AJLS

Asean Journal of Life Sciences, Vol 1(1), 2021, pp 9-20

Accepted date: 28 May 2021
Publish date: 21 June 2021

Immunological Cross-Reactivity Between Anti-Vitellogenin Antibodies and Vitellogenin in Three Species of Catfish

Roshani Othman[1*], Sharr Azni Harmin[1], Siti Hasmah Mohtar[1], Moohamad Ropaning Sulong[1], Mohd Syahril Mohd Zan[2] and Zarirah Mohd Sulperi[3]

[1]Department of Science and Biotechnology, Faculty of Engineering and Life Sciences, Universiti Selangor, Bestari Jaya Campus, Jalan Timur Tambahan, 45600 Batang Berjuntai, Selangor, Malaysia
[2]School of Biological Sciences, Faculty of Applied Sciences, Universiti Teknologi MARA, Negeri Sembilan Branch, Kuala Pilah Campus, 72000 Kuala Pilah, Negeri Sembilan, Malaysia
[3]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang Selangor, Malaysia
roshani@unisel.edu.my

Abstract

Vitellogenin (Vtg) is a specific biomarker of egg yolk precursor protein that is abundant in circulatory blood. This protein indicates levels of ovarian development in female fish and endocrine disruption in male fish based on its expression level. The protein highly conserved in various forms of Vtg in specific species. The aims of this study was to evaluate the cross-reactivity between *Hemibagrus nemurus* Vtg polyclonal antibody and vitellogenin from other catfish, *Hemibagrus wyckiodes*, *Pangasius pangasius* and *Clarias gariepinus* by enzyme-linked immunosorbent assay (ELISA). The plasma of three species of catfish was characterized using 7.5% SDS-PAGE and revealed a major polypeptide band corresponding to 100-175 kDa in *H. wyckiodes*, 70-160 kDa in *Pangasius pangasius* and 95-160 kDa in *Clarias gariepinus* and the existence of Vtg has been confirmed. The optimal detection of the *H. nemurus* Vtg was at dilution of 1/1000 polyclonal antibody. Furthermore, cross-reactivity tests showed that polyclonal antibody specific to *H. nemurus* Vtg reveals a cross-reaction with the plasma Vtg from three species of catfish. Thus, the polyclonal antibody can be used to detect and quantify Vtg in the three species of catfish that are closely related in order to examine the vitellogenesis periods and oocyte maturation.

Keyword: Vitellogenin; *Hemibagrus nemurus*; catfish; polyclonal antibody; ELISA

INTRODUCTION

Vitellogenin is a female-specific reproductive protein in fish and reliably associated with the vitellogenesis process in oocytes that indicates ovarian development. The Vtg molecule has multi-functional functions such as protein nutrition, amino acids, various lipids and carbohydrates during oocyte development and roles in immunological defences (Sun et al., 2020). It acts as an antimicrobial and antioxidant (Park et al., 2018) and provides nutrition for embryo development (Zou et al., 2020). This protein produced by the liver into the bloodstream under estrogen influence, sequestered by the ovaries into the Vtg oocytes by the receptor-mediated endocytosis, and enzymatically abolished by the serine protease and cathepsins into the lipovitellin, phosvitin and β-component deposits (Mao et al., 2020). In female fish, Vtg transmitted to the ovaries in the circulatory system during the development of ovarian follicles and incorporated as a yolk. However, in male fish, Vtg is not expressed due to poor endogenous estrogens but can be synthesized in the presence of external estrogen stimulation (Soliman et al., 2020).

The molecular weight of Vtg varies significantly between species of fish from different families as well as their biochemical properties (Reading et al., 2017). The expression of Vtg is slightly different depending on the area of the fish body, different stages of the life cycle, different levels of gonad maturity and different environmental conditions (Azad & Al-Jandal, 2020) that indicate the complexity of the reproductive protein. However, Vtg is highly conserved in some regions of the gene, depending on the species, and produces multiple forms of specific Vtg species (Sanchis et al., 2020). Thus, antibodies that have been raised from a particular species of Vtg could have special characteristics and cross-reactivity across species subjected by enzyme immunoassay techniques due to Vtg have some conserve region.

