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Production of a monoclonal antibody against of muskellunge (*Esox masquinongy*) IgM heavy chain and its use in development of an indirect ELISA for titrating circulating antibodies against VHSV-IVB

Mohamed Faisal[a,b,*], Isaac F. Standish[a], Mary Ann Vogelbein[c], Elena V. Millard[a], Stephen L. Kaattari[c,1]

[a] Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, 784 Wilson Road, East Lansing, MI 48824, USA
[b] Department of Fisheries and Wildlife, College of Agriculture and Natural Resource, Michigan State University, 480 Wilson Road, East Lansing, MI 48824, USA
[c] Department of Aquatic Health Sciences, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA

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ABSTRACT

This study reports the development of a monoclonal antibody (designated 3B10) against the muskellunge (*Esox masquinongy*) IgM. The 3B10 monoclonal antibody (mAb) belongs to the IgG3 kappa isotype. Western blotting demonstrated that 3B10 mAb reacted primarily to muskellunge IgM heavy chain. 3B10 also reacted strongly with the IgM heavy chain of other esocids, including the northern pike (*Esox lucius*), tiger muskellunge (*E. masquinongy x E. lucius*), and, to a much lesser extent, the chain pickerel (*E. niger*). The 3B10 mAb did not bind to IgM from 10 other fish species resident in the Great Lakes basin. Using the 3B10 mAb, it was possible to determine the muskellunge Ig ability to bind to antigens. Using trinitrophenyl hapten conjugated to keyhole limpet hemocyanin (TNP-KLH) as the eliciting antigen, muskellunge Ig subclasses exhibited a range of affinities with log aK values 5.56–6.25 that is considered intermediate compared to other fish species. 3B10 mAb was used to develop and evaluate an indirect ELISA for the detection and quantitation of circulating antibodies against the viral hemorrhagic septicemia virus genotype IVb (VHSV-IVb). Using the newly optimized assay, anti-VHSV-IVb antibodies were detected in sera of VHSV-IVb vaccinated muskellunge as well as from those of wild muskellunge sampled from an endemic waterbody. In addition to its use in immunoassays, the developed 3B10 mAb will enable future investigation aiming at deciphering immune mechanism of this important fish species to pathogens.

1. Introduction

A decade ago, the Laurentian Great Lakes (LGL) of North America was invaded by a highly pathogenic sublineage (b) of VHSV genotype IV [1]. Within five years, VHSV-IVb spread to the five Great Lakes and to a number of inland lakes and rivers leaving behind major fish kills and ecologic devastation [2]. The first isolation of VHSV-IVb was from muskellunge (*Esox masquinongy*) [1], a common species in eastern and central North America [3]. Field observation and experimental infection studies demonstrated that muskellunge exhibit the highest susceptibility to this virus sublineage as compared to other LGL representative fish species [2,4,5]. VHSV-IVb was found to also infect 28 other LGL wild fish species and two macroinvertebrates, a matter that raises serious concerns about the future of LGL fisheries [2,6,7]. In order to protect Great Lakes wild and cultured fish from the threat posed by the emergence of this highly pathogenic sublineage of the OIE reportable VHSV, regulatory agencies in North America implemented strict control measures including general and targeted surveillance as recommended by the OIE Aquatic Code [8]. Unfortunately, there is a relatively short period of time during the course of the disease in which VHSV can be isolated on tissue culture [9,10] or detected by molecular assays [11–14]. While the developed molecular assays increased the likelihood of virus detection in infected tissues, they failed to identify fish that survived the infection with the virus cleared from tissues or remained below the assay detection limit, a matter that impinged on the accuracy of surveillance studies and zoning delineation efforts and a

* Corresponding author. Department of Pathobiology and Diagnostic Investigation, 1129 Farm Lane, Room 174 Food Safety and Toxicology Building, Michigan State University, East Lansing, MI 48824, USA.
E-mail address: faisal@cvm.msu.edu (M. Faisal).
[1] Deceased 11 November 2014.

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need to develop sensitive and specific immunoassays emerged.

The lack of species-specific monoclonal antibodies (mAbs) against indigenous fish immunoglobulins dictated the use of other more laborious serological assays such as the serum neutralization [15,16] tests and competitive enzyme-linked immunosorbent assays (cELISA) [17–19]. While these assays have already proven beneficial in the detection of VHSV-IVb humoral immune responses, they fail to detect all antibody subpopulations. For example, when Encinas et al. [20] compared anti-VHSV antibodies prevalence and levels in surviving rainbow trout (*Oncorhynchus mykiss*) by using both the serum neutralization assay and indirect ELISA (using recombinant viral G protein fractions), no correlation could be found. Serum neutralizing antibodies in surviving fish declined with time to reach as little as 10%, while the binding antibodies, as measured by indirect ELISA, increased as the fish survived the disease and were long lasting. The authors concluded that the absence of anti-VHSV neutralizing antibodies does not mean the lack other antibody subpopulations in survivor fish sera [20,21].

