

# Antibody response of brown trout *Salmo trutta* injected with pathogenic *Saprolegnia parasitica* antigenic extracts

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**ABSTRACT:** Brown trout *Salmo trutta* injected with antigenic extracts from a pathogenic isolate of *Saprolegnia parasitica* developed specific antibodies that were detected by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF) and Western blotting (WB), but not by immunodiffusion (ID). Three groups of five 2 yr old brown trout were injected intraperitoneally with 3 different antigenic extracts: small hyphal fragments (HF) and soluble extracts from sonicated mycelia grown in medium with or without  $\beta$ -sytosterol (SEB and SE, respectively). In the 2 groups injected with SE and SEB, antibodies were found in 66.7% of the serum samples by ELISA, 54.5% by IF and 48.5% by WB. In the group injected with HF, only 1 trout survived the experiment, and in this fish only 1 sample was positive by ELISA. The results obtained by ELISA and IF were similar and show that there is cross-reaction between the antigens used. By WB, the proteins most frequently recognised were 2 proteins of 25 and 29 kDa. No significant differences were found in the groups injected with SE or SEB.

**KEY WORDS:** *Saprolegnia parasitica* · Brown trout · Antibody response · Immunological techniques

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## INTRODUCTION

Saprolegniosis caused by *Saprolegnia* species is an important freshwater fish disease, which often affects wild and farmed salmonids. In salmonid fish farms, saprolegniosis mainly affects broodfish and incubating eggs, causing major financial losses (Pickering & Willoughby 1982, Noga 1993, Bruno & Wood 1999). The disease appears as cotton-wool-like tufts on the body surface causing destruction of the skin and/or fins due to cellular necrosis by hyphal penetration that is generally restricted to the epidermis and dermis. Once infected, fish do not recover unless treated, and death occurs due to osmoregulatory failure caused by destruction of the superficial tissues (Pickering & Willoughby 1982, Hatai & Hoshiai 1994).

It is thought that an important defence mechanism of fish against saprolegniosis is the mucus layer of the epidermis, which acts as a physical barrier, and inter-

nal cellular responses based on non-specific phagocytic activity of inflammatory cells; it is, however, uncertain whether the specific immune response is involved (Hodkinson & Hunter 1970, Sohnle & Chusid 1983, Wood et al. 1986, Pickering 1994). Recently, Szalai et al. (1994) have suggested that reduced levels of a humoral factor (phosphorylcholine-reactive protein, PRP) play an important role in increasing the susceptibility of channel catfish *Ictalurus punctatus* to infection by *Saprolegnia* spp. Moreover, Thompson et al. (1997) have shown an antibody response by snakehead *Channa striata* to *Aphanomyces invaderis*, another important oomycete fish pathogen. However, knowledge about the production of specific antibodies against *Saprolegnia* infection in fish, particularly in brown trout *Salmo trutta*, is scarce, and the results are contradictory, depending on the species studied, water temperature and the antigens and serological techniques used (Hodkinson & Hunter 1974, El-Feki 1987,

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Sohnle & Chusid 1983, Bly et al. 1993). Hodkinson & Hunter (1974) investigated the influence of culture conditions on the antigenic products of *Saprolegnia* and found that precipitating reactions only occurred between salmon serum and antigens from *Saprolegnia* grown in medium containing  $\beta$ -sytosterol.

The aim of the present research was to study the capacity of brown trout injected with antigenic extracts of *Saprolegnia parasitica* pathogenic to salmonids to produce specific serum antibodies.