Vitellogenin quantification can help improve the efficiency of induced spawning techniques to produce high-quality seed by first establishing the oocyte quality (Rawung & Saruan, 2020). *Hemibagrus nemurus* is one of the species known as asynchronous fish (Adebiyi et al., 2011) and the molecular weight of Vtg reproductive protein in this species is 130 kDa (Othman et al., 2015). In addition, the specific sites of the Vtg protein could recognize the polyclonal antibody of Vtg in different epitopes of the similar biomarker by binding sites (Caza et al., 2020). In this perspective, the production of polyclonal antibody has characteristics related to the immunogenicity of phylogenetically conserved proteins by cross-reaction in immunoassays. The polyclonal antibody of *H. nemurus* Vtg was produced in the previous study (Othman, 2019), is used on the determination of cross-reactive epitopes by immunoassay, among other species of catfish, for the broad application of the tool.

The aim of this study is to evaluate the polyclonal antibody of *H. nemurus* Vtg towards Vtg from other species of catfish (red tail catfish; *Hemibagrus wyckiodes*, river catfish: *Pangasius pangasius* and African catfish; *Clarias gariepinus*) which may provide useful information to the aquaculture industry in Malaysia. In addition, the study can contribute to the sustainable management of reproduction in catfish species and indirectly ensure the quality of the aquatic environment.

METHODOLOGY

Samples Collection and Blood Sampling

A total of 28 fish, consisting of three species of catfish, was used in this study to evaluate the polyclonal antibody of *H. nemurus* Vtg in cross-reaction to Vtg of the three species of catfish. Adults *P. pangasius* with a body weight of 1.36 ± 0.21 kg and *H. wyckiodes* with a body weight of 0.73 ± 0.10 kg, consisting of five female samples and two male samples (as control) were collected from the Pahang fish farm. For adults *C. gariepinus* with body weight range from 0.6 kg to 2.0 kg and immature *C. gariepinus* with a body weight range of 0.1 kg to 0.3 kg were purchased from a local fish farm near Bestari Jaya, Selangor. *H. nemurus* was used as a control and purchased from a local fisherman in Bestari Jaya. All fish were brought to the Aquaculture Laboratory, Science and Biotechnology Department, Universiti Selangor (UNISEL) and acclimatized within one week for the experiment. Water parameters were maintained at a mean temperature of 29±2°C, pH of 8.23-8.70, and dissolved oxygen at 6.5 mg/mL. The fish were fed at 3% of body weight with commercially formulated feed (Cargill, Malaysia). The fish were anaesthetized with clove oil for blood collection from the caudal tail vein using 5 mL syringe. All live fish experiments have been approved by the Research Ethics Committee of the Universiti Selangor (UNISEL/CRIL/EC/0001) and have been conducted in accordance with the Research Ethics Guidelines.

Protein Assay

The blood plasma of the three species of catfish was used to measure the total protein in the blood to indicate the presence of proteins that may consist of the reproductive protein. The protein quantification of blood samples was determined and the Bovine Serum Albumin BSA (BioRad) was used as the standard by applying of a dye-binding method (Bradford, 1976). The blood plasma sample (200 µL, unknown concentration) was diluted in the Bradford reagent at a 1:1 to 1:7 ratio. Negative control (0 µg/mL) was prepared with distilled water and Bradford reagent. The protein assay was measured using Microplate Reader (Biochrom Asys UVM340, UK) with an absorbent wavelength of 595 nm. The standard absorbance curve at 595 nm vs. BSA concentration was plotted. The protein content of the unknown sample was estimated from the standard BSA curve.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to examine the presence of crude Vtg protein and was conducted according to Laemmli et al., (1970) on BioRad Mini-PROTEAN II (BioRad, Hercules, CA) electrophoresis gel via 4% stacking gel and 7.5% resolving gel (1.0 mm gel thickness). Prior application to the gel, the samples were diluted at a ratio of 1:1 in the SDS sample buffer (125mM Tris-HCl, 10% SDS, 20% v/v glycerol, 5% v/v β-marcapcoethanol, 0.02% bromophenol blue) (15 µg/mL) and heated to 95°C for five minutes in the Thermomixer comfort 1.5 mL (Eppendorf). The gel was loaded with 20 µL samples and the molecular weight standard (Thermo Scientific PageRuler Plus Prestained Protein Ladder; 10-250 kDa) was included as a reference. Electrophoresis was performed on buffer ice (50 mM Tris, 192 mM glycine and 0.1% SDS) at a constant current of 100 mA, 50 V for three hours. It was then stained with 0.1% Coomassie Brillant Blue R250 (Hercules, Canada) for 30 minutes with a slow shaker agitation and immersed in a destaining solution for three hours until clear bands were visible.