Our previous studies demonstrated that among representative LGL fish species collected from endemic waters, muskellunge exhibited the highest prevalence and titers of circulating antibodies against VHSV-IVb [16]. This finding was further confirmed by experimental studies [22,23], a matter that made muskellunge an ideal target wild species for use in VHSV-IVb serosurveys and virus-free zone delineation. Unfortunately, there are no available antibodies against the muskellunge immunoglobulin that can be used in the development of sensitive and specific immunoassays. The absence of this anti-Ig is also impairing studies aiming at determining the affinity of muskellunge immunoglobulin to bind and sequester antigens. The degree of antibody affinity toward influences the functionality of antibodies and consequently their antigen binding *in vitro* [24]. To this end, the present study reports on the development of a monoclonal antibody that recognizes and binds to the heavy chain of muskellunge IgM. This mAb (designated 3B10) was successfully used to develop and evaluate an indirect ELISA for the detection of VHSV-IVb circulating antibodies in muskellunge.

2. Material and methods

2.1. Muskellunge sera collection

All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Michigan State University (MSU) as per the National Institutes of Health guide for the care and use of laboratory animals (NIH Publication# 8023, revised 1978) and all experimental fish were housed at the MSU Research Containment Facility (East Lansing, MI, USA). In addition to muskellunge, several fish species were used in this study (Supplemental Table 1). All muskellunge used in experiments of this study were certified free of VHSV and other reportable diseases, and were acclimated in an aerated flow-through 500-L tank at 11 °C. Fish were fed certified disease-free live fathead minnows obtained from Anderson Farms, Inc. (Lonoke, AR, USA). All blood samples were collected by caudal venipuncture from fish anesthetized with 0.1 g L^{-1} of tricaine methanesulfonate (MS-222, Western Chemical, Ferndale, WA, USA) buffered with 0.3 g L^{-1} sodium bicarbonate. Obtained sera were aliquoted and stored at −80 °C till analyzed.

Experimental work was performed on several groups of muskellunge. Fish of the first group were used as serum donors for the generation of the anti-muskellunge IgM mAb. Muskellunge of this group were acquired as four months post-hatch, with average total length 17.45 ± 1.5 cm and weight 22.52 ± 5.4 g from Harrison Fishery Inc. (Hurdland, MO, USA). This group of muskellunge was divided into two subgroups; one remained naïve and its sera was used for the mAb generation (detailed under 2.1.2), while the other was hapten vaccinated and its sera used in assessing the mAb binding affinity with muskellunge IgM (detailed under 2.3).

2.2. Production of mAbs

Pooled sera from eight naïve muskellunge (~150 µL $fish^{-1}$) were concentrated using saturated ammonium sulfate (SAS) precipitation with 20–40% (v/v) concentrations of SAS solution. SAS precipitated muskellunge immunoglobulin (Ig) from each cut was analyzed by running 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Sigma-Aldrich, St. Louis, MO, USA) using the EZ-Run™ Prestained *Rec* Protein Ladder (Thermo-Fisher Scientific, Waltham, MA, USA).

Balb/c mice (17-week old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). A pristane-primed male was immunized IP with 50 µg of the 20% SAS purified muskellunge Ig in 500 µL sterile phosphate buffered saline (PBS) and emulsified 1:1 in Freund's complete adjuvant (FCA). Approximately four weeks following the first immunization, the mouse received another 10 µg of purified Ig in 50 µL of RPMI-1640 (Hyclone Laboratories Inc., Logan, UT, USA) via tail vein injection. Three days later, the mouse was euthanized after blood was collected, and the spleen aseptically removed. A single splenocyte suspension was prepared then fused with SP2/0 myeloma cells (American Type Culture Collection, Manassas, VA, USA) at a 5:1 ratio with 50% (w/v) polyethylene glycol-1,450 Hybri-Max™ (PEG; Sigma-Aldrich) as previously described [25]. For selection of myeloma-lymphocyte hybrids, RPMI medium was supplemented with 16.5% fetal bovine serum (Hyclone Laboratories Inc.) and L-glutamine (Hyclone Laboratories Inc.) and mixed 1:1 with hypoxanthine-aminopterin-thymidine medium (Sigma-Aldrich), 1 x oxaloacetate-pyruvate-insulin (Sigma-Aldrich) and 26% conditioned RPMI medium (i.e., supernatant from previously cultured SP2/0 myeloma cells). Cells were then dispensed into five 96 well Costar® cell culture plates (Corning, NY, USA) at a volume of 150 µL $well^{-1}$ and the plates were incubated at 37 °C under 5% CO_2 tension.

2.3. Selection and characterization of the mAb

After colony formation, antibody-producing hybridomas were screened against 30% SAS precipitated muskellunge immunoglobulin using an endpoint ELISA [26]. Based on this screening, the positive hybridoma cells (3B10) were selected, plated using limiting dilutions, and cloned three times using the limiting dilution method [25].

2.3.1. Western blotting

Following cloning of the 3B10 cells, 14 healthy colonies were screened for anti-muskellunge Ig activity using western blot. Sera was added to 2x reducing sample buffer [RSB; 4XRSB with 30% sucrose (Sigma), 8% SDS (Fisher Scientific), 10% β-mercaptoethanol (Sigma), 0.25 M Tris-HCl (Fisher Scientific), a few granules of bromophenol blue (Sigma), pH 6.8], boiled for 8 min and cooled prior to loading into a 10% SDS-PAGE gel and run alongside the EZ-Run™ Prestained *Rec* Protein Ladder. Ig was transferred overnight at 4 °C and 30 V to a methanol-activated Immobilon-P polyvinylidene difluoride - FL membrane (PVDF-FL; Millipore, Billerica, MA) following the manufacturer's protocol. The membrane was subsequently washed twice for 15 min each with 0.1% Tween-PBS (Sigma) and blocked with TTBS for 40 min for 1 h in 4% casein solution containing 5% sucrose (Sigma), 150 nM NaCl, 50 mM Tris Base and 1 mmolL^{-1} EDTA, (Sigma), pH of 7.6, followed by four washes as previously stated. The blot was then incubated for 1.5 h in 20 mLs of the 3B10 mAb (1:2000 dilution in PBS), followed by the four washes then a 1:8000 dilution of the goat anti-mouse IgG secondary antibody Alexa Fluor 680 conjugate (Invitrogen, Eugene, OR) in PBS. After another four, 4 min washes, the membrane was air dried in the dark and scanned using a Li-Cor Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE).