## MATERIALS AND METHODS

**Fish and antigenic extracts.** Three different groups of five 2 yr old brown trout ( $225 \pm 70$  g weight and  $26.5 \pm 2.8$  cm length) were injected intraperitoneally with 0.2 ml of antigenic extracts in phosphate-buffered saline (PBS) (approximately 200  $\mu$ g of total protein) twice at 2 wk intervals. The extracts were mixed with Freund's complete adjuvant at a ratio of 3:1 in the first injection and with Freund's incomplete adjuvant (1:1) in the second injection. A fourth group was injected only with PBS and adjuvant as a negative control group. Three different antigenic extracts were prepared using the pathogenic isolate TRU 8 of *Saprolegnia parasitica* (formerly *Saprolegnia* sp.; Fregeneda-Grandes et al. 2000, 2003): (1) small hyphal fragments (HF) obtained after sonication of formalised-mycelial mats growing on glucose-yeast broth (GY: 1% glucose, 0.25% yeast extract); (2) soluble extract (SE) from sonicated formalised-mycelia growing on GY; and (3) SE from sonicated formalised-mycelia growing on GY supplemented with 20 mg l<sup>-1</sup> of  $\beta$ -sytosterol (SEB). The experiment was carried out between April and June 2004 at a fish farm in small raceways situated in the open and with a continuous supply of well water. The water temperature varied from 9 to 14°C. Two wk after the second injection, blood samples were obtained by puncturing the caudal vein of all surviving fish 4 times at 2 wk intervals. Fish were anaesthetised with 50 mg l<sup>-1</sup> of MS 222 (tricaine methane sulphonate). After clotting overnight at 4°C, the blood samples were centrifuged at 1000 *g* for 45 min to obtain the serum. The sera were stored at -20°C until use.

**Antibody detection.** The presence of antibodies in the serum was analysed by enzyme-linked immunosorbent assay (ELISA), immunodiffusion (ID) and Western blotting (WB) using SE and by immunofluorescence (IF) with HF extract. In all these techniques mouse or rabbit polyclonal antiserum (obtained after immunisation with HF or SE antigens) and a pre-immune serum were used as positive and negative controls, respectively.

ELISA was performed in 96-well polystyrene plates (Costar, Corning), which were coated with SE diluted in PBS (20  $\mu$ g ml<sup>-1</sup>) overnight at 4°C and blocked with PBS plus 1% of bovine serum albumin (PBS-BSA) for 1 h at 37°C. Serial 2-fold dilutions (1:20 to 1:2560) of trout serum in PBS-BSA were added and incubated for 3 h at room temperature; then the plates were washed 3 times with PBS plus 0.05% Tween-20. The plates were then incubated for 1 h at room temperature with anti-rainbow trout IgM monoclonal antibody (Mab 4C10) (Thunvander et al. 1990) diluted 1:1000 in PBS-BSA; a further washing cycle was carried out and the plates were incubated for 1 h at room temperature with anti-mouse immunoglobulins conjugated with peroxidase (Sigma-Aldrich) diluted 1:1000 in PBS-BSA. After a final washing step, OPD substrate (DakoCytomation) was added, and the reaction was stopped after 15 min at room temperature by addition of 0.5 M H<sub>2</sub>SO<sub>4</sub>. Optical density (OD) was measured at 492 nm, and ELISA titre was defined as a reciprocal value of the highest serum dilution giving an OD value >0.5 (mean OD values of the negative control sera plus 3 times the standard deviation). Only titres of 40 or higher were considered positive.

ID was performed on microscope slides, which were coated with 4 ml of 1% veronal-buffered agarose (Pronadisa-Hispanlab). Groups of 7 holes (1 central and 6 peripheral) of 3 mm diameter were cut in the gel. Various distances between neighbouring holes and dilutions of SE and trout sera were used in different experiments. Holes were filled with 12  $\mu$ l of antigen or serum dilution, and the slides were incubated in a wet chamber for 24 to 72 h. Following incubation, the slides were washed first with 5% sodium citrate for 1 h, then with PBS for 24 h and finally with distilled water for 2 h. Slides were then stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

WB analysis was performed in order to identify individual proteins of SE recognised by the trout sera. Purification of SE, SDS-PAGE and sample transfer to nitrocellulose membranes were made as described by Lilley et al. (1997), except that Proteinase K solution was not used and protein concentration was determined by Branford's method and adjusted to 1 mg ml<sup>-1</sup>. Following transfer, the nitrocellulose membrane was washed with distilled water for 5 min and then blocked with 5% dry skimmed milk in tris-buffered saline (TBS). After washing 3 times for 5 min with TBS, the nitrocellulose membranes were cut into strips of 3 mm width. Trout sera diluted 1:20 in TBS were added and incubated for 3 h at room temperature. After a further washing cycle, the strips were incubated for 1 h at room temperature with Mab 4C10, followed by washing



