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Systemic and mucosal immune response of rainbow trout to immunization with an attenuated *Flavobacterium psychrophilum* vaccine strain by different routes

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ABSTRACT

Teleosts possess three immunoglobulin (Ig) heavy chain isotypes viz., IgM, IgT and IgD and all three isotypes are reported in rainbow trout. The expression of these Ig isotypes in response to different immunization routes was investigated and results provide a better understanding of the role these Igs in different tissues. Rainbow trout (*Oncorhynchus mykiss*) were immunized with an attenuated *Flavobacterium psychrophilum* strain, 259-93-B.17 grown under iron limiting conditions, by intraperitoneal, anal intubation and immersion routes. Serum, gill mucus, skin mucus and intestinal mucus samples were collected at 0, 3, 7, 14, 28, 42 and 56 days post immunization by sacrificing four fish from each treatment group and the unimmunized control group, and the IgM levels were estimated by an enzyme linked immunosorbent assay (ELISA). In addition, blood, gill, skin and intestinal tissue samples were collected for Ig gene expression studies. The secretory IgM, IgD and IgT gene expression levels in these tissues were estimated by reverse transcription quantitative real time PCR (RT-qPCR). Levels of IgM in serum, gill and skin mucus increased significantly by 28 days after immunization in the intraperitoneally immunized group, while no significant increase in IgM level was observed in fish groups immunized by other routes. Secretory IgD and IgT expression levels were significantly upregulated in gills of fish immunized by the immersion route. Similarly, secretory IgT and IgD were upregulated in intestines of fish immunized by anal intubation route. The results confirm mucosal association of IgT and suggest that IgD may also be specialized in mucosal immunity and contribute to immediate protection to the fish at mucosal surfaces.

1. Introduction

Aquaculture of salmonid species is a high value fish production activity in Europe, North America, Chile, Japan, Australia and other parts of the world. Bacterial cold water disease (CWD) and rainbow trout fry syndrome (RTFS) caused by *Flavobacterium psychrophilum* are major diseases very often resulting in severe production and economic losses in salmonid aquaculture facilities, especially those producing rainbow trout *Oncorhynchus mykiss* (Walbaum), coho salmon *Oncorhynchus kisutch* (Walbaum) and steelhead [1,2]. Trout farming often suffers substantial economic loss due to problems associated with CWD epizootics including high mortality, increased susceptibility to other diseases, use of chemotherapeutants, high costs of treatment and skeletal deformities resulting in quality reduction in recovering fish [1]. Despite tremendous efforts, a vaccine to control CWD is yet to be approved and available. A major constraint to the development of an efficacious CWD vaccine is our inadequate understanding of the innate and adaptive immune responses of the host fish to the pathogen, especially at the mucosal surfaces.

Immunoglobulins (Igs) comprised of heavy and light chain molecules, are the mediators of adaptive immunity in fish and other vertebrates. Mammals have five different heavy chain isotypes of Ig namely IgM, IgG, IgA, IgD and IgE each with distinct functions while teleosts were long considered to have only two Ig isotypes, IgM and IgD. Teleosts were thought to lack specialized mucosal antibodies equivalent to IgA of mammals. However, recent

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discovery of other isotypes viz. IgT/IgZ [3,4] and IgM-IgZ chimera [5], the functions of which are not fully characterized, has thrown open the research to elucidate the role of these newly discovered Ig isotypes.

IgM is the most ancient and the only isotype functionally conserved in all jawed vertebrates (reviewed by Flajnik [6]). Serum IgM, a pentamer in mammals, birds and cartilaginous fish, is secreted as a tetramer in teleosts [7] in response to infections. IgM is considered to protect the fish against pathogens both systemically and at mucosal surfaces [8].

Recent studies provide evidence that IgD is also a primordial antibody isotype present in all jawed vertebrates including elasmobranchs [9], acipenseriformes [10], and teleosts except birds [11] and some mammalian species. Among teleosts, IgD is reported in almost all species examined including channel catfish (*Ictalurus punctatus*) [12], Atlantic salmon (*Salmo salar*) [13], Atlantic cod (*Gadus morhua*) [14], Atlantic halibut (*Hippoglossus hippoglossus*) [15], Japanese flounder (*Paralichthys olivaceus*) [16], fugu (*Takifugu rubripes*) [17], grass carp (*Ctenopharyngodon idella*) [18], three-spined stickleback (*Gasterosteus aculeatus*) [19,20], and rainbow trout (*O. mykiss*) [21]. IgD exists as multiple structural variants and splice forms exist in different vertebrates. Teleost IgD is characterized by long chimeric molecules consisting of repeated domains compared to shorter hinge containing molecules in mammals [22]. It is monomeric [21] and is found as membrane bound on B cells as well as a secreted form [23]. In humans, IgD secreted by IgD^+ cells in the upper respiratory mucosa mediate mucosal immunity by binding to respiratory pathogens. In addition, IgD activated basophils trigger innate antimicrobial response. Binding of IgD to channel catfish granulocytes has been reported [24], and this function is believed to be evolutionarily conserved. However, the exact function of IgD in teleost is not clearly understood.

