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*Research Article*

# Production, characterization, and evaluation of polyclonal antisera specific to stellate sturgeon (*Acipenser stellatus*) immunoglobulin

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**Abstract**

Sturgeon species are commercially important species in finfish aquaculture industry. Till now, a small number of studies have been reported on the immune responses in sturgeon species against infectious diseases. Monoclonal and polyclonal antibodies in fish immunoglobulins (Igs) are essential to evaluate the humoral immune response. This study aimed to produce, characterize, and evaluate the polyclonal antisera against stellate sturgeon Igs. For the production of polyclonal antibodies against fish Igs, stellate sturgeon serum Igs was isolated using the ammonium sulfate precipitation method. The purified Igs with the Freund adjuvant was injected subcutaneously in to sheep (*Ovis aries*). Then, anti- stellate sturgeon Ig antibody titer and specificity were determined using ELISA and Western blot techniques. Stellate sturgeon Ig antibodies were detected in the immunized animals and were significantly higher in the immunized group compared to the non-immunized group. The polyclonal antisera showed a high specificity to the immunoglobulin heavy (H) chain of stellate sturgeon and moderately cross-reacted with the immunoglobulin H chain in Siberian (*Acipenser baerii*) and ship (*A. nudiventris*) sturgeons but did not show reactivity with rainbow trout (*Oncorhynchus mykiss*) serum Igs. It was indicated that the produced polyclonal antisera should be further investigated to be used for immune-diagnostic purposes in sturgeon species upon exposure to various infectious diseases.

## Introduction

Sturgeons such as stellate sturgeon (*Acipenser stellatus*) are important fish species both in wildlife and aquaculture industry. In recent years, the sturgeon culture has led to the outbreak of several diseases caused by various bacterial and viral pathogens resulting in economic losses. Fish can generate humoral antibody response during infection or immunization with any antigen. The main class of immunoglobulin in fish is immunoglobulin M (IgM) which is tetrameric and each monomer contained a heavy (H) chain and light (L) chain with a molecular weight of 60–77 and 23–26 kDa respectively (Koumans-van Diepen *et al.,* 1995; Van Muiswinkel, 1995). Immunoglobulins have been isolated and characterized from important aquaculture species (Lim *et al.*, 2009). Specific polyclonal and monoclonal antibodies to fish Ig have been important for developing immunological tools to monitor specific antibodies produced in response to pathogens and to evaluate the efficacy of vaccines on disease protection. The production of PAbs and MAbs specific to Igs and in different fish species have been reported, including sea bass (Romestand *et al.*,1995), European eel (Van der Heijden *et al.*,1995), salmon (Magnadottir *et al.*,1996), Torafugu (Miyadai *et al.,* 2004), common carp (Vesely, *et al.*, 2006), Japanese flounder (Li, *et al.,* 2007; Kim *et al*., 2017), rohu (Rathore *et al.*,2008), black rockfish (Shin *et al.,* 2006), walking catfish (Sood *et al.*, 2012), and Nile tilapia (Al-Harbi *et al.*,2000; Soonthonsrima *et al.,* 2019).

In recent years, (PAbs) can be considered a convenient and cost-effective tool to success fully apply for immunodiagnostic purposes (Crosbie *et al.,* 2002; Lim *et al.,* 2009; Purcell *et al.,* 2012). The generation of novel tools such as polyclonal and monoclonal antibodies to study the immune response of sturgeons has great importance in fish immunology. Studies on *A. stellatus* Ig and reacting anti-Ig antibodies have not been reported due to the lack of reagents for evaluation of the humoral immune response following various infectious diseases and vaccination programs on this fish species. A better understanding of the fish immune system and immune responses is needed to design strategies for the prevention of infectious diseases. Antibodies will be used for immune-diagnosis of infectious diseases and facilitate accurate monitoring of immune responses for the control of diseases. In the case of the PAbs against the *A. stellatus* Igs, a commercialized PAbs is not also available for the monitoring of stellate sturgeon immune responses. Therefore, this study aimed to produce, characterize, and evaluate polyclonal antibody specific to stellate sturgeon (*A. stellatus*) immunoglobulin.