Three clones that exhibited the strongest anti-Ig activity were allowed to grow and then injected intraperitoneally (IP) into 8, 7 and 3 pristane-primed Balb/c mice for each clone, respectively. Mice were

monitored for the development of ascites for 14 days. Ascitic fluid was purified using SAS precipitation, analyzed for purity with SDS-PAGE, diluted to 1 mg mL^{-1} in PBS with 50% glycerol (Sigma-Aldrich), and stored at −20 °C until use.

2.3.2. Determination of 3B10 mAb species and chain specificity

The species specificity of the 3B10 mAb was assessed by conducting immunoblots against sera from 13 Great Lakes fish species (Supplemental Table 1). Each blot also contained a raw serum and SAS precipitated IgM sample from naïve muskellunge. Immunoblotting and visualization of bands was performed as previously described.

2.3.3. Determination of 3B10 mAb isotype

The determination of a monoclonal antibody isotype is important in selecting the secondary antibody to be used for its detection [27]. The specific IgG isotype of the final selected mAb was verified using the Mouse Monoclonal Sub-Isotyping Kit (American Qualex International, San Clemente, CA, USA) following the manufacturer's instructions.

2.4. Determination of the muskellunge Ig affinity

Determining the affinity of an Ig toward an eliciting antigen sheds light on the strength of antigen-antibody binding *in vitro* [24]. To assess muskellunge Ig affinity, nine muskellunge (from muskellunge Group 1) were immunized IP with 100 µg of trinitrophenylated-keyhole limpet hemocyanin (TNP –KLH) in sterile phosphate buffered saline (PBS; Sigma-Aldrich), prepared as previously described [28] and emulsified 1:1 with Freund's Complete Adjuvant FCA (Sigma-Aldrich). Blood was collected before and once monthly following the TNP-KLH injection, and the anti-TNP sera was assessed using a kinetic ELISA employing as described elsewhere [26]. A unit of anti-TNP antibody activity was defined as the volume of sera required to produce an OD rate of half the maximum rate [26]. Immunized muskellunge sera showing the highest anti-TNP activities were pooled and divided into aliquots; 250 µL for immunoadsorption and 400 µL for SAS precipitation. Immunoadsorption of pooled TNP-KLH immunized muskellunge sera was performed as previously described [28] using TNP_{13}-BSA linked to cyanogen bromide-activated sepharose beads (Sigma-Aldrich) and Bio-Spin chromatography column (Bio-Rad Laboratories, Hercules, CA, USA). Adsorbed antibodies were eluted with TNP-lysine (ICN Biochemicals, Cleveland, OH, USA) and concentrated with PEG 20,000 (Sigma-Aldrich) [25]. Meanwhile, a SAS precipitation of the pooled anti-TNP serum was conducted as previously described and fractions analyzed for purity by running SDS-PAGE under reducing conditions as described above.

The ELISA affinity-partitioning method as per the protocols originally outlined by Neito et al. [29] and later modified by Shapiro et al. [23] was employed to determine affinity constant of muskellunge Ig, a measurement of the average affinity of the various antibody subpopulation using the equation: $aK = \log (1/[H_{50}]_i)$, where $[H_{50}]$ is the concentration of TNP-lysine required for a 50% reduction in the maximum O.D. rate for the coating solution "i" [26].

2.5. Development and application of an indirect ELISA using 3B10 mAb for anti-VHSV-IVb antibody detection in muskellunge serum

2.5.1. Virus and plate coating

The VHSV strain used throughout the study was the Great Lakes index strain MI03GL [1]. The isolate has been maintained by continuous subculture in the *Epithelioma papulosum cyprini* (EPC) cell line. Virus titration and purification was performed as described by Millard et al. [17]. Protein concentration of the final preparation was determined using a Qubit® Fluorometer with the Qubit® Protein Assay Kit (Life Technologies, Carlsbad, CA, USA).

2.5.2. Indirect ELISA procedure

In order to establish the positive/negative threshold cutoff value to be used in the indirect ELISA, positive and naïve muskellunge sera were used. For the positive control serum, we used a hyperimmune serum that was obtained from muskellunge that survived multiple experimental exposures to VHSV-IVb in a previous study performed in our laboratory and was proved to have high titers of neutralizing and binding anti-VHSV antibodies [9]. These certified VHSV-free juvenile muskellunge were originally obtained at 4 months post-hatch from the Rathbun National Fish Hatchery, Iowa, USA (average total length 17.2 cm ± 1.9 cm, average weight 23.9 cm ± 5.0 cm) and were maintained in our laboratory for up to three years. Donor for the ELISA negative serum control was a group of 77 naïve, juvenile muskellunge (average length 14.2 cm ± 1.4 cm, average weight 11.9 g ± 3.8 g) acquired from Chautauqua State Fish Hatchery (Chautauqua, NY, USA).

