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Antibacterial activity of rainbow trout plasma: *in vitro* assays and proteomic analysis

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Motivation and Aim: The ability of body fluids and secretions to kill bacteria is an important aspect of immune defence. Although many antimicrobial factors have already been discovered in fish plasma, the biochemical and physiological consequences of contact between bacteria and the complex of bacteria-binding proteins present in the blood remain poorly understood. Previously, a simple approach for screening potential immunomodulators was developed using live bacteria as bait for affinity-bound proteins from biological fluids [1–3]. The high-throughput mass spectrometry used in these studies provides unique information on protein–protein interactions between microorganisms and the host blood proteins as well as real-time monitoring of adaptive metabolic rearrangements of pathogens during invasion. This approach is therefore very promising for screening previously uncharacterised proteins with antimicrobial activity in biological fluids from different species.

In this study, we investigated the natural antibacterial activity of blood plasma of cultured rainbow trout *Oncorhynchus mykiss*. First, we studied the growth dynamics, morphology, and protein profile of the fish pathogen *Aeromonas hydrophila* when incubated in plasma from trout from two different fish farms. Second, a comparative analysis of fish plasma samples with different levels of antimicrobial activity was performed. To identify molecules with signaling functions and bactericidal activity, the study design also included an investigation of trout proteins interacting with live *A. hydrophila in vitro*. Finally, changes in the pathogen proteome in response to contact with the host blood plasma were studied to elucidate the mechanisms of action of fish antimicrobial factors.

Methods and Algorithms: Fish blood plasma was collected from two independent populations of rainbow trout *O. mykiss.* Fish from the first farm (Farm A, Lake Onego, Northwest Russia) were characterised as relatively healthy. Fish from the second fish farm (farm N, Angara River, Eastern Siberia) have chronic bacterial disease associated with intestinal inflammation, rectal prolapse, and skin hyperemia. Sequencing of the

hypervariable V3-V4 region of the 16S rRNA gene in inflamed tissue revealed an overwhelming number of reads belonging to the salmonid pathogen *F. psychrophilum*.
Native blood plasma from fish caught in farm A was pooled and further referred to as AP and plasma from fish caught in farm N was referred to as NP. The low molecular weight fraction (< 10 kDa) was obtained by plasma filtration using Amicon Ultra-0.5 Millipore concentrators. Denatured plasma was obtained by heating at 56 °C for 30 min.
The antimicrobial activity of native, filtered and denatured AP and NP trout plasma was assessed by the growth inhibition zone at the plasma application sites on Mueller–Hinton agar (MHA) seeded with a bacterial culture of *Aeromonas hydrophila*. The growth dynamics of *A. hydrophila* incubated in fish plasma was assessed by the optical density of the bacterial biomass at 620 nm. The viability of *A. hydrophila* after 4 h incubation in fish plasma was assessed by counting the subsequent growth of bacterial colonies on 1.5 % MHA. Morphology and cell integrity of *A. hydrophila* after incubation with fish plasma were assessed by fluorescence microscopy using the LIVE/DEAD® BacLight Bacterial Viability Kit (Invitrogen).
For the proteomic assay, the native plasma was analysed as is, while the bacterial cells were pre-treated for protein isolation. After 4 h incubation in AP, NP or denatured AP plasma, suspensions of *A. hydrophila* were washed in buffer to remove unbound trout proteins by a triple sedimentation-resuspension procedure, followed by cell lysis in 2.5 % SDS. The collected samples were homogenised and trypsinised according to standard procedures.
Proteomic analysis of trout and bacterial peptides was performed using an Ultimate 3000 RSLCnano chromatographic HPLC system (Thermo Scientific) coupled to a Q-exactive HFX mass spectrometer (Thermo Scientific). Protein identification in the mass spectra was performed using MaxQuant v. 1.6.3.4 via the Andromeda search algorithm. The UniProt sequence databases UP000193380 (*O. mykiss*) and UP000000756 (*A. hydrophila*) were used to identify proteins in the samples. Protein abundance analysis was based on label-free quantification (LFQ). Differential expression between samples was analysed using the *Limma* R package.
Results: The study demonstrated the inhibition of *A. hydrophila* growth by native blood plasma obtained from trout infected with *F. psychrophilum* (antimicrobial plasma, AP), suggesting the production of bacteriostatic humoral immune factors and/or antibodies in diseased fish. No antimicrobial activity against *A. hydrophila* was observed in the plasma from healthy trout (non-antimicrobial plasma, NP), demonstrating that antigen loading is necessary for the humoral immune response in cultured rainbow trout. The level of bactericidal activity of the 10-kDa cut-off filtered or denaturated AP and NP plasma was low, suggesting that the inhibitory effect of AP is related to the protein fraction.
After 4 hours of incubation in AP, but not NP plasma, *A. hydrophila* lost its ability to divide on the culture media, although, according to lifetime staining, most of the cells were not destroyed. Microscopy revealed changes in the morphology of AP-treated bacterial cells, which were irregular in length, chained together and agglutinated.
Circulating immunoglobulins have been suggested as candidates for the major antimicrobial factor in the AP plasma. Immunoglobulins isoforms were shown to differ in their ability to bind bacterial cells. The isoform abundant in AP plasma was found to adhere to *A. hydrophila* cells treated with both AP and NP. In turn, another two Ig isoforms were identified exclusively in the lysate of bacteria treated with bacteriostatic AP. These proteins were not detectable in the mass spectra of native plasma, suggesting

that minor but highly selective components of AP could play role in pathogen recognition.

Increased level of the circulating isoform of C-type lectin was found in AP plasma, but not in the washed lysate of either AP- or NP-treated bacteria. In turn, another C-type lectin (ladderlectin) was found to be retained exclusively in the lysate of *A. hydrophila* treated with antimicrobial AP plasma. Thus, lectins selectively retained in bacterial lysates can be suggested as another candidate for the role of the antimicrobial factor in trout challenged with an *F. psychrophilum* infection.

Finally, we have shown that treatment with AP plasma severely affects protein biosynthesis in bacteria, including the loss of components of the translation machinery. This led to the disorganisation of core cellular processes and to the observed inhibition of cell growth, although it is not yet clear which host molecules might be the trigger for this process.

Conclusion: In our screening study, we found that antimicrobial proteins in the plasma of cultured rainbow trout infected with *F. psychrophilum* inhibit the growth of *A. hydrophila in vitro* by arresting protein biosynthesis in the bacteria rather than by causing cell perforation. Cross-reactive antibodies and the antimicrobial protein ladderlectin, which is capable of cell agglutination, are thought to be responsible for the bacteriostatic properties of the fish plasma. The applied affinity approach, in which bacterial cells were used as bait, greatly expanded the range of plasma proteins analysed, and allowed the capture of minor components that selectively bind to antigens and are thus directly involved in immune defence.

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