and Pilström (1994, 1995). Eels whose sera showed the highest OD values had worms in their swimbladders, and fish with minor swimbladder lesions (stages 2–4) gave higher OD values in our ELISA. The means of OD values obtained in the spring were lower than those found in the summer months (Table 1); however, Molnár (1994) found no seasonality in *A. crassus* larval invasion in Lake Balaton.

Hung et al. (1996) studied humoral antibodies of eels naturally infected by the microsporean parasite *Pleistophora anguillarum* by an ELISA and stated that the lower level of antibodies detected in some sera and mucous samples was evidently not caused by a lack of immunoglobulins *per se*. The recent findings of Haenen et al. (1996) suggest that humoral antibodies play a minor role in the development of immunity against *A. crassus*. This fact could be another reason for the low reactions obtained for naturally infected eels by ELISA. Van der Heijden et al. (1996) suspect the superiority of cellular reactions in the development of immunity against *A. crassus*.

In the case of recurrent infection, Molnár (1994) found that the majority of 3rd and 4th stage larvae do not reach the lumen of the swimbladder but undergo larval development in the oedematous subserosa of the organ and in the intestine without giving rise to a significant cellular host reaction. However, in advanced cases immobilised necrotic larvae were found, sometimes surrounded by a fibrous capsule. Fibrinous degeneration of collagenous fibres of the swimbladder wall, resembling an immunocomplex reaction, was observed by Csaba et al. (1993), who reported excessive thickening and oedema of swimbladder wall. This may be due to a hyperergic reaction of the organism challenged by the antigen stimulus from decomposing dead worms. The reactions are possibly accelerated by high water temperature, where the mortality of *A. crassus* infected eels generally occurs (Molnár et al., 1993). It is not clear whether the primary *A. crassus* invasion and the necrotic worm debris make the swimbladder unsuitable for further invasion (Van Banning and Haenen, 1990) or the specific host reaction is responsible for the different inflammatory reactions around the abnormally located adults and larvae stuck in several organs (Molnár, 1994). The correlation found between anguillicolosis and water oxygen content (Molnár, 1993) and the secondary bacterial infections diagnosed in infected eels with different swimbladder lesions (Csaba et al., 1993) suggest the possible involvement of other factors in the aetiology of eel mortality in Lake Balaton.

Studies on the cellular immune response of *A. crassus* infected eels could lead to a better understanding of the pathobiology of this parasitosis.

Acknowledgements

We thank Ms Cs. Takács for preparing the pilot design of the work. The authors wish to thank Dr. K. Molnár for useful advice during the work and manuscript preparation. We are grateful to Ms E. Papp, Z. J. Széll and Ms Zs. Csonka for their skilful technical assistance.

The work was supported by the Fisheries Fund of the Hungarian Ministry of Agriculture and partly by the National Research Fund of Hungary (OTKA), project no. 020044.

References

Bradford, M. M. (1976): A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Boon, J. H., Lokin, C. J. A., Ceusaters, R. and Ollevier, F. (1989): Some properties of the blood of European eel (*Anguilla anguilla*) and the possible relationship with *Anguillicola crassus* infestation. Aquaculture **76**, 203–208.

Buchmann, K., Pedersen, L. O. and Glamann, J. (1991): Humoral immune response of European eel *Anguilla anguilla* to a major antigen in *Anguillicola crassus* (Nematoda). Dis. Aquat. Org. **12**, 55–57.

Csaba, Gy., Láng, M., Sályi, G., Ramotsa, J., Glávits, R. and Rátz, F. (1993): The nematode *Anguillicola crassus* (Nematoda, Anguillicolidae) and its role in the death of eels in Lake Balaton during 1991 (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **48**, 11–21.

Haenen, O. L. M., Van Banning, P. and Dekker, W. (1994): Infection of eel *Anguilla anguilla* (L.) and smelt *Osmerus eperlanus* (L.) with *Anguillicola crassus* in the Netherlands from 1986-1992. Aquaculture **126**, 219–229.