Indirect ELISA was performed as described by Crowther [30] using microtiter plates (Greiner Bio-One, Monroe, NC, USA) coated with 100 µL $well^{-1}$ of purified VHSV-IVb, followed by the addition of muskellunge test serum. The 3B10 mAb was used as the primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse monoclonal IgG as the secondary antibody (Invitrogen). Optical density (OD) was read at 490_{nm} on a BioTek, ELx808™ plate reader (BioTek Instruments, Inc., Winooski, VT, USA) and analyzed using the Gen5 software (BioTek Instruments, Inc.). The average value of blank wells was subtracted from test and control wells prior to analysis.

Initial optimization of reagents was conducted using standard checkerboard titration as detailed in Ref. [30]. The coating antigen (purified VHSV-IVb) was titrated using serial dilutions ranging from 8 µg mL^{-1} to 0.25 µg mL^{-1}. Similarly, different sera dilutions ranging from 1:5 to 1:80 were examined. Serial dilutions of the newly developed 3B10 mAb (1:2,500 to 1:240,000) and HRP-conjugated goat anti-mouse IgG (1:2,000 to 1:64,000) were also titrated. Conditions that resulted in the highest OD_{490} ratio between positive and negative serum (P/N) OD values while maintaining a maximum OD_{490} value close to 1.0 were considered optimal and selected for use in subsequent assays.

The detection limit of the assay was determined by measuring the maximum dilution of a 1:4 dilution series of the positive control that remained above the established positive/negative threshold. Positive control (n = 3) and 77 naïve serum samples, tested individually in triplicate, were used to examine intra and inter-assay variability.

Specificity of muskellunge antibodies to VHSV-IVb was tested by indirect ELISA utilizing plates coated with VHSV-IVb (MI03) and five other pathogenic fish viruses. Viral isolates included a VHSV genotype IVa (Makah isolate), VHSV genotype IVc (2000-149 isolate), infectious hematopoietic necrosis virus (IHNV) genotype M (220-90 strain), the infectious pancreatic necrosis virus (IPNV), and the largemouth bass virus (LMBV). After determining the $(TCID)_{50}$, viral stocks were adjusted to 10^3 $TCID_{50}$ $well^{-1}$ in cold PBS and coated to plates as previously described. ELISA was then conducted as previously described using two naïve and three positive serum samples obtained from pVHSivb-G vaccinated fish.

2.6. The use of the optimized indirect ELISA in detecting and quantitating circulating anti-VHSV-IVb antibodies in muskellunge

2.6.1. Following vaccination

An additional muskellunge group consisting of 32 naïve muskellunge (14.2 ± 1.4 cm, 11.9 ± 3.8 g, Chautauqua State Fish Hatchery) received DNA plasmid containing the VHSV-IVb glycoprotein (G) gene that was prepared and administered as detailed in our previous studies [22,23]. Each fish of this group was individually pit-tagged with 9 mm tags (Biomark© Inc.) two weeks prior to vaccination so that they can be tracked over time. Prior to vaccination, a serum sample was non-lethally collected from each fish to establish the antibody baseline value. Sera were collected 28 days post-vaccination, then at 14-day intervals up to Day 70 post-vaccination. At that day, fish also

received a booster dose of the same vaccine and sera were collected 35, 56 and 70 days following the second vaccine administration. Indirect ELISA was performed on each of the samples collected and paired *t*-tests were performed to determine significant changes in the OD values of the fish over the study period.

2.6.2. Wild muskellunge residing in VHSV-IVb-endemic waterbody

Indirect ELISA was conducted on serum samples collected from wild mature muskellunge collected by the Michigan Department of Natural Resources personnel from the VHSV-IVb endemic Detroit River [1,2], Michigan from 2012 to 2015. The number of muskellunge sampled varied each year, with 26 in 2012, 27 in 2013, 38 in 2014 and 45 in 2015, for a total of 134. Fish length averaged 105.2 cm ± 11.5 cm while their weight averaged 7143.2 g ± 2017.1 g. Blood was collected non-lethally and serum samples stored individually at −80 °C until assayed. Indirect ELISA was performed on each of these serum samples and trends of the OD values analyzed by year using a general linear model procedure (PROC GLM) in SAS (Statistical Analysis Software; version 9.2, SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Production of 3B10 mouse anti-muskellunge Ig mAb

The 20% SAS cut of the precipitated muskellunge Ig appeared to be have the least impurities with prominent bands at the molecular weights ~73 kDa and ~26 kDa, corresponding to the heavy and light chains of the muskellunge IgM molecule, respectively (Fig. 1). This fraction was used for mice immunization. The endpoint ELISA led to the selection of one of the hybridoma clones; the 3B10 clone. Western blotting of supernatants of selected 14 3B10 clones yielded strong bands at ~73 kDa corresponding to muskellunge IgM heavy chain (Fig. 2). Three clones were selected (depicted in *lanes* 8, 10, and 14 in Fig. 2) for mice inoculation. The ascites fluid collected from a single inoculated mouse then underwent SAS precipitation. The 20% SAS cut demonstrated the least impurities, and was selected for use to determine muskellunge Ig maturation affinity and to develop and optimize an indirect ELISA for the detection of anti-VHSV antibodies in muskellunge sera.

3.2. Characterization of the 3B10 mAb

The isotype of the 3B10 mAb was determined to be IgG3-*kappa*.