Sensitivity Test by ELISA

The minimum detection of purified *H. nemurus* Vtg and anti-*H. hemurus* Vtg in rabbit serum dilution was optimized and used in the immunoassay test (Othman, 2019). Three different concentrations of purified Vtg (125, 250, 500 ng/mL) and five serial dilutions of polyclonal antibody (1/500, 1/1000, 1/2000, 1/4000 and 1/8000) were examined. The three different concentrations of purified *H. nemurus* Vtg was diluted in a 50 mM carbonate buffer pH 9.6 and incubated at 4°C overnight. For non-specific binding (NSB), only three wells were filled with a buffer coating. Microplates were then blocked with 200 µL of 1% skimmed milk in PBST (phosphate buffered saline-tween 20) for two hours at 37°C. Then, 96 microplates were washed with PBST four times (pH 7.4, with 0.1% Tween 20). Polyclonal antibody of *H. nemurus* Vtg diluted in different dilutions (200 µL per well) was added to the wells and incubated at 37°C for two hours. After four times wash with PBST, all wells received goat anti-rabbit IgG conjugated with HRP at 1:2000 dilution in blocking buffer, incubation at 37°C for two hours followed by four washes with PBST. Finally, the microplates received 100 µL of 3,3,5,5'-Tetramethylbenzidine (TMB) substrate solution and were incubated for 30 minutes for substrate development. The reaction was stopped by adding 100 µL of 1M H_2SO_4 to each microplate. The absorbance at 450 nm was read with a Microplate Reader (Biochrom Asys UVM340, UK).

Vitellogenin Quantification and Data Analysis

A competitive ELISA method was used for the quantification of Vtg in three species of catfish. The *H. nemurus* Vtg was used to capture an antigen. Microplates were blocked for 45 minutes at 37ºC with a blocking buffer (1% skimmed milk in PBST, 200 µL per well) to reduce non-specific binding. Samples (dilution ranging from 1:100 to 1:1000000) and standards (31.2, 62.5, 125, 250, 500 and 1000 ng/mL) were diluted in an assay buffer (1% skimmed milk in PBST). Standards and samples consisting of female and male plasma were mixed (1:1. v/v) with the primary antibody that was diluted to a minimum concentration as a result of optimization. Secondary antibody horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and TMB substrate solution were used to evaluate peroxidase activity. Color development was stopped by the addition of 100 µL of 0.05 M sulphuric acid, H_2SO_4. The 450 nm absorbance was read with the Microplate Reader. Data analysis was performed using One Way Variance Analysis (ANOVA) followed by Tukey's post-hoc test using IBM SPSS statistic 19.0 software and the significance was calculated at $P<0.05$.

RESULTS AND DISCUSSION

Total Protein in Blood Plasma Samples

The concentration of proteins in blood plasma samples of the three species of catfish was quantified and the BSA (bovine serum albumin) was used as the standard (Bradford, 1976). Table 1 shows the concentration of plasma protein, body weight and standard length in males and females of three species of catfish. The distribution of the total protein in the blood plasma of all species of catfish in this study showed a range between 0.0987 and 0.1240 mg/mL, indicating that almost all samples were not significantly different (Table 1). The total protein concentration may be influenced by the presence of various proteins such as dissolved nutrients, metabolites, hormones, immunological proteins to counteract infection and haemorrhage, and other proteins in blood plasma samples (Mahavidyalaya et al., 2020).