## Material and methods

### *Animal ethic*

All methods were performed in accordance by the relevant guidelines and regulations of the Ethical Committees of Shiraz University.

### *Isolation of stellate sturgeon immunoglobulin*

Ten healthy adult stellate sturgeon (*Acipenser stellatus*) (5.47±0.34 kg) were obtained from a fish farm in Kamfiruz city, Fars province, Iran. Blood was drawn from

the caudal vein and the collected blood was allowed to clot at room temperature (RT). The blood samples were centrifuged at 3000×g for 15 min and the serum was separated and stored at - 20°C. Stellate sturgeon serum immunoglobulin was purified by salting out with ammonium sulfate. Serum was precipitated with an equal volume of 50% saturated ammonium sulfate. The suspension was centrifuged at 9000×g for 15 min at 4°C. The pellets were resuspended in PBS and dialyzed against phosphate buffer (pH 7.2) at 4 °C overnight. The protein content of the dialyzed sample was determined by Bradford protein assay (Bradford, 1976). The purity of Ig was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving and 5% stacking gels.

*Production of anti- stellate sturgeon Ig antibody*

The purified stellate sturgeon Ig (0.1 mg/ml) was mixed with complete Freund's adjuvant (Sigma Aldrich, USA) (1:1) and was subcutaneously injected into two of the 8-month-old sheep. Booster immunizations were conducted at the one week interval with the incomplete Freund's adjuvant (Sigma Aldrich, USA). Blood samples were collected before inoculation and at the time of antigen injections to monitor the production of specific antibodies. Final bleeding was performed 14 days after the last immunization. The blood samples were centrifuged at 2500 g for 10 min and the serum was collected and stored at -20°C for further use.

*Enzyme linked Immunosorbent assay (ELISA)*

The anti- stellate antibody titers and specificity were determined by enzyme-linked immunosorbent assay. ELISA plate (Greiner, Bio-One, Austria) was coated with 100 µL/well of purified stellate sturgeon serum Ig (0.1 µg/ml in 50 mM carbonate/bicarbonate buffer pH 9.6) and incubated at 4 °C overnight. Blocking solution (5% skim milk powder in PBS) was added at 200 µL per well and incubated at 37 °C for 2 hr. The plate was washed three times with washing buffer PBST [PBS at pH 7.2 with 0.05% Tween 20]. Anti- stellate sturgeon Ig polyclonal antibody was added at 1:50 dilution with [PBS at pH 7.2 with 1% bovine serum albumin] in 100 µL volume to each well and incubated for 1 h at 37°C. The plate was washed again 3 times with PBST and then horseradish peroxidase-conjugated donkey-anti-sheep IgG (Sigma-Aldrich, USA) was added to the wells at 1:6 000 and incubated for 1 h at 37 °C. After washing, 100 µL of O-Phenylenediamine (OPD) (Sigma Aldrich, USA) substrate was added to all the wells for 15 min. The optical density (OD) of each well was determined at 450 nm by a microplate reader (Laemmli, 1970):

S/P ratios= (OD of sample − OD of negative control)/ (OD of positive control − OD of negative control)

*Western blotting*

The reactivity of anti- stellate sturgeon Ig antibody was evaluated against the whole stellate sturgeon serum and the heterologous serum from other fish species such as Siberian sturgeon (*Acipenser baerii*), ship sturgeon (*Acipenser nudiventris)* and rainbow trout (*Oncorhynchus mykiss)* using Western blot analysis. Proteins were electrophoresed on 12% SDS-PAGE polyacrylamide gel. Protein bands were transferred onto a nitrocellulose membrane under semi-dry conditions for 1.5h. After blocking overnight at room temperature (RT) with 5% skimmed milk in PBS, nitrocellulose membrane stripes were cut, washed with PBST and incubated with a serum sample at RT for 1h. Sera samples from the immunised sheep were diluted at 1:50. After washing, anti-sheep conjugate peroxidase (Sigma, USA) diluted in PBS-T (1:1000) was added and incubated with shaking for 1h at RT. Finally, the membrane stripes were washed and placed into a substrate solution of 0.05% diaminobenzidine in 50mM Tris pH7.4 containing 0.05% H2O2. (DAB/ $H_2O_2$) (Sigma, USA) (Fig. 1).

