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Production and characterization of monoclonal antibodies to IgM of Pacific herring (*Clupea pallasii*)

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ABSTRACT

Pacific herring (*Clupea pallasii*) have a central role in the North Pacific ecosystem as a forage fish species and are natural reservoirs of several important finfish pathogens, including *Viral hemorrhagic septicemia virus* (VHSV). Here, we report the identification of the gene encoding the immunoglobulin mu (IgM) heavy chain, as well as the development and characterization of monoclonal antibodies (MAbs) that specifically react with Pacific herring IgM. Pacific herring immunoglobulin was purified and consisted of heavy and light chains of approximately 80 and 25 kDa. Three hybridoma clones were initially identified by ELISA as reactive with purified immunoglobulin but only one clone was able to detect an 80 kDa protein in Pacific and Atlantic herring (*Clupea harengus*) whole plasma by denaturing western blot. However, all three MAbs were able to precipitate an 80 kDa protein from Pacific herring and LCMS sequencing of peptide fragments derived from this protein matched the predicted amino acid sequence of the cloned, heavy chain gene. In addition, two of the MAbs stained cells within the putative lymphocyte gates for the spleen, anterior kidney and posterior kidney but were not reactive for myeloid/granulocyte gates, which is consistent with these MAbs reacting with surface IgM^+ B-cells. To our knowledge, this is the first report of IgM-related gene sequences and anti-IgM monoclonal antibodies from any member of the family *Clupeidae*. The antibodies produced in this study are critical for achieving our long-term goal of conducting serological surveillance to assess pathogen exposure in natural populations of Pacific herring.

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1. Introduction

Pacific herring (*Clupea pallasii*) are distributed in coastal regions throughout the North Pacific, and have a critical ecosystem role in the flow of energy and nutrients between primary/secondary production and higher predators [1]. Pacific herring are also important natural reservoirs for several commercially important finfish pathogens including *Ichthyophonus hoferi* and *Viral hemorrhagic septicemia virus* (VHSV) [2]. Throughout the NE Pacific Ocean, periodic viral hemorrhagic septicemia (VHS) epizootics are accompanied with fish kills in free-ranging and confined populations [3–5]. It is hypothesized that pathogens such as *Ichthyophonus* and VHSV contributed to the decline and failed recovery of certain Pacific herring populations, including the population of herring in Prince William Sound Alaska [6]. A previous study of Prince William Sound herring found a positive correlation between *Ichthyophonus*-associated heart lesion scores and circulating immunoglobulin mu (IgM) levels, with serum IgM being detected using a rabbit polyclonal antiserum [7].

Quantification of the exposure history of Pacific herring to *Ichthyophonus*, VHSV and other pathogens represents a critical first step toward forecasting the potential for disease epizootics among wild populations and developing adaptive fisheries management strategies intended to prevent or mitigate future population-level impacts. Toward this long-term goal, here we report the development of three monoclonal antibodies (MAb) that specifically recognize Pacific herring IgM and can be used to characterize the Pacific herring antibody response. Additionally, we report the identification of the gene encoding the constant domains of the Pacific herring IgM heavy chain.

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2. Methods

2.1. Fish

Specific pathogen free (SPF) Pacific herring were created as previously described [8,9]. Herring were reared in tanks supplied with single pass, sand filtered, particle filtered to 10 μm, and ultraviolet-irradiated seawater and fed a commercially-available pellet feed (BioOlympic, Bio-Oregon® line, produced by Skretting) after metamorphosis.

2.2. Identification of Pacific herring IgM heavy chain cDNA sequence

Extraction of total RNA and cDNA synthesis from VHSV infected herring spleen tissue was previously described [9]. Combinations of degenerate and non-degenerate PCR primers (Table 1) were used to amplify fragments of the herring IgM heavy chain. Amplification was achieved using the *Taq* PCR Core Kit (Qiagen Inc.) following manufacturer's recommendation for a 25 μl final volume. The reactions contained either 100 pmol of degenerate or 50 pmol of non-degenerate primers. Reaction conditions were 1 cycle of 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s followed by 1 cycle of 72 °C for 10 min. PCR reactions were cloned into the Topo TA Cloning Kit for Sequencing (Invitrogen Inc.) following manufacturer's instructions. The full-length heavy chain gene was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) (Invitrogen Inc.). Sequencing was performed using the ABI Big Dye Terminator V1.1 and an ABI 3700 automated sequencer (Applied Biosystems, Inc.; Foster City, CA). Sequence chromatograms were visually assessed and edited using the Sequencher V.4.5 software (Gene Codes Corporation, Ann Arbor, MI). Amino acid sequences were broken down into individual Ig superfamily constant heavy chain (CH) domains using SMART analysis (www.smart.embl-heidelberg.de) and then aligned using ClustalX [12] for phylogenetic analyses using the MEGA V5.0 software package [13]. Phylogenetic interferences of the CH1 domain were performed using the maximum likelihood method [10] in the MEGA V5.0 software; tree topologies were validated by bootstrapping 1000 times. Putative N-linked glycosylation sites for the herring IgM heavy chain were predicted using NetNGlyc v1.0 (www.cbs.dtu.dk/services/NetNGlyc).