Haenen, O. L. M., van Wijgaarden, T. A. M., van der Heijden, M. H. T., Höglund, J., Cornelissen, J. B. J. W., van Leengoed, L. A. M. G., Borgsteede, F. H. M. and van Muiswinkel, W. B. (1996): Effect of experimental infection with different doses of *Anguillicola crassus* (Nematoda, Dracuncoloidea) on European eel (*Anguilla anguilla*). Aquaculture **141**, 41–57.

Hartmann, S. (1987): Schwimmblasenwürmel beim Aal. Fischer and Teichwirt **38**, 2–3.

Höglund, J. and Pilström, L. (1994): Purification of adult *Anguillicola crassus* whole-worm extract antigens for detection of specific antibodies in serum from European eel (*Anguilla anguilla*) by ELISA. Fish & Shellfish Immunol. **4**, 311–319.

Höglund, J. and Pilström, L. (1995): Mechanical isolation and characterisation of antigens from adult *Anguillicola crassus*. Fish & Shellfish Immunol. **5**, 51–60.

Hung, H. W., Lo, C. F., Tseng, C. C. and Kou, G. H. (1996): Humoral immune response of Japanese eel, *Anguilla japonica* Temminck and Schlegel, to *Pleistophora anguillarum* Hoshina, 1951 (Microspora). J. Fish Dis. **19**, 243–250.

Molnár, K. (1993): Effect of decreased oxygen content on eels (*Anguilla anguilla*) infected by *Anguillicola crassus* (Nematoda: Dracunculoidea). Acta Vet. Hung. **41**, 349–360.

Molnár, K. (1994): Formation of parasitic nodules in the swimbladder and intestinal walls of the eel *Anguilla anguilla* due to infections with larval stages of *Anguillicola crassus*. Dis. Aquat. Org. **20**,163–170.

Molnár, K., Székely, Cs. and Baska, F. (1991): Mass mortality of eel in Lake Balaton due to *Anguillicola crassus* infection. Bull. Eur. Ass. Fish Pathol. **11**, 211–212.

Molnár, K., Baska, F., Csaba, Gy., Glávits, R. and Székely, Cs. (1993): Pathological and histopathological studies of the swimbladder of eel *Anguilla anguilla* infected by *Anguillicola crassus* (Nematoda: Dracunculoidea). Dis. Aquat. Org. **155**, 41–50.

Moravec, F. and Taraschewski, H. (1988): Revision of the genus Anguillicola Yamaguti, 1935 (Nematoda, Anguillicolidae) of the swimbladder of eels, including descriptions of two new species *A. novazelandiae* sp. n. and *A. papernae* sp. n. Folia Parasitol. **35**, 125–146.

Sarti, M., Giorgetti, G. and Brisinello, W. (1985): A new problem for intensive eel rearing in Italy: *Anguillicola australiensis*. Proc. Eur. Ass. Fish Pathol. Congr., Montpellier, September 1985, p. 95.

Székely, Cs., Láng, M. and Csaba, Gy. (1991): First occurrence of *Anguillicola crassus* in Hungary. Bull. Eur. Ass. Fish Pathol. **11**, 162–163.

Van Banning, P. and Haenen, O. L. M. (1990): Effect of the swimbladder nematode *Anguillicola crassus* in wild and farmed eel *Anguilla anguilla.* In: Perkins, F. O. and Cheng, T. C. (eds) Pathology in Marine Science. Academic Press, New York, pp. 317–330.

Van der Heijden, M. H. T., Roijakkers, J. B. M. A., Booms, G. H. R., Rombout, J. H. W. M. and Boon, J. H. (1996): Production, characterisation and applicability of monoclonal antibodies to European eel (*Anguilla anguilla* L., 1758) immunoglobulin. Vet. Immunol. Immunopathol. **15**, 151–164.

Van Willingen, J. and Dekker, W. (1989): 1988 update on Anguillicola in Dutch outdoor waters. European Inland Fisheries Advisory Commission (FAO) Working Party of Eel. Oporto, Portugal, 30 May to 5 June 1989, FAO, Rome.

Yamaguti, S. (1935): Studies on the helminth fauna of Japan. Pt. 9. Nematodes of fishes, 1. Jap. J. Zool. **6**, 338–386.