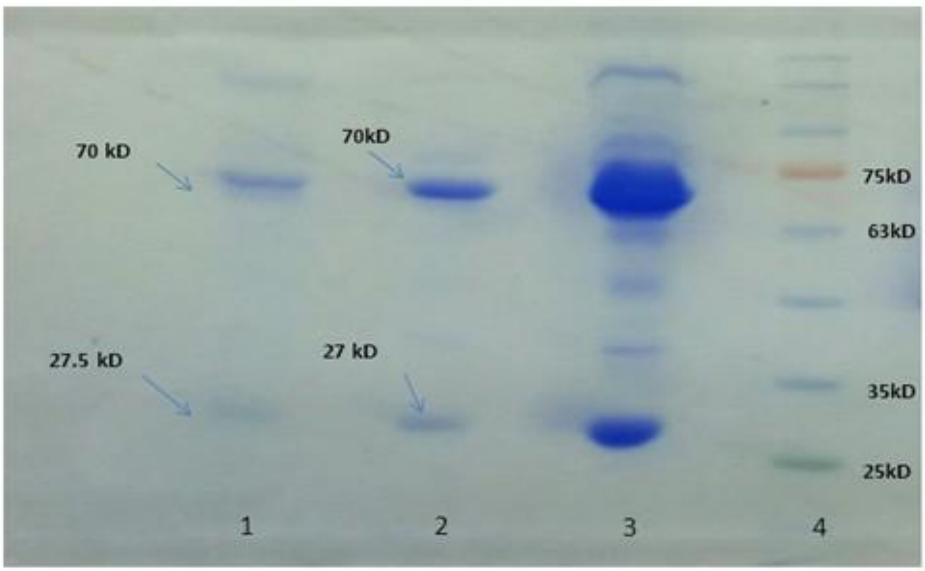


**Figure 1: SDS-PAGE under reducing conditions for estimation of molecular weight of Ig of the sturgeons. Lanes (1-2): Purified Igs from *Acipenser stellatus* and *Acipenser baerii* respectively, Lane (3): Unpurified Ig from *Acipenser baerii*, Lane (4): Marker (Cinnagen [SL7012 ] prestained protein ladder).**

*Statistical analysis*

Statistical analysis was carried out using SPSS version 16. All data were represented as the mean±standard deviation (SD). Differences among. *P*-values <0.05 were considered statistically significant.

## Results

The specificity of anti- stellate sturgeon Ig polyclonal antibody was determined using ELISA and Western blot analysis.

*ELISA*

Specific antibody against stellate sturgeon Ig resulted from toward the second week after the first immunization. High anti-

stellate sturgeon Ig antibody were significantly detected in immunized animal throughout the experiment which confirmed the immunization procedure ($p$<0.05). Antibody responses started to increase from the 7th day after the first immunization. Antibody level increased from day 14 to 42 in groups immunized. Antibody level reached a peak at day 42, following the 6th immunization (Fig. 2). Western blot analysis also confirmed the specificity of produced anti- stellate sturgeon Ig antibody in all immunized groups (Fig. 3).