2.3. Purification of immunoglobulin and production of monoclonal antibodies

To obtain Pacific herring plasma containing elevated levels of circulating IgM, age 5+ yr SPF Pacific herring [8] were given an intraperitoneal injection (75 μl volume) of trinitrophenyl hapten-conjugated keyhole limpet hemocyanin (TNP-KLH; Biosearch Technologies) emulsified in Freund's complete adjuvant (Thermo Fisher Scientific Inc.) to achieve a final dose of 37.5 μg/fish. Injected herring were euthanized and sampled at 42 d and 69 d ($n = 2$/day) post-injection. Fish were anesthetized using sodium bicarbonate-buffered tricaine methane sulfonate (MS-222; Western Chemical) for injections and were euthanized by an overdose of buffered MS-222. Whole blood was collected from the caudal vein into heparin-treated capillary tubes for all sampled fish and plasma was separated by centrifugation at 12,700× *g* for 2 min and stored at −80 °C until used.

Plasma from TNP-KLH immunized herring was purified using a size exclusion chromatography as previously described [11]. Briefly, 500 μl of plasma was added to a sephracryl S300 column (16 mm × 1 m), and 1 ml fractions were collected. Fractions containing purified antibody were determined by the presence of a high molecular weight protein (>700 kDa) on sodium dodecyl sulfate (SDS)-composite agarose gel electrophoresis (CAGE) [12], which dissociated into putative antibody heavy and light chains when processed using reducing SDS-polyacrylamide gel electrophoresis (PAGE). Six week old BALB/c mice were immunized with 100 μg of the purified herring IgM fraction emulsified in Freund's complete adjuvant. A 50 μg booster injection of purified herring IgM in Freund's incomplete adjuvant was given at four and eight weeks post-injection. A final intravenous injection (5 μg of purified IgM in PBS) was given four days before the fusion (Week 12). A standard 50% PEG fusion was performed [13], the cells were plated into 96 well culture plates and resultant hybridoma supernatants were screened via ELISA and western blot using both the purified antibody and whole serum (as described below). The Pierce Rapid ELISA Mouse Antibody Isotyping Kit (Thermo Scientific) was used to determine the MAb isotypes.

2.4. Hybridoma screening

Preliminary hybridoma screens were conducted by coating a Corning® 96-well flat-well ELISA microplate (Sigma Aldrich) with 2 μg/ml of purified herring IgM or 10 μg/ml of whole serum diluted in carbonate coating buffer, followed by 1 h incubation. Next, the plates were blocked with 240 μl of TTBS+1% BSA (0.05 M Tris-buffered saline (TBS), pH 8.0; 0.05% Tween-20; and 1% bovine serum albumin (BSA)). Between each of the following steps the plates were washed three times with 200 μl TTBS. To each well of the ELISA plate, diluted cell culture supernatant (5 μl supernatant in 95 μl of TTBS) was added and incubated for 1 h. A 0.2 μg/ml solution of goat anti-mouse HRPO (Jackson Immunoresearch) was added for 1 h followed by addition of substrate chromogen (ABTS Peroxidase Substrate System, KPL Inc.). Wells demonstrating a strong color change to both purified and whole serum were then screened via western blot.

Candidate MAbs were clonally selected by limiting dilution passage and were additionally screened using an antigen-specific ELISA to ensure that the MAb recognition site was not occluded following antigen-binding. ELISA plates were coated overnight with 10 μg/ml of TNP-BSA diluted in carbonate coating buffer (100 μl/well). Wells were blocked and washed as described above. To test each MAb supernatant, 10 μl of TNP-KLH immunized and control herring plasma samples were titrated on the plate in TTBS + 1% BSA. Following 2 h incubation the plates were washed, and a 1:10 dilution of MAb supernatant was added to each well. The remaining steps were as described above.

Ten μg of purified antibody or 50 μg of whole plasma was added to 100 μl of reducing sample buffer and boiled for 8 min. The entire volume of each sample was added to 10% SDS-PAGE gels containing an IPG well and the proteins separated by the addition of current. The contents of the gel were transferred to Immobilon-FL PVDF (EMD Millipore) and then the membrane was blocked with 3%

Table 1
Primers used to clone and sequence Pacific herring IgM.

Purpose	Primer name	Sequence (5′ → 3′)
Degenerate PCR	IgM CH1 F1 (deg)	ACIGAYKYNGTICARTAYCC
	IgM CH3 F1 (deg)	TAYGANGARTGGWBIAAYGG
	IgM CH3 R1 (deg)	CCRTTIVWCCAYTCNTCRTA
	IgM CH4 R2 (deg)	GCNACIGGYTCRTCRTCNGC
Sequence walking	Cp-IgM F	CAAAGATTTCCACCCCAAG
	Cp-IgM R	CCTGAAGGAACCTTTTTATGA
5′ RACE	Cp-IgM 5′ RACE R1	CAGCAGCAAAGCAGGCA
	Cp-IgM 5′ RACE R2	CGCTCGTGGGTTTCGT
3′ RACE	Cp-IgM 3′ RACE F1	ACGAAACCCACGAGCG
	Cp-IgM 3′ RACE F2	TGCCTGCTTTGCTGCTG