Short communication

Exclusion of IgD, IgT and IgM positive immune cells in *Ichthyophonus* induced granulomas in rainbow trout *Oncorhynchus mykiss* (Walbaum)

A Al-Jubury^a, S LaPatra^b, N D Christensen^a, S Zuo^a, C Tafalla^c and K Buchmann^a

^aDepartment of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark

^bClear Springs Foods, Inc., Research Division, Buhl, Idaho, USA

^cCentro de Investigación en Sanidad Animal, (CISA-INIA), Valdeolmos, Madrid, Spain

Key words: Rainbow trout, *Ichthyophonus*, immune response, immune evasion

Running head: *Ichthyophonus* and immunity

Correspondence

Kurt Buchmann, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 7, DK-1870 Frederiksberg C, Denmark. (e-mail: kub@sund.ku.dk)

Abstract

Infections with *Ichthyophonus* (Plehn & Mulsow) cause a serious and granulomatous disease in rainbow trout *Oncorhynchus mykiss* (Walbaum) affecting several organ systems. The pathology associated with infection of this protist is known but the specific cellular reactions associated with this pathogen are less well known. We investigated the cellular immune reaction towards the parasite by employing immunohistochemical techniques showing to what extent IgD, IgT and IgM positive lymphocytes were involved. Samples from heart, liver and head kidney were collected from naturally infected rainbow trout. Positive immune cells were present in all organs but it was found that a wide zone around the parasite was devoid of Ig positive cells. This suggests that the parasite induces some immune-suppression in the host securing a local antibody poor microenvironment.

Ichthyophonus (Plehn & Mulsow) is a protistan fish parasite with a broad host range (Ragan *et al.* 1996). It infects marine and freshwater fish species worldwide and produces a granulomatous systemic disease in vascularized organs such as heart, liver and kidney (Spanggaard *et al.* 1994; McVicar 1999). The susceptibility of various fish species is considerably variable. Atlantic herring (*Clupea harengus* L.) are highly susceptible (Rahimian & Thulin 1996) while rainbow trout *Oncorhynchus mykiss* (Walbaum) show a medium level of susceptibility and catfish (*Ameiurus nebulosus* Lesueur) are relatively resistant to this parasite (McVicar 1982; 1999). The main characteristics of the infection are multinucleated spherical parasite cells with a double wall and the tissue around the parasite contains inflammatory cells early in the infection whereas fibrous tissue dominates late in the infection (McVicar & McLay 1985; Franco-Sierra & Alvarez-Pellitero 1999; McVicar 1999). Despite the numerous histopathological studies conducted we have no information

about specific lymphocytes involved with *Ichthyophonus* in rainbow trout. In teleost fish, different B lymphocyte subsets can be found defined by their expression of immunoglobulin types (Igs) present in fish. IgD⁺IgM⁺ B cells are present in all teleost species analyzed thus far and represent the majority of B lymphocytes in fish. In addition, IgD⁺IgM⁻ B cells have been reported in channel catfish *Ictalurus punctatus* Rafinesque) (Edholm et al. 2010) and in rainbow trout gills (Castro et al. 2014), but their role in immunity has not been yet clarified. Finally, a lineage of B cells uniquely expressing IgT has been reported in some species (Schorpp et al. 2006; Zhang et al. 2010), where they seem particularly important for mucosal responses. In this study, immunohistochemical techniques were applied in order to characterize to what extent B lymphocytes take part in the immune response against *Ichthyophonus* in naturally infected rainbow trout. For this, cells bearing the different immunoglobulin (Ig) types present in rainbow trout (IgM, IgT and IgD) were recorded in infected fish.