Another antibody isotype, IgT is the latest antibody class discovered in vertebrate species [25]. The IgT or its equivalent (IgZ) is reported in many teleosts including Zebrafish (*Danio rerio*) [4], common carp (*Cyprinus carpio*) [5], fugu (*T. rubripes*) [26], rainbow trout (*O. mykiss*) [3], grass carp (*C. idella*) [18], three-spined stickleback (*G. aculeatus*) [19], and Atlantic salmon (*S. salar*) [27]. Most of these species possess more than one subclass of IgT [28]. Evidences show that IgT is involved in gut [29] and gill [30] mucosal immunity. The finding has challenged the paradigm that the specialization of immunoglobulin isotypes into mucosal and systemic arose during tetrapod evolution [28]. IgT is expressed as a monomer in serum and as a tetramer in gut mucus [29]. All the three Ig isotypes (IgM, IgD and IgT) are expressed both as membrane bound and secretory form [21,23,28]. Measurement of IgM, IgD and IgT expression in teleosts will improve our understanding of the role of these immunoglobulins in combating invading pathogens not only through the systemic circulation, but at specific mucosal surfaces as well.

A live attenuated strain of *F. psychrophilum* (259-93-B.17) has been developed at the Department of Fish and Wildlife Sciences at the University of Idaho [31]. The efficacy of this vaccine has been improved by growing the bacteria under iron limiting conditions and has recently been shown to provide significant protection against CWD in coho salmon [32]. A deeper understanding of the immune response of fish to *F. psychrophilum* especially at the mucosal surfaces, which is the natural route of infection is important for further fine tuning the efficacy of the CWD vaccine and for better health management of farmed salmonids. The expression of different immunoglobulin isotypes of fish in response to infection and vaccination is not fully understood. In this study the expression of all three isotypes of Ig was characterized in blood and different mucosal organs following immunization with *F. psychrophilum* by different routes.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*O. mykiss*) (mean weight 35 g) were procured from the University of Idaho's Aquaculture Research Institute (ARI) and were maintained in 500 L tanks with continuous aeration in a flow through water system supplied with dechlorinated municipal water maintained at 15 ± 1 °C. The fish were fed a commercial pellet feed (Rangen EXTR 450 1/16) at 1% of body weight divided into two equal doses. All experimental procedures with live fish were carried out with prior approval from the Institutional Animal Care and Use Committee, University of Idaho (IACUC # 2012-30).

2.2. Bacteria

A live attenuated vaccine strain of *F. psychrophilum*, 259-93-B.17 [31] maintained at Department of Fish and Wildlife Sciences, College of Natural Resources, University of Idaho, Moscow, USA was used for immunizing the fish.

2.3. Culture of F. psychrophilum

Glycerol stocks of *F. psychrophilum* strain 259-93-B.17 were revived by inoculating in to tryptone yeast extract salts broth (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% $MgSO_4$, 0.05% $CaCl_2$, pH 7.2) and incubated at 15 °C shaking at 80 rpm. Once visible turbidity was observed in the broth, the culture was streaked on TYES agar (1.5% agar in TYES broth) and incubated at 15 °C. The vaccine strain *F. psychrophilum* 259-93-B.17 was grown under iron limited condition (B.17-ILM) in TYES broth in the presence of an iron chelator, 2′,2′ Bipyridine (50 μM) under shaking conditions at 15 °C for 4 days. Bulk culture was produced by inoculating a single colony of *F. psychrophilum* in TYES broth containing 2′,2′Bipyridine in 10 mL and scaled up to produce the desired volume of culture. The bacterial cells were pelleted by centrifuging at 5000 × *g* for 20 min at 4 °C. The cells were washed twice in phosphate buffered saline (PBS), finally resuspended in desired volume of PBS and stored at 4 °C. The colony forming units (CFU) in the culture was estimated by standard plate count method. The plate count of the vaccine strain, *F. psychrophilum* B.17-ILM obtained was 3×10^8 CFU mL^{-1}.

2.4. Immunization

Fishes were divided into four groups and were immunized with *F. psychrophilum* B.17-ILM as shown in Table 1. Fishes were anaesthetized with tricaine methanesulfonate (100 mg L^{-1}) before immunization. Intraperitoneal (IP) injection was administered using tuberculin syringe and anal intubation (AI) was done using a micropipette attached with a 200 μL microtip and bacteria were administered into the hind gut. A 100 μL dose of the stock culture containing 3×10^7 CFU was used to immunize each fish by IP and AI

Table 1
Table showing different treatment groups, route and dose of immunization of rainbow trout.

Group	Number of fish immunized	Route of immunization	Dose
A	55	Unimmunized control	Nil
B	40	Intraperitoneal	3.0×10^7 CFU $fish^{-1}$ in 100 μL
C	40	Anal intubation	3.0×10^7 CFU $fish^{-1}$ in 100 μL
D	40	Bath	3.0×10^6 CFU mL^{-1} water for 1 h