However, one of the major proteins in plasma is the reproductive protein or vitellogenin and has been confirmed as a precursor to the main protein in egg yolk (Deeley et al., 1975). Other variables that may affect protein concentrations in fish are distinct due to variations in body

weight, diet, feed supplements and the environment (Higgs et al., 2009). Furthermore, ecological components (stress), food and pathogens may affect the health and reproductive status of fish. Stress in fish can be caused by various biotic environmental factors, such as changes in water temperature, pH, oxygen fixation and water pollution, including pesticides and bug sprays (Bal et al., 2021). Thus, a specific examination is essential to ensure that the fish is suitable for spawning purposes.

Table 1 The standard length, body weight and total protein concentration in plasma for female *Clarias gariepinus*, *Pangasius pangasius*, *Hemibagrus wyckiodes* and *Hemibagrus nemurus*

Catfish species	Body Weight (g)	Standard Length (cm)	Total protein concentration (mean± SE) (mg/mL)
Pangasius pangasius	1400±200^{c}	46.64±2.46^{c}	0.1029±0.0074^{ab}
Hemibagrus wyckiodes	685±57.66^{ab}	36.3±0.57^{ab}	0.1044±0.0039^{ab}
Clarias gariepinus (matured)	1560±178.19^{c}	55.4±5.81^{d}	0.0987±0.0052^{a}
*Clarias gariepinus (*immature)	234±72.66^{a}	30.3±4.02^{a}	0.1175±0.0081^{ab}
Hemibagrus nemurus	1168±831.25^{bc}	38.00±7.42^{b}	0.1240±0.0200^{b}

Note: Means with the same superscript letter (^{a,b,c}) within column are not significantly different p>0.05 by Tukey's post-hoc test

Characterization of Plasma Protein in Blood

The molecular weight of the three species of catfish was evaluated between male and female fish in order to characterize the Vtg protein, which should be present only in female fish, and to differentiate the band in the SDS-PAGE gel. In this study, non-purified Vtg blood plasma was used to screen the presence of the reproductive protein. The results showed that, under the reduced condition of the SDS-PAGE, lanes 1, 2, 3 and 4 consist of female *H. wyckiodes* samples, a major band between 100 to 175 kDa (Figure 1a). Lane 5 and Lane 6 consisting of male *H. wyckiodes* samples appeared thinner and slightly no intact bands of similar size (Figure 1a) and were not well expressed. There are several factors that have not contributed to intact bands produced, such as blood plasma samples that have not been purified and fish samples from cages in the river that have been exposed to environmental conditions. Previous study reported the molecular weight of purified Vtg of closely related species, *H. nemurus* appeared at 130 kDa (Othman et al., 2015). However, male fish may have Vtg expressed as a result of exogenous estrogen in the aquatic environment. Previous study shows that the polluted area containing xenoestrogens may affect the endocrine system of fish making male fish produce female exclusive proteins such as Vtg. (Badamasi et al., 2020; Wang et al., 2019)

The result for SDS-PAGE of crude plasma from *P. pangasius* is shown in Figure 1b. Plasma was obtained from female *P. pangasius* (lanes 1 and 2; Figure 1b) and it was shown to contain Vtg protein. The band in both lanes has a high concentration of 70 to 160 kDa. Compared to lane 3, a similar range of band concentrations shows there is less protein present in the plasma of the male *P. pangasius* specimen. Figure 1c shows the result of the SDS-PAGE for *C. gariepinus* plasma. The plasma obtained from female *C. gariepinus* is shown in lanes 1 and 2 while lane 3 was a male specimen. The band in lane 3 is lower than the band in lane 1 and 2. The first two lanes have a band higher than, 95 to 160 kDa. In this study, the reproductive proteins of all male specimens are not well expressed. The molecular weight of catfish Vtg may vary according to the size of the different bands between catfish species, as agreed in the

previous study (Pamungkas, Jusadi, Junior, et al., 2019; Thanomsit et al., 2020). In this study, it was shown that no major intact band appears in the gel loaded with male fish plasma. However, some minor bands appearing on the gel may have been the result of the use of raw fish plasma and other proteins may have contributed to the presence of the band. In addition, SDS-PAGE was a reliable method to be used in the determination of protein molecular weight of the purified Vtg.