Fish and sampling: Samples were collected at a commercial freshwater trout farm located in Idaho, USA, during the monthly health routine monitoring. It was known that the pathogen could occur in the farm but there was no external signs or mortality recorded. From experience it was known that older fish had a higher probability of infection and three infected rainbow trout (age 500 days old, 7250 degree days, body weight 450 - 550 g) were sampled from three different raceways at the farm. Small white spots or granuloma formations were visible on the heart and liver and an *Ichthyophonus* infection was confirmed in all fish by heart explant culture and by observing the germination of spherical bodies. These fish were being reared in raceways with a constant ambient water temperature at 14.5 °C. Fish were fed a dry pelleted diet that contained 45% protein and 20% fat. Following euthanization heart, liver and head kidney were collected and fixed in 10% neutral buffered formalin for 24 h.

IHC: After fixation tissue specimens were transferred to 70 % ethanol and preserved for two months at RT before processing. Briefly, the tissue went through dehydration and paraffin embedding. Tissue

sections (4 μm) were deparaffinized with xylene, and subjected to immunohistochemical staining (Deshmukh *et al.* 2013). Specific monoclonal antibodies reacting against trout either IgM, IgT or IgD (Olsen *et al.* 2011; Jørgensen *et al.* 2011; Ramirez-Gomez *et al.* 2012) were applied. HiDef Detection™ HRP polymer system (Cell Marque, US) was then used and the bound antibody was visualized by incubating slides in carbazole solution for 10-12 min. Slides were counter-stained with Mayer's hematoxylin (Dako, Denmark), mounted in a water soluble mounting medium (Aquamount, Merck, UK) and observed under a compound microscope (Leica DM5000B) and digital images created using a Leica MC170 HD camera.

Measuring cell numbers around the parasite: Enumeration of Ig-positive cells was conducted at 400x magnification by counting the total number of Ig positive cells in four concentric zones (width 10 μm) (zone 1-4) around the *Ichthyophonus* spores (Fig. 1). Cells encircling 10 parasite spores (with diameters ranging from 18-142 μm , mean $71.2 \pm \text{SD } 39.98$) were counted in each section, and 3 different sections were used from each organ from each fish (total three fish) except for head kidney which was only recovered from one fish. Thus, for liver and heart cells surrounding 90 parasites were enumerated and cells surrounding 30 parasites in head kidney were counted. The spore density in different organs and fish did not differ significantly.

An IHC approach was used to investigate the cellular immune reaction involving Ig-positive lymphocytes towards *Ichthyophonus* in rainbow trout. IgD, IgT and IgM positive cells were detected in all organs investigated (Fig. 2) but the cells became increasingly less frequent near the parasite surface. A few IgD and IgM positive cells were noticed in Zone 1, 2 and 3 whereas no IgT-positive cells at all were found in that area. Significantly ($P < 0.05$) more IgD, IgT and IgM positive cells were detected in zone Zone 4 compared to the three inner zones (Table 1).

Previous histopathological studies have noted that *Ichthyophonus* in fish tissue often is associated with occurrence of melanomacrophages, giant cells, melanin and a thick layer of fibrocysts (Rahimian 1998; McVicar 1999). In this study it was shown that IgD, IgT and IgM positive cells are widely distributed in rainbow trout liver, heart and head kidney. It is noteworthy that the number of IgM and IgD positive cells differed which may suggest that not all IgM cells carry IgD. However, these cells were absent or very rare in a wide zone around the parasite spanning 30 µm from the surface of the parasite in the same tissue. It is well documented that fish hosts respond against the ciliate *Ichthyophthirius multifiliis* by eliciting an antibody production (Clark *et al.* 1992; Jørgensen *et al.* 2011; Olsen *et al.* 2011; Xu & Klesius 2013; Xu *et al.* 2013) and IgM and IgT producing cells are often found near this ciliate in infected trout tissue. In addition, direct binding of trout IgM and IgT to *Ichthyophthirius* has been demonstrated by the IHC technique by several authors (Jørgensen *et al.* 2011; Olsen *et al.* 2011; Xu *et al.* 2013). However, such a binding of trout Ig to *Ichthyophonus* was not recorded in the present work by the same IHC method and our observations may suggest that this parasite has the capability to repel or evade Ig positive cells. *Ichthyophonus* induced depression of protein production in rainbow trout has in fact been described previously by Rand and Cone (1990). As seen in Fig. 2 the *Ichthyophonus* parasite is surrounded by a thick wall that may be composed of 1-7 layers (McVicar 1982; Rahimian 1998). Such a thick cell wall may have evolved to isolate and protect the delicate membrane and cytoplasmic elements of the parasite from immune molecules including Ig, complement and various reactive cells. In addition, a wide zone around the parasite appears to be depleted of Ig positive cells and this suggests that the parasite may apply mechanisms for disguise or evasion in its protection against host attacks.