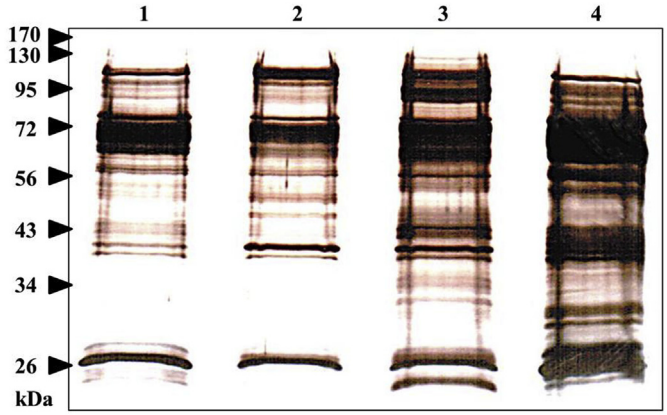

Fig. 1. A 10% SDS-PAGE followed by silver staining. Gel contains saturated ammonium sulfate (SAS) precipitation of the pooled naïve muskellunge serum to be used for mice immunization. *Lane 1*: 2 µg of the 20% SAS; *Lane 2*: 2 µg of the 30% SAS; *Lane 3*: 2 µg of the 40% SAS; *Lane 4*: 2 µg of the remaining SAS supernatant. Note the band at ~73 kDa (heavy chain) and ~26 kDa (light chain).

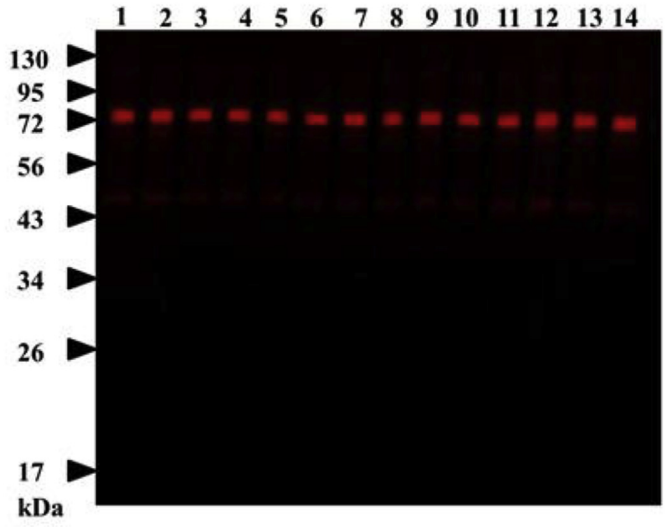

Fig. 2. Immunoblot of SAS purified muskellunge Ig examining mAb production in 14 3B10 clones. *Lanes 1–14*: 10 µg lane^{-1} of the purified muskellunge Ig followed by 1:25 dilution of supernatant from 14 individual colonies.

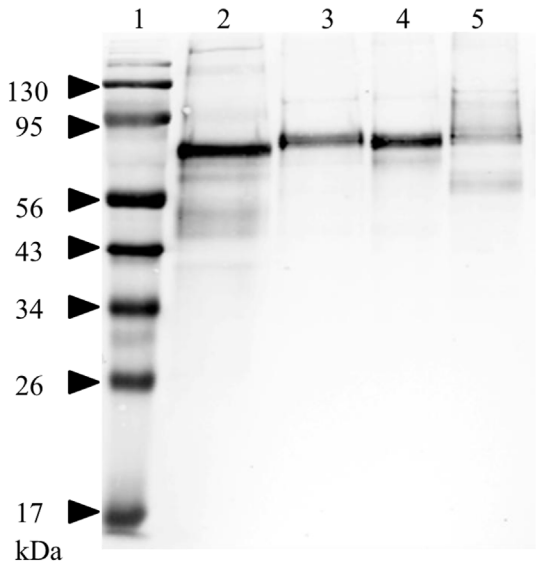

Fig. 3. Immunoblots of 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels examining 3B10 monoclonal antibodies (mAb) against different fish species' immunoglobulin (Ig). *Lane 1*: 3 µL of EZ-Run™ Prestained *Rec* Protein; *Lane 2*: 0.75 µg lane^{-1} of the saturated ammonium sulfate (SAS) precipitated muskellunge Ig. Other *Lanes* are each loaded with 0.75 µg lane^{-1} of unpurified sera from different species, unless otherwise noted. *Lane 3*: northern pike (*Esox lucius*); *Lane 4*: 0.5 µg tiger muskellunge (*E. masquinongy x E. lucius)*; *Lane 5*: chain pickerel (*E. niger*).

As depicted in Fig. 3, 3B10 bond strongly to the heavy chain of IgM from muskellunge and northern pike, as well as to their hybrid, the tiger muskellunge. Though, we note that the IgM heavy chain of both the northern pike and tiger muskellunge appears to be slightly larger than the muskellunge IgM heavy chain ~73–75 kDa. Relatively less reactivity was observed with the IgM of chain pickerel, another esocid species. No reactivity was observed with the Ig or sera from any of the other species that were examined (Fig. 3).

3.3. Determination of the muskellunge Ig affinity

Serum sample of TNP-KLH immunized muskellunge that exhibited the highest circulating anti-TNP antibody titers (> 100,000 U mL^{-1})

was selected for IgM concentration and then used to assess 3B10 mAb affinity with the kinetic ELISA. Using the 3B10, the average affinity (log aK) of the 3B10 mAb against the two elutions of chromatography purified high tittered pooled muskellunge sera were 5.96 and 6.08, while the average affinity against a 35% SAS precipitation of the same serum pool was 6.25. These consistent values indicate the mAb has a rather constant, but moderate affinity profile.