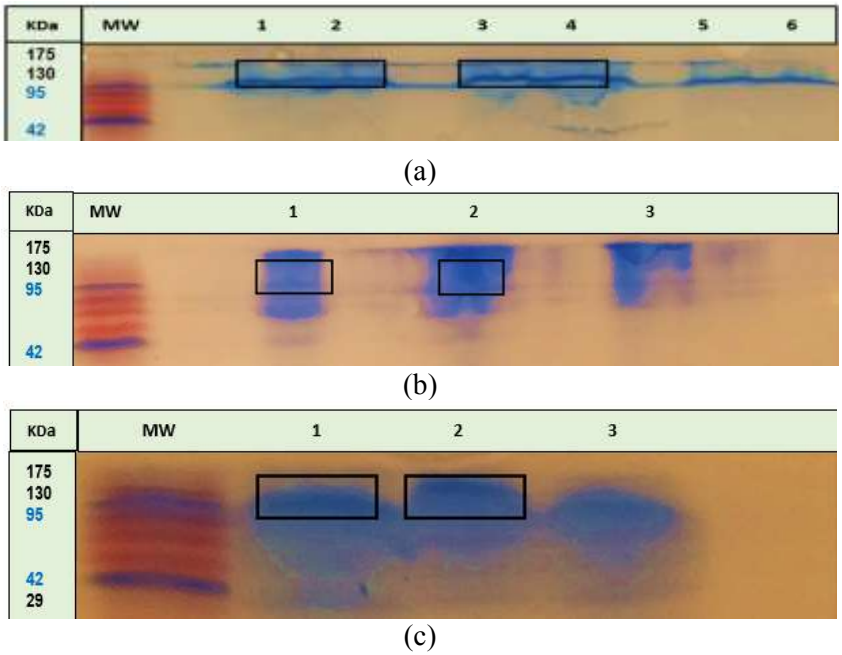

Figure 1 SDS-PAGE electrophoretic pattern of crude Vtg from blood plasma (a) *Hemibagrus wyckiodes*: MW, Molecular mass marker; Lane 1,2,3,4: female plasma; Lane 5 and 6: male plasma (b) *Pangasius pangasius*: MW, Molecular mass marker; Lane 1,2: female plasma; Lane 3: male plasma (c) *Clarias gariepinus* (matured): MW, Molecular mass marker; Lane 1,2: female plasma; Lane 3: male plasma

Antigen and Antibody Sensitivity Test

An antigen and antibody dilution test was performed to select the optimum dilution. The optimal anti-Vtg dilution of 1:2000 and Vtg concentration of 250 ng/mL showed the best performance due to OD readings close to one (0.938± 0.044 at 450 nm; Figure 2) after 30 minutes of colour development (Swart & Pool, 2009). As a result, a purified *H. nemurus* Vtg concentration of 250 ng/mL and 1:2000 anti-Vtg dilution were used for the application of working ELISA. In addition, the presence of IgG antibody against Vtg can be confirmed by ELISA, which has revealed the antigen-antibody reaction specificity (Amano et al., 2019; Sanchis et al., 2020; Wang et al., 2019). Almost similar findings have been reported for antigen-antibody binding concentrations such as in mahseer, *Tor tambroiders* (Muhammad et al., 2011) Vtg with a combination of 253 ng/mL and 1:500 dilution of anti-carp monoclonal antibody, Asian sea bass, *Lates calcarifer* (Fazielawanie et al., 2011) with a combination of 250 ng/mL and 1:1000 dilution of primary antibody and Asian catfish, *Clarias batrachus* (Garnayak et al., 2013) with combination of 100 ng/mL and 1:100 000 of primary rabbit antiserum dilution.