Acknowledgements

The present work was supported by the European Commission through the Horizon 2020 grant H2020-634429 ParaFishControl – Advanced tools and research strategies for parasite control in European farmed fish.

References

- Castro R., Bromage E., Abos B., Pignatelli J., Gonzalez Granja A., Luque A. & Tafalla C. (2014) Ccr7 is mainly expressed in teleost gills, where it defines an Igd+Igm- B lymphocyte subset. *Journal of Immunology* **192**, 1257-1266.
- Clark T.C., McGraw R.A. & Dickerson H.W. (1992) Developmental expression of surface antigen genes in the parasitic ciliate *Ichthyophthirius multifiliis*. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6363-6367.
- Deshmukh S., Kania P.W., Chettri J.K., Skov J., Bojesen A.M., Dalsgaard I. & Buchmann K. (2013) Insight from molecular, pathological, and immunohistochemical studies on cellular and humoral mechanisms responsible for vaccine-induced protection of rainbow trout against *Yersinia ruckeri*. *Clinical and Vaccine Immunology* **20**, 1623-1641.
- Edholm E.S., Bengten E., Stafford J.L., Sahoo M., Taylor E.B., Miller N.W. & Wilson M. (2010) Identification of two Igd+ B cell populations in channel catfish, *Ictalurus punctatus*. *Journal of Immunology* **185**, 4082-4094.
- Franco-Sierra A. & Alvarez-Pellitero P. (1999) The morphology of *Ichthyophonus* sp. in their mugilid hosts (Pisces: Teleostei) and following cultivation *in vitro*. A light and electron microscopy study. *Parasitology Research* **85**, 562-575.
- Jørgensen L.V.G., Heinecke R.D., Skjødtt K., Rasmussen K.J. & Buchmann K. (2011) Experimental evidence for direct *in situ* binding of IgM and IgT to early trophonts of *Ichthyophthirius multifiliis* (Fouquet) in the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **34**, 749-755.

- McVicar A.H. (1982) *Ichthyophonus* infections of fish. In: *Microbial diseases of fish* (ed by R.J. Roberts), pp. 243–269. Academic Press, London.
- McVicar A. H. & Maclay H.A. (1985) Tissue response of plaice, haddock, and rainbow trout to the systemic fungus *Ichthyophonus*. In: *Fish and shellfish pathology* (ed by A.E. Ellis), pp. 329–346. Academic Press, London
- McVicar A.H. (1999) *Ichthyophonus* and related organisms. In: *Fish Diseases and Disorders 3*, Viral, bacterial and fungal Infections (ed by P.T.K. Woo & D.W. Bruno), pp. 661–687 CABI Publishing, New York.
- Olsen M.M., Kania P.W., Heinecke R.D., Skjødtt K., Rasmussen K.J. & Buchmann K. (2011) Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: molecular and immunohistochemical studies. *Fish & Shellfish Immunology* **30**, 859–869.
- Ragan M.A., Goggin C.L., Richard J.C., Cerenius L., Jamieson A.V.C., Plourde S.M., Rand T.G. & Soderhall K. (1996) A novel clade of protistan near the animal-fungal divergence. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 11907-11912.
- Rahimian H. & Thulin J. (1996) Epizootiology of *Ichthyophonus hoferi* in herring populations off the Swedish west coast. *Diseases of Aquatic Organisms* **27**, 187-195
- Rahimian H. (1998) Pathology and morphology of *Ichthyophonus hoferi* in naturally infected fishes off the Swedish west coast. *Diseases of Aquatic Organisms* **34**, 109-123.
- Rand G. T. & Cone D. K. (1990) Effects of *Ichthyophonus hoferi* on condition indices and blood chemistry of experimentally infected rainbow trout (*Oncorhynchus mykiss*). *Journal of Wildlife Diseases* **26**, 323-328.