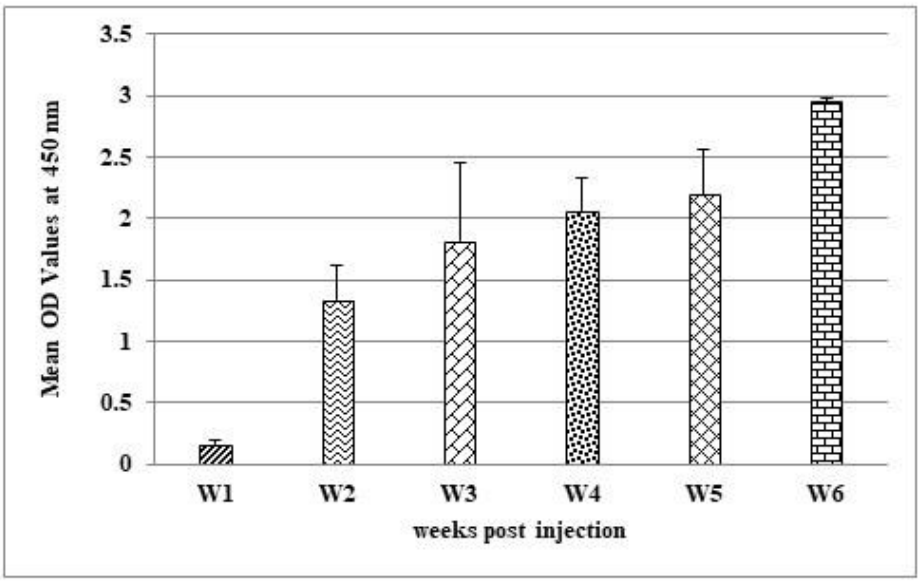


**Figure 2: Detection of antibody titers by Indirect ELISA. Sera collected from sheep before or at 7, 14, 21, 28, 35 and 42 days post-immunization. Averages of duplicate samples were represented with standard deviations.**

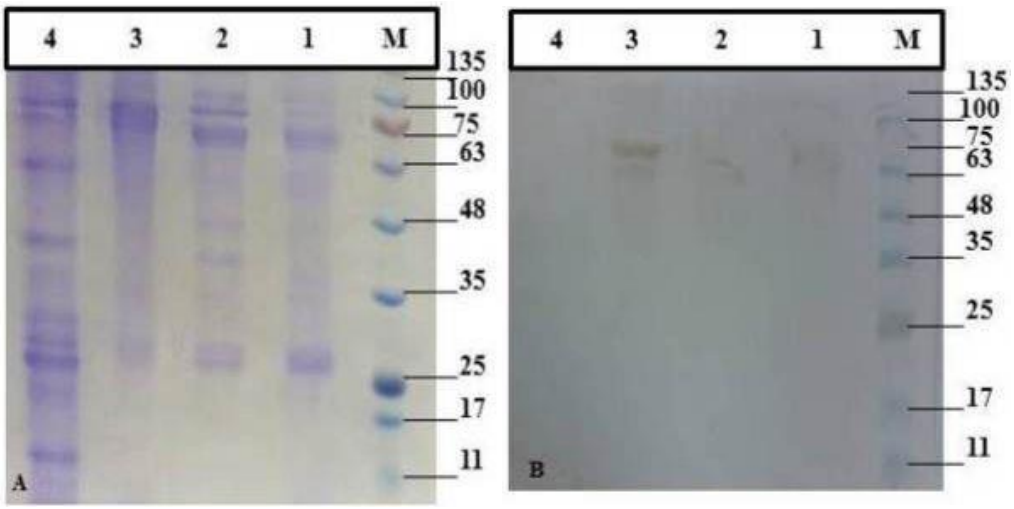


**Figure 3: (A) Denaturing SDS-PAGE analysis of fish serum immunoglobulin indicating the location of immunoglobulin H and L chains and (B) Western blot analysis of polyclonal antibodies (PAbs) specific to *Acipenser stellatus* immunoglobulin to check the specificity and cross-reaction. Panel A and B: Lane M: Size of molecular weight marker (Cinnagen [SL7012] protein ladder). Lane 1: Ship sturgeon *(Acipenser nudiventris)* serum. Lanes (2-3): Correspond to Siberian sturgeon (*Acipenser baerii)* and stellate sturgeon (*Acipenser stellates)* sera, respectively. Lane 4: Rainbow trout (*Oncorhynchus mykiss)* serum.**

*Western blot analysis*

The SDS-PAGE analysis showed stellate sturgeon Ig with a molecular weight of 27.5 kDa as a light chain and 70 kDa as a heavy chain (Fig. 1). In western blot analysis, the specificity of sheep sera against stellate sturgeon Ig was confirmed by a lack of any cross reactions with rainbow trout *(Oncorhynchus mykiss).* The anti- stellate sturgeon Ig antibody revealed strong reactivity with the serum Ig H chain of stellate sturgeon (*A. stellatus*). A slight reaction was found with the serum Ig H chains of Siberian sturgeon (*A. baerii)* and ship sturgeon *(A. nudiventris)* (Fig. 3).