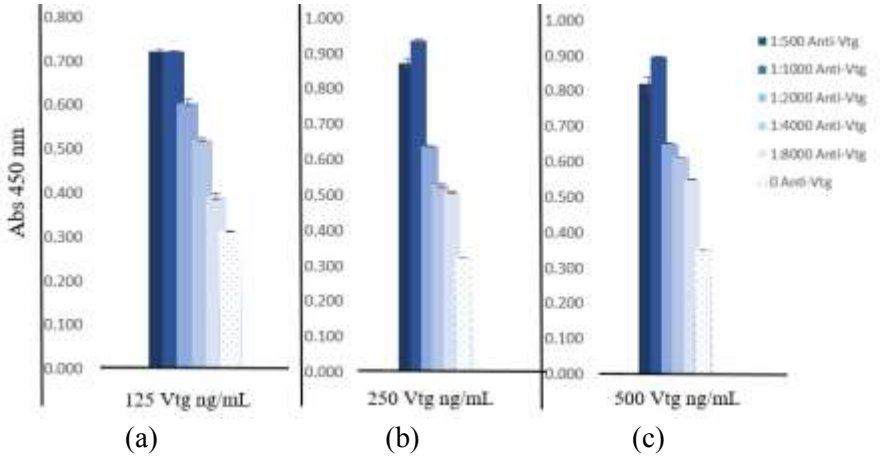

Figure 2 Absorbance values of competitive ELISA with different dilution of anti-Vtg (polyclonal antibody IgG) and different concentration of *H. nemurus* Vtg (a) 125 ng/mL (b) 250 ng/mL and (c) 500 ng/mL

Evaluation of Anti-Vtg towards Catfish Species Vtg

The purified *H. nemurus* Vtg and anti-*H. nemurus* Vtg serum was used to evaluate cross reactivity among catfish species Vtg as well to measure the levels of Vtg in blood plasma samples. The specificity of anti-*H. nemurus* Vtg serum against Vtg in females of three species of catfish was evaluated. Data obtained based on the presence of cross-reactivity between three species of catfish (Figure 3) using a standard curve (Figure 4; R squared = 0.9745, y=0.0003x + 0.3113). The result showed a cross-reaction between the blood plasma of female fish *C. gariepinus* (matured), *C. gariepinus* (immature), *H. wyckiodes* and *P. pangasius*, which serve as an antigen to anti-Vtg rabbit serum. Although ELISA is an excellent immunological method for the detection of Vtg, previous studies have suggested that the method may have the limitation of relying on a specific antiserum that may be species-specific (Heppell et al., 1999). The study suggested that the immunological and structure of Vtg may differ between fish species and therefore a specific antiserum may be required for each species. However, in the present study, the anti-Vtg of *H. nemurus* is recognized in the three species of catfish. The lack of anti-Vtg binding to any protein in normal mature and immature male fish and non-vitellogenic females clearly indicates that the antibody is Vtg-specific.

Each antiserum was highly reactive with its specific antigen, and polyclonal antibodies typically recognize cross-reactive epitopes between related species using competitive ELISA (Watts et al., 2003). However, there was also a lack of cross-reactivity between unrelated species in the previous study (Amthauer et al., 2021). The concentration of plasma Vtg in mature female *P. pangasius*, *H. wickiodes* and *C. gariepinus* was found to be significantly different $p<0.05$. It indicates that fish have different seasonal profiles and different reproductive strategies (Zhang et al., 2009). Other factors affecting this are the duration of the vitellogenic process and the size of the Vtg reached by the oocytes during the growing phase.

