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Optimisation and standardisation of functional immune assays for striped catfish (*Pangasianodon hypophthalmus*) to compare their immune response to live and heat killed *Aeromonas hydrophila* as models of infection and vaccination

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ABSTRACT

Aquaculture production of *Pangasianodon hypophthalmus* is growing rapidly in South East Asia, especially in Vietnam. As it is a relatively new aquaculture species there are few reports evaluating its immune response to pathogens. Thus, functional assays for *P. hypophthalmus* were optimised to evaluate both innate and adaptive immune responses, and were then used to examine immune response following stimulation with live and heat-killed *Aeromonas hydrophila*. These were used as models of infection and vaccination, respectively.

Four treatment groups were used, including a control group, a group injected intraperitonally (IP) with adjuvant only, a group injected with heat-killed *A. hydrophila* (1×10^9 cfu ml^{-1} mixed with adjuvant), and a group injected with a subclinical dose of live *A. hydrophila*. Samples were collected at 0, 1, 3, 7, 14 and 21 days post-injection (d.p.i.) to assess their immune response. The results indicated that challenge with live or dead bacteria stimulated the immune response in *P. hypophthalmus* significantly above the levels observed in control groups with respect to specific antibody titre, plasma lysozyme and peroxidase activity, and phagocytosis by head kidney macrophages at 7 or/and 14 d.p.i. At 21 d.p.i., total and specific antibody (IgM) levels and plasma lysozyme activity in fish injected with either live or dead *A. hydrophila* were significantly different to the control groups. Differential immune responses were observed between fish injected with either live or dead bacteria, with live *A. hydrophila* significantly stimulating an increase in WBC counts and plasma peroxidase activity at 3 d.p.i., with the greatest increase in WBC counts noted at 21 d.p.i. and in phagocytosis at 14 d.p.i. By 21 d.p.i. only the macrophages from fish injected with dead *A. hydrophila* showed significantly stimulation in their respiratory burst activity. This study provides basic information on the immune response in pangasius catfish that can be useful in the health control of this species.

1. Introduction

Pangasianodon hypophthalmus is the most important fish species currently cultured in Vietnam. Aquaculture production of pangasius is also developing in other Asian countries such as Bangladesh, India, Indonesia, Malaysia, Myanmar, the Philippines and Thailand, however Vietnam still remains the largest global producer [1]. Outbreaks of bacterial diseases in *P. hypophthalmus* culture have a significant impact on production. Two of the main problems reported are bacillary necrosis of *Pangasianodon* (BNP) [2] caused by *Edwardsiella ictaluri*, and motile aeromonas septicaemia (MAS) [3] caused by *Aeromonas hydrophila*.

A. hydrophila vaccine preparations based on dead bacterial preparations, killed using formalin or heat (i.e. whole bacterial

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cells) or isolated non-replicating pathogen subunits such as outer membrane proteins, lipopolysaccharide (LPS), extra-cellular proteins, recombinant S-layer protein, biofilms and or live attenuated *A. hydrophila* have been investigated by numerous researchers, with varying degrees of protection against experimental challenge observed [4–8]. Efficacy testing of potential vaccines and immune response to *A. hydrophila* have been conducted in a variety of fish species, including Nile tilapia (*Oreochromis niloticus*) [4], rainbow trout (*Oncorhynchus mykiss*) [9,10], roho (*Labeo rohita*) [11], Indian carp i.e. *Catla catla, Labeo rohita* and *Cirrhunas mrigala* [12], Asian catfish (*Clarias batrachus*) [13], zebrafish (*Danio rerio*) [14], carp (*Cyprinus carpio*) [5,7,15] and European eel (*Anguilla anguilla*) [16], but few of these studies have compared the effect of live and dead *A. hydrophila* on the immune response of fish, and none have used striped catfish as their model fish species.

As *P. hypophthalmus* is a relatively new aquaculture species, with little published on its immune response to pathogens, this study optimised and standardised functional assays to evaluate their innate and adaptive immune responses and then these assays were applied to compare immune response to live and heat-killed *A. hydrophila* as models of infection and vaccination, respectively. The ultimate aim of this work was to obtain basic information on the immune response of *P. hypophthalmus* that could be used to help in the development of products to improve their resistance to disease, such as vaccines and immunostimulants.

2. Materials and methods

2.1. Experimental animals

P. hypophthalmus, with no history of disease problems, were purchased from a local fish farm in Nakhon Sawan Province, Thailand and transported to the Aquatic Animal Laboratory, Faculty of Veterinary Sciences, Mahidol University, Nakhon Pathom Province. The fish were quarantined and acclimated to laboratory conditions for two weeks prior to use, and inspected daily for signs of disease. They were fed with a commercial diet (Inteqc Feed Company, Thailand) at 3% body weight per day. The weight of the fish at the start of the experiment was 53.2 ± 14.8 g.