3.4. Establishment of indirect ELISA

The VHSV coating concentration, serum dilution, 3B10 mAb concentrations, as well as the conjugated goat anti-mouse HRP antibody concentrations, were optimized using standard checkerboard titration. The optimal concentration of purified VHSV-IVb and serum dilution were determined to be 0.1 μg well^{-1} and 1:10, respectively (Supplemental Table 2). The optimal concentrations of the 3B10 mAb and the goat anti-mouse HRP secondary antibody were determined to be 1:30,000 (3.3 ng well^{-1}) and 1:4,000, respectively (37.5 ng well^{-1}) (Supplemental Table 3). Conditions were optimized when coating took place overnight at 4 °C, and all other incubations were conducted for 1 h at room temperature, with the exception of blocking, which was conducted at 37 °C.

Following condition optimization, the positive/negative threshold was determined using the individual OD values from 77 naïve muskellunge, which ranged from 0.025 to 0.15, with a mean of 0.025. Using the common method of threshold determination, i.e., the mean + 3 standard deviations [0.025 + (3 × 0.033)], the positive/negative threshold was determined to be 0.124. However, we also applied the more stringent approach of calculating the 95% confidence limit of the 95th quartile in SAS 9.2 [31], as previously described [17]. The threshold calculated using this second method was 0.163, which we adopted as the final threshold.

3.5. Indirect ELISA evaluation

The reproducibility of the indirect ELISA was examined using triplicates of five samples (Supplemental Table 4). The intra-assay coefficient of variation (CV) ranged from 1.56% to 6.54% with a mean of 2.85%. The inter-assay CV ranged from 1.50% to 5.06% and a mean of 3.23%. These values indicate low variation and strong reproducibility, both within and between plates. The sera minimum detection limit determined by the maximum dilution of positive sera that remained above the established threshold (0.163) was 1:1024.

The viral specificity of the assay was determined by coating plates with standardized concentration (10^3 $TCID^{50}$ well $^{-1}$) of other pathogenic fish viruses and comparing the OD values. Throughout this analysis, the OD values for the blank well (containing no virus) and naïve muskellunge sera were all less than 0.089, indicting minimal non-specific binding (Table 1). In the case of the three muskellunge sera obtained from vaccinated fish, reactivity was only observed against the two VHSV-IVb preparations and there was no reactivity with either VHSV-IVa or IVc (OD ranged from 0.001 to 0.015). Similarly, no reactivity was observed against the other viral coating solutions, with OD values ranging from 0.000 to 0.018.

3.6. Detection of circulating anti-VHSV-IVb antibodies in muskellunge using the newly optimized ELISA

3.6.1. In vaccinated fish

Prior to vaccination with the pVHSivb-G (n = 32), the mean OD value was 0.010 (SD = 0.008) (Fig. 4). By 28 days following immunization, the OD value increased significantly in serum samples of all individual fish samples (average of 0.244 ± 0.161, paired t-test; $p < 0.0001$; $t = 8.207$; df = 28). At 42 days post administration, the mean OD value continued its increase to 0.388 ± 0.301 and peaked in sera obtained 56 days following exposure (0.395 ± 0.267). OD values had substantially decreased by 70 days post vaccination (0.215 ± 0.150), at which point muskellunge were given a second administration of the pVHSivb-G vector. Thirty-five days following a second administration, mean OD values had significantly increased (1.098 ± 0.506) when compared to the 70 day OD values ($p < 0.0001$; $t = 9.084$; df = 28). By 56 and 70 days after the second exposure, the mean OD values had again decreased to 0.293 ± 0.174 and 0.274 ± 0.158, respectively.

Table 1
Viral specificity of muskellunge (*Esox masquinongy*) sera using three positive sera obtained from pVHSivb-G vaccinated fish and two sera from naïve muskellunge against different viral coating preparations. Antigen coating preparations included 0.1 μg well^{-1} of purified viral hemorrhagic septicemia virus genotype IVb (VHSV-IVb) (MI03) and 10^3 $TCID_{50}$ well^{-1} of the following: VHSV-IVb (MI03), VHSV-IVa (Makah), VHSV-IVc (2000-149), infectious hematopoietic necrosis virus (IHNV) (220-90), infectious pancreatic necrosis virus (IPNV) and largemouth bass virus (LMBV).

Coating preparation	mean muskellunge sera OD490 values				
	Positive serum samples obtained from three pVHSivb-G vaccinated fish			Negative control	
	1	2	3	1	2
Purified VHSV-IVb (MI03)[a]	1.017	0.429	0.424	0.003	0.062
VHSV-IVb (MI03)[a]	0.854	0.375	0.345	0.065	0.011
VHSV-IVa (Makah)[b]	0.015	0.001	0.001	0.07	0.001
VHSV-IVc (2000-149)[b]	0.014	0.004	0.001	0.011	0.001
IHNV (220-09, genogroup M)[c]	0.001	0.016	0.018	0.089	0.001
IPNV[a]	0.004	0.000	0.004	0.044	0.011
LMBV[a]	0.034	0.005	0.003	0.033	0.011
Blank (coating solution with no virus)	0.008	0.005	0.002	0.001	0.006

[a] Isolated in our laboratory from infected fish.
[b] Obtained from the National Veterinary Service Laboratory, Animal and Plant Health Inspection Services, United States Department of Agriculture, Ames, Iowa, USA.
[c] Obtained from Dr. Scott LaPatra, Clear Spring Foods Inc., Buhl, Idaho, USA.