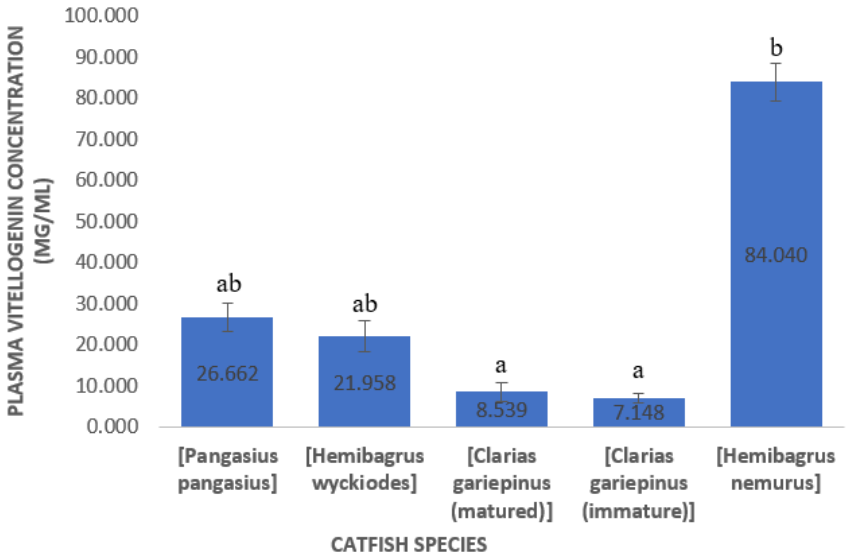

Figure 3 Plasma vitellogenin concentration for female *Clarias gariepinus*, *Pangasius pangasius*, *Hemibagrus wyckiodes* and *Hemibagrus nemurus*. Bar represents the mean ± standard deviation. Bars showing similar superscript letters are not significantly different at $p > .05$.

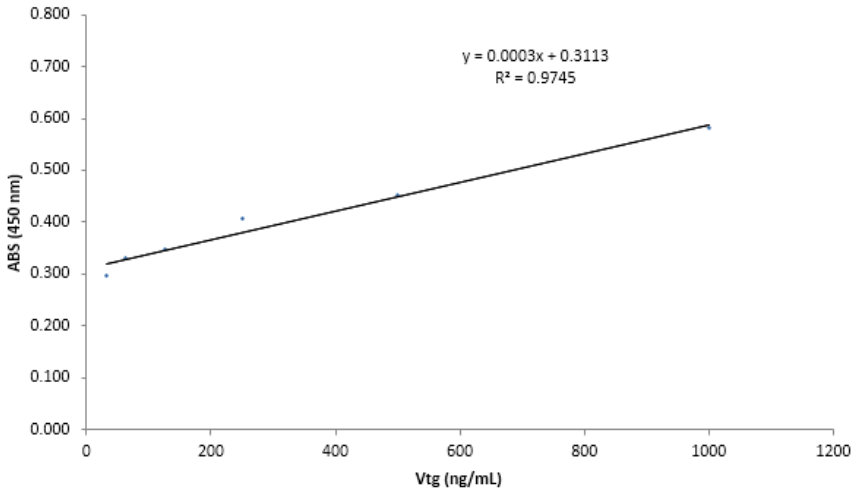

Figure 4 A standard curve of *Hemibagrus nemurus* vitellogenin concentration (ng/mL).

CONCLUSION

The present study provides a better understanding of the cross reactivity of polyclonal antibodies from *H. nemurus* Vtg towards Vtg from *P. pangasius*, *H. wyckiodes*, and *C. gariepinus*. This finding suggests that *H. nemurus* Vtg antibody can be used as a probe for detecting and quantifying Vtg in blood plasma in these catfish species, which can be carried out successfully in the lab. Therefore, the Vtg immunological assay as an alternative in determining the reproductive status of female fish, potentially leading to the reproductive success of these catfish species.

ACKNOWLEDGEMENT

This research is funded by the Selangor State Research Grant "Geran Penyelidikan Negeri Selangor", GPNS (Grant No: GPNS-01/UNISEL/18-053).