**Discussion**

Anti-Ig monoclonal and polyclonal antibodies, in marine and freshwater fish species, are widely applied for developing immunoassays to investigate the immunological studies (Beelen *et al.,* 2004; Grove *et al.,* 2006; Tian *et al.,* 2009; Swennes *et al.,* 2007; Rathore *et al.,* 2008; Bag *et al.,* 2009). In this study, the possible production of polyclonal antibodies specific to stellate sturgeon Ig and its use as a diagnostic tool were investigated by ELISA and Western blot. The SDS-PAGE analysis of purified antibodies revealed high purity and quantity of the product at about 27.5 kDa as a light chain and a 70 kDa as a heavy chain. These results are consistent with those of Lim *et al.* (2009), Rathore *et al.* (2008), Babu *et al.* (2008), Uchida *et al.* (2000), Rajavarthini *et al.* (2000), Bag *et al.* (2009), and Covello *et al.* (2009) in several fish species, such as, Sea bass, Japanese eel, Tilapia, African catfish, Rohu, Indian major carps, striped trumpeter and Guppy. To our best knowledge, there are no previous reports of production of polyclonal antibodies specific to stellate sturgeon Ig. This study is a first description of production of polyclonal antibodies against stellate sturgeon serum Ig, which can be considered as an excellent tool that can be used for immunoassay purposes. The present study results showed the highest cross-reactivity of anti- stellate sturgeon Ig polyclonal antibodies with serum Ig H chain of stellate sturgeon and the lowest with serum Ig H chains of Siberian sturgeon (*A. baerii)* and ship sturgeon *(A. nudiventris)* but not with that of rainbow trout *(Oncorhynchus mykiss).* The reactivity with these species H chain is a reflection of the phylogenetic relationship between the stellate sturgeon and both species (being of the family sturgeon) and is not unexpected. This indicates that there is epitope sharing among the serum Ig H chains of all the three species of sturgeon. This suggests that the L chain may undergo antigenic modification during the preliminary processing. Few studies have explored the production and utilization of specific Abs against serum Igs of fish. Crosbie and Nowak (2002) reported that antiserum raised against Barramundi (*Lates calcarifer* Bloch) immunoglobulin (Ig) was specific for HC and LC Ig components using Enzyme-linked immunosorbent assay (ELISA) and Western blot analyses. Jang *et al.* (2004) examined the production and specificity of monoclonal antibodies against heavy and light chains of flounder (*Paralichthys olivaceus*) immunoglobulin. In a Western blot analysis, the produced MAbs recognized both the 72 and 77 kDa H chains, 26 kDa, and 28 kDa L chain, respectively. Li *et al.* (2007) also

investigated the production, characterization and applicability of monoclonal antibodies to immunoglobulin of Japanese flounder (*Paralichthys olivaceus*). Under reducing conditions in western blotting, MAbs were specific for the heavy chain 74 kDa of Japanese flounder Ig and 24 kDa (light chain). In conclusion, this is the first study describing the indigenous production of specific antibody against stellate sturgeon Ig. As indicated by the above-mentioned findings, anti- stellate sturgeon Ig polyclonal antibodies have great potential immunodiagnostic applications as an effective tool for examination of stellate sturgeon humoral antibody response in vaccination trails and infectious diseases causing mortality and economic losses in commercially important aquaculture species such as sturgeon species. These Polyclonal antibodies can be considered as an inexpensive source of specific antibodies that can be used for immunoassay purposes to investigate the humoral immune responses of stellate sturgeon to various pathogens in order to evaluate the efficacy of vaccines on disease protection.

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**Conflict of interest**

The authors do not have any conflicts of interest to declare.

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