- Ramirez-Gomez F., Greene W., Rego K., Hansen J.D., Costa G., Kataria P. & Bromage E.S. (2012) Discovery and characterization of secretory IgD in rainbow trout: secretory IgD is produced through a novel splicing mechanism. *Journal of Immunology* **188**, 1341–1349.
- Schorpp M., Bialecki M., Diekhoff D., Walderich B., Odenthal J., Maischein H.M., Zapata A.G. & Boehm T. (2006) Conserved functions of ikaros in vertebrate lymphocyte development: Genetic evidence for distinct larval and adult phases of t cell development and two lineages of b cells in zebrafish. *Journal of Immunology* **177**, 2463-2476.
- Spanggaard B., Gram L., Okamoto N. & Huss H. H. (1994) Growth of the fish-pathogenic fungus, *Ichthyophonus hoferi*, measured by conductimetry and microscopy. *Journal of Fish Diseases* **17**, 145–153.
- Xu D.H. & Klesius P.H. (2013) Comparison of serum antibody responses and host protection against parasite *Ichthyophthirius multifiliis* between channel catfish and channel × blue hybrid catfish. *Fish & Shellfish Immunology* **34**, 1356-1359.
- Xu Z., Parra, D., Gomez D., Salinas I., Zhang Y.-A., Jørgensen L. v. G., Heinecke R. D., Buchmann K., LaPatra S. & Sunyer J. O. (2013). Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proceedings of the National Academy of Sciences of the United States of America* **110** (32), 13097-13102.
- Zhang Y.A., Salinas I., Li J., Parra D., Bjork S., Xu Z., LaPatra S.E., Bartholomew J. & Sunyer J.O. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature Immunology* **11**, 827-835.

Table 1. Enumeration of IgM, IgT and IgD positive cells in concentric zones around *Ichthyophonus* in liver, heart and head kidney of rainbow trout. Mean $\pm$ SD of Ig positive cells per zone (width 5 μ m). A total of 90 zones were counted for liver and heart and 30 zones for headkidney.*: P<0.05

Liver	Zone 1	Zone 2	Zone 3	*Zone 4
IgM	0.44 $\pm$ 0.83	0.56 $\pm$ 1.07	0.67 $\pm$ 1.25	5.73 $\pm$ 10.30
IgT	0	0	0	2.49 $\pm$ 3.12
IgD	0.22 $\pm$ 0.63	0.33 $\pm$ 0.94	0	1.46 $\pm$ 1.42
Heart				
IgM	0	0	0	6.92 $\pm$ 7.42
IgT	0	0	0	3.43 $\pm$ 3.55
IgD	0.11 $\pm$ 0.31	0.07 $\pm$ 0.19	0.11 $\pm$ 0.31	4.34 $\pm$ 9.32
Head kidney				
IgM	0	0	0	0
IgT	0	0	0	3.17 $\pm$ 0.45
IgD	0	0	0	0.9 $\pm$ 0.14

Figure legends

Fig. 1. Counting of total numbers of Ig positive cells was conducted in four concentric zones (width 10 μm) around the parasite

Fig. 2. Immunohistochemical detection of Ig positive cells in rainbow trout tissues infected with *Ichthyophonus hoferi* tissues. (A) Infected heart tissue showing thick walled parasites. Absence of positive cells. (B) IgT positive cells in head kidney tissue. (C) IgD positive cells in head kidney tissue. (D) IgM positive cells in head kidney. Scale bar 50 μ m.