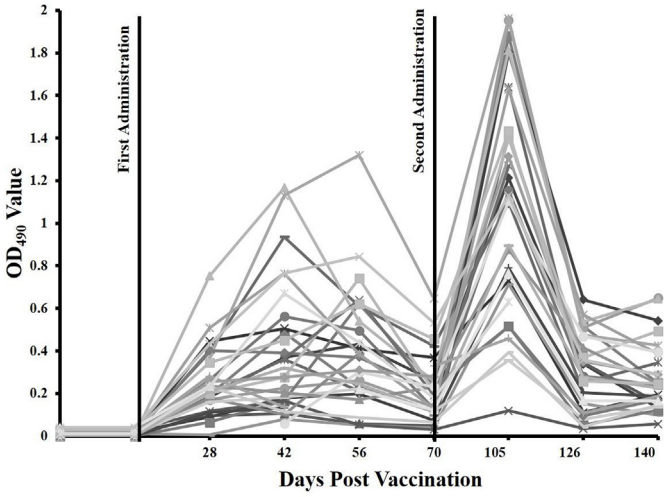

Fig. 4. Levels of circulating anti-VHSV antibodies of 32 individual juvenile muskellunge that were vaccinated with the pVHSivb-G vector on two occasions and antibody titers assessed by the newly developed and optimized ELISA using the 3B10 monoclonal antibody developed against the heavy chain of muskellunge immunoglobulin. The cut off value was estimated to be 0.163, above which a sample was considered positive.

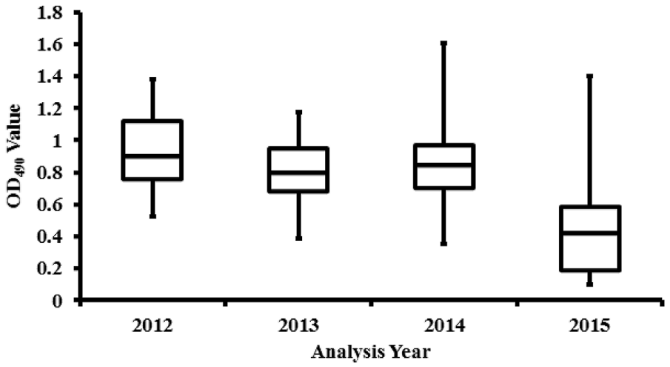

Fig. 5. Levels of circulating anti-VHSV antibodies in Detroit River, Michigan, muskellunge (*Esox masquinongy*). Sera were collected between 2012 and 2015 and assessed by the newly developed and optimized ELISA using the 3B10 monoclonal antibody developed against the heavy chain of muskellunge immunoglobulin. Data are presented as box plots of OD_{490} value displaying the mean and upper/lower quartiles; extended lines indicate the maximum and minimum observed values obtained for the respective populations. Cut off value was estimated to be 0.163, above which a sample was considered positive.

3.6.2. In wild muskellunge caught from an endemic waterbody

Indirect ELISA results demonstrated the presence of relatively high levels of anti-VHSV-IVb antibodies. OD values ranged from 0.089 to 1.61, with a mean of 0.703 ± 0.341 in 134 wild adult muskellunge collected from the Detroit River, a VHSV-IVb endemic water body in Michigan which connects Lake St. Clair to Lake Erie. Results also showed that OD values of the Detroit River muskellunge population have changed over time (Fig. 5). For example, in 2012, the mean OD value for the sampled population was 0.899 ± 0.250, 0.796 ± 0.229 in 2013, and 0.845 ± 0.257 in 2014. However, in 2015, when the greatest number of individual muskellunge were examined ($n = 45$), the mean OD value had decreased to 0.422 ± 0.321. Statistical analyses of sera collected in all four years indicated significant downward trend in antibody levels with an estimated slope of −0.152 ($p < 0.0001$; df = 1; F = 42.4).

4. Discussion

Specific antibodies (polyclonal or monoclonal) to species immunoglobulins and their subunits constitutes a fundamental step for better understanding of the antigen-antibody interactions and in the development of diagnostic assays. Monoclonal antibodies are particularly valuable since their hybridomas can provide a continuous supply of these antibodies. Herein, we report the development of hybridoma clones that secrete anti-muskellunge IgM specific monoclonal antibodies. Evidence that 3B10 is specific for muskellunge Ig is provided by its reactivity with serum IgM in western blot, in partition ELISA of TNP-KLH primed fish, and anti-VHSV antibodies in vaccinated and wild muskellunge. The fact that 3B10 recognizes the IgM heavy chain is advantageous as it is class specific as opposed to light chain since all immunoglobulin classes use the same kappa or lambda light chains. To our knowledge, this is the first report of mAbs available for muskellunge IgM. Our ultimate goal is to use the developed mAb to detect and quantitate circulating antibodies against muskellunge pathogens generated by vaccination or after surviving a natural infection.