2.2. Experimental design

Four treatment groups with three replicate tanks (40 fish/replicate) per group were used. Fish were maintained in 500 L recirculation tanks with a water temperature of 28 °C ± 2 °C and a photoperiod consisting of a 12:12 h artificial light regime. Water quality parameters i.e. dissolved oxygen, NH_3, pH and temperature were checked daily, and 20–30% water exchanges were also made each day. The fish were injected intraperitoneally (0.1 ml per fish) with either tryptic soya broth (TSB; Merk-Datmstadt) as negative control group, adjuvant (Montanide ISA 760 VG), or heat-killed *A. hydrophila* at 1×10^9 cfu ml^{-1} mixed with the adjuvant. Another group was injected with a subclinical dose of live *A. hydrophila* (i.e. 2.7×10^5 cfu ml^{-1}) [7]. The fish were acclimated for 21 days prior to experimentation Two fish per tank (6 fish per group) were sampled to assess their immune response on 0, 1, 3, 7, 14 and 21 days post-injection (d.p.i.). Blood and head kidney samples were collected from these fish for the various haematological and immunological analyses.

2.3. Haematological analysis

Blood samples (1 ml) were taken from the caudal vein of fish using disposable syringes (3 ml) flushed with heparin (Sigma, UK). These were divided into two aliquots, one sample for white and red blood cell (RBC) counts and differential white blood cell (WBC) counts, and the other for plasma collection. For the plasma collection, blood was centrifuged at 3000× *g* for 5 min and once separated stored at −70 °C for further analysis (i.e. lysozyme activity, total plasma IgM, complement activity, plasma peroxidase activity and specific antibody titre against *A. hydrophila*).

2.3.1. Haematocrit values

Capillary tubes were filled with blood and sealed at one end with clay. The filled tubes were then centrifuged at 10,000 to 15,000× *g* for 5 min in a micro-haematocrit centrifuge. The haematocrit values were expressed as a percentage of the packed cell volume, while the Mean Corpuscular volumes (MCV) were expressed in fL.

2.3.2. White blood cell and red blood cell counts

White blood cell and RBC counts were measured as described by Natt and Herrick [17]. Blood (20 μl) was added to 4 ml Natt-Herricks's solution and mixed thoroughly. A haemocytometer was filled with the blood suspension (10 μl), which was allowed to settle for 2–3 min before counting the RBCs and the WBC.

2.3.3. Differential WBCs counts

Differential WBC counts were made according to Nussey et al. [18]. Blood smears were prepared and allowed to air dry, before fixing with methanol for 3–5 min. The slides were then stained with Giemsa (IVD, UK) [5% (v/v) Giemsa's azure eosin methylene blue solution in buffer solution supplied with the stain] for 30 min, rinsed two times with buffer solution for 1 min, before air drying the slides and mounting them with Pertex® (Cellpath, UK). The cells were examined under a light microscope (×100 magnification) and the number of different WBCs present in 200 cells determined.

2.4. Head kidney macrophage activity

2.4.1. Isolation of P. hypophthalmus head kidney macrophages

Fish were killed by overdosed anaesthesia (200 mg l^{-1} Benzocaine; Sigma, UK) and bled to reduce red blood cell contamination. Head kidney macrophages were isolated using the method of Secombes [19]. The cell suspension was placed into Bijoux bottles (Sigma, UK) and kept on ice until used in the phagocytosis activity and respiratory burst activity assays outlined below.

2.4.2. Phagocytosis by P. hypophthalmus head kidney macrophages

Phagocytosis by head kidney macrophages was performed using the method of Thompson et al. [20], but slides were incubated at 28–30 °C throughout the procedure. The cells were finally stained with 5% (v/v) Giemsa stain for 30 min, air dried and mounted with Pertex. The cells were examined under oil immersion (×100 magnification) and the number of macrophages containing yeast counted out of a total of 200 macrophages. The phagocytic activity (PA), phagocytic index (PI) and phagocytic capacity (PC) were determined according to [21], using the equations outlined below.

Equation 1: Phagocytic Activity (PA)

$$\mathrm{PA} = \frac{\text{Mean average of yeast cells engulfed by active macrophage}}{\text{Total number of macrophages with engulfed}} \times 100$$

Equation 2: Phagocytic Index (PI)

$$\mathrm{PI} = \frac{\text{Total number yeast cells engulfed}}{\text{Number of macrophage counted}} \times 100$$

Equation 3: Phagocytic Capacity (PC)