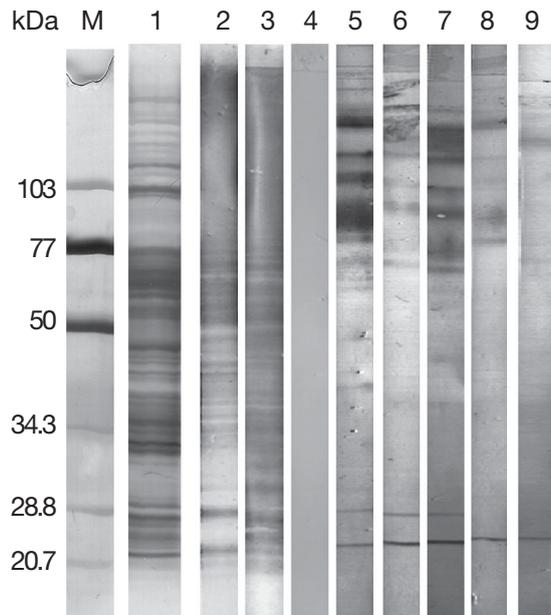


Fig. 1. Western blot on nitrocellulose membrane after SDS-PAGE (Gel 4 to 20%) of *Saprolegnia parasitica* soluble extract. Lane M: low-range molecular weight protein marker (Bio-Rad, Cat. No. 161-0304); Lane 1: silver-stained SDS-PAGE of soluble extract, SE; Lane 2: rabbit polyclonal positive serum; Lane 3: mouse polyclonal positive serum; Lane 4: negative trout serum; Lanes 5 to 9: positive trout sera

## DISCUSSION

The results obtained by other authors in relation to the detection of anti-*Saprolegnia* serum antibodies are contradictory. Thus, El-Feki (1987) did not find any serological response in carp intraperitoneally injected with soluble or hyphal extracts of *S. diclina* or in carp experimentally infected with *S. diclina*, but did detect antibodies in rainbow trout with saprolegniosis from a fish farm. Moreover, Sohnle & Chusid (1983) and Bly et al. (1993) did not find precipitating antibodies in rainbow trout or channel catfish, respectively, experimentally inoculated with *Saprolegnia* sp. However, Hodkinson & Hunter (1970) found antibodies in 93% of the wild salmon studied, but only 66% of these were colonised by *Saprolegnia* sp. In all these studies, the technique used was a double-diffusion immunoprecipitation test. In the present study no positive sample was found by ID in the immunised brown trout. However, when ID was performed using serum samples from mice and rabbit immunised with HF or SE, several positive samples were found (and used as positive controls). These findings may reflect differences in the immune response against *Saprolegnia* sp. antigens between fish and mice or rabbit. Thus, in WB analysis, we found that mice and rabbit sera recognised more proteins (17 to 18) than trout sera (1 to 8).

In contrast to the results obtained by Hodkinson & Hunter (1974), no significant differences were found when using soluble extracts from culture media with or without  $\beta$ -sytosterol. The response to SE and SEB may be better than to HF extract, although it is difficult to prove in our study because only 1 trout survived 4 wk in the group immunised with the HF antigen.  $\beta$ -glucans are one of the major components of the *Oomycetes* hyphal wall. Moreover,  $\beta$ -glucans have been shown to stimulate non-specific immunity in fish such as lysozyme, complement activity and phagocytic activation (Robertsen et al. 1994), which may be involved in the immune response of fish to fungal infections. Thus, Álvarez et al. (1988, 1995) found increased phagocytic activity in macrophages, but also in the sinusoidal endothelial cells of the peripheral lymphoid organs (spleen, kidney and thymus) of *Saprolegnia*-infected wild brown trout. In some cytoplasmic vesicles of these cells they also found an electro-dense content that may represent components of the fungal wall. They speculated that the hypertrophic aspect and degeneration of the endothelial cells observed in the lymphoid organs of sick trout would appear to indicate a possible toxic effect of this material. These findings may explain the high mortality rate observed in the current study in the group of trout injected with HF extract.

In conclusion, the present work has shown that brown trout injected with antigenic extracts from a pathogenic isolate of *Saprolegnia parasitica* developed specific antibodies that can be detected by standard immunological techniques. These are initial experiments, so further studies will be necessary, involving a greater number of serum samples from fish infected both experimentally and naturally with *Saprolegnia* for a better understanding of the specific immune response against *Saprolegnia* infections. These studies will include characterisation of the more antigenic proteins of *Saprolegnia* extracts and their usefulness for fish vaccination.

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