Though the initial plan was to use immunopurified Ig from high-titered TNP-KLH immunized muskellunge, the 20% SAS precipitate of naïve muskellunge serum possessed enough IgM for the development of 3B10 hybridomas with a strong binding to IgM heavy chain. No reactivity was seen with the light chain. Most of the other fish anti-IgM mAbs that have been developed also target the IgM heavy chain [32–36], although a few mAbs have also targeted the IgM light chain [37,38]. This may be attributed to the fact that the heavy chain is more antigenic in nature as compared to IgM light chain. Following reduction of the muskellunge IgM with β-mercaptoethanol and separation by SDS-PAGE, muskellunge IgM heavy and light chains were estimated to be 73 and 26 kDa, respectively. Considering that IgM found in serum and mucus of teleosts is a tetramer with each subunit composed of 2H- and 2L-chains, the molecular weight of muskellunge IgM molecule can be calculated to be ~792 kDa, which is within the range reported from other fish species [32,35,37–41].

Though the monoclonal antibodies are highly specific and can differentiate small variations in epitopes, yet close structural similarity in heavy or light chains of immunoglobulins can result in cross reactivity. Findings of this study suggest that the epitope of muskellunge IgM heavy chain recognized by 3B10 is partially conserved among the four esocid species tested in this study which led to varying degrees of cross reactivity. As such, 3B10 can be used in future studies aiming at exploring the host defense mechanisms of esocids. It has been reported that antigenic cross reactivity exists among teleostean IgM molecules from fish species within the same genus [34,37, G.W. Warr, Medical University of South Carolina, personal communication] and in some cases, across different genera and families [35,42]. In this context, it was noted that IgM molecular weights of the four esocid species were slightly different from each other in size, which is most likely due to variation in the degree of glycosylation of the heavy chain.

Like in other vertebrates, the ability of a fish to mount an effective adaptive humoral immune response is controlled by the antibody ability to recognize, attach, sequester, and then facilitate the elimination of the eliciting antigens. In the present study, the newly developed 3B10 mAb allowed us to determine the avidity of muskellunge IgM against an antigen that elicited its induction (TNP-KLH). To do that we employed a partition-based immunoassay that permits the dissection of a single antiserum into discrete, affinity-based antibody subpopulations. The average affinity (log aK) of muskellunge IgM ranged from 5.96 to 6.25. These consistent values are equivalent to those reported for the rainbow trout [reviewed in 43] and further attest to the suitability of muskellunge antibodies for use in indirect ELISA whose protocol involves multiple washing steps.

Armed with the newly developed mAb, an indirect ELISA for the detection of circulating muskellunge anti-VHSV-IVb was optimized and its protocols established. All reagents used in this assay were titrated (Supplemental Tables 1–3). Because the generated 3B10 belongs to the immunoglobulin subclass IgG3 with a kappa light chain, we were able to use any anti-mouse IgG secondary antibody as suggested by the manufacturer's protocol. The indirect ELISA proved to be effective in deciphering some details of the humoral immune response that attest to the sensitivity and specificity of the assay. For example, when the developed assay was applied to post-vaccination serum samples it was obvious that a second administration of the vaccine induced a sharp increase in antibody titers in all primed fish (Fig. 4). This finding was surprising since DNA-vaccines are administered only once and are supposed to confer long-term protection [44]. It was also of interest to notice that all fish showed a peak of their antibody titers, at the 35th day following the second vaccine administration, in contrast to the variation in the number of days to achieve the antibody peaks observed following administration of the first vaccine dose. Determining the kinetics of antibody production following vaccination with such precision in important in designing vaccine regimen.

The effectiveness of the optimized indirect ELISA protocol using 3B10 was obvious when it detected circulating anti-VHSV antibodies in almost all (96%) muskellunge sampled from Detroit River and Lake St Clair, a waterbody that is known to be VHSV endemic. The widespread presence of anti-VHSV-IVb, despite the fact that the last time that this virus was isolated in this area was in 2009 [2], underscores the importance of using muskellunge in serosurveys. Given the decline in antibody titers that we detected between previous years and 2015, it does seem that anti-VHSV antibody levels do not persist for the entire fish life. Indeed, VHSV mortalities in muskellunge and other fish species

in the waterbody were observed in 2017 (Faisal and Loch, unpublished data). Optimizing the indirect ELISA protocol yielded an interesting observation in muskellunge vaccinated against VHSV-IVb glycoprotein (G) antigen; their antibodies recognized only VHSV-IVb and not the IVa or the IVc sublineages of the same virus or other its other closely related novirhabdovirus; the infectious hematopoietic virus (IHNV, Table 1). While this is surprising since the G antigens of VHSV and IHNV cross react and vaccination of rainbow trout against VHSV conferred transient protection (4 days) against IHNV, yet it is advantageous for the specificity of VHSV serosurveys using muskellunge [45].

5. Conclusion

This study details the successful development of a monoclonal antibody (designated 3B10) against the heavy chain of muskellunge IgM. The developed 3B10 appeared to be useful in a variety of assays that were used successfully in shedding light on the humoral immune responses of this species. First, 3B10 allowed the determination of the average muskellunge antibody affinity in a partioning ELISA assay. Second, an indirect ELISA employing 3B10, allowed the examination of circulating antibody kinetics in muskellunge following administration (s) of a VHSV-IVb DNA vaccine. Last, the optimized indirect ELISA confirmed the wide spread prevalence of anti-VHSV-IVb antibodies in muskellunge residing VHSV-endemic area in the Detroit River and Lake St. Clair, Michigan. The development of 3B10 will definitely enable studies to better understand the intricacies of muskellunge immune responses mounted against its pathogens.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.002.

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