REFERENCES

Adebiyi, F. A., Siraj, S. S., Harmin, S. A., & Christianus, A. (2011). Ovarian development of a river catfish *Hemibagrus nemurus* (Valenciennes, 1840) in captivity. *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology*, *315*(9), 536–543. https://doi.org/10.1002/jez.702

Amano, H., Uno, S., Koyama, J., Hiramatsu, N., Todo, T., & Hara, A. (2019). Development of specific enzyme-linked immunosorbent assays for multiple vitellogenins in marbled sole, *Pleuronectes yokohamae*. *General and Comparative Endocrinology*, *281*, 67–72. https://doi.org/10.1016/j.ygcen.2019.05.012

Amthauer, R., Cárdenas, F., Reyes, A., Valenzuela, A., Dantagnan, P., Vidal, R., & Vargas-Chacoff, L. (2021). Vitellogenesis in the Patagonian toothfish (Dissostichus eleginoides) conditioned to a recirculating aquaculture system. *General and Comparative Endocrinology*, *307*, 113768. https://doi.org/10.1016/j.ygcen.2021.113768

Azad, I. S., & Al-Jandal, N. (2020). Induction of vitellogenin in cultured fish: Vitellogenin gene expression response of blue fin and yellow fin breams to injected 17 -estradiol. *Journal of Environmental Chemistry and Ecotoxicology*, *12*(1), 8–16. https://doi.org/10.5897/JECE2019.0448

Badamasi, I., Odong, R., & Masembe, C. (2020). Threats posed by xenoestrogenic chemicals to the aquatic ecosystem, fish reproduction and humans: A review. *African Journal of Aquatic Science*, *45*(3), 243–258. https://doi.org/10.2989/16085914.2020.1746233

Bal, A., Panda, F., Pati, S. G., Das, K., Agrawal, P. K., & Paital, B. (2021). Modulation of physiological oxidative stress and antioxidant status by abiotic factors especially salinity in aquatic organisms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *241*, 108971. https://doi.org/10.1016/j.cbpc.2020.108971

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Caza, J., Fernández, R., Arias, M. T., Muñoz, D., Tirado, J. O., Manjunatha, B., & Kundapur, S. I. M. R. R. (2020). The development, purification and characterization of anti-vitellogenin antibodies raised in hens (*gallus gallus domesticus*) against tilapia (*Oreochromis niloticus*) vitellogenin protein. *Journal of Microbiology, Biotechnology and Food Sciences*, *2020/21*(vol. 10), 1165–1168.

Deeley, R. G., Mullinix, D. P., Wetekam, W., Kronenberg, H. M., Meyers, M., Eldridge, J. D., & Goldberger, R. F. (1975). Vitellogenin synthesis in the avian liver. Vitellogenin is the precursor of the egg yolk phosphoproteins. *The Journal of Biological Chemistry*, *250*(23), 9060–9066.

Fazielawanie, N. M. R., Siraj, S., Harmin, S., Ina-Salwany, M. Y., & Nik-Daud, N. S. (2011). Development and Validation of Enzyme-Linked Immunosorbent Assay (ELISA)

Vitellogenin in *Lates calcarifer*. *Journal of Fisheries and Aquatic Science*, *6*, 715–727. https://doi.org/10.3923/jfas.2011.715.727

Garnayak, S. K., Mohanty, J., Rao, T. V., Sahoo, S. K., & Sahoo, P. K. (2013). Vitellogenin in Asian catfish, *Clarias batrachus*: Purification, partial characterization and quantification during the reproductive cycle by ELISA. *Aquaculture*, *392–395*, 148–155. https://doi.org/10.1016/j.aquaculture.2013.02.020

Heppell, S. A., Jackson, L. F., Weber, G. M., & Sullivan, C. V. (1999). Enzyme-Linked Immunosorbent Assay (ELISA) of Vitellogenin in Temperate Basses (*Genus Morone*): Plasma and In Vitro Analyses. In *Transactions of the American Fisheries Society* (Vol. 128).

Higgs, D. A., Sutton, J. N., Kim, H., Oakes, J. D., Smith, J., Biagi, C., Rowshandeli, M., & Devlin, R. H. (2009). Influence of dietary concentrations of protein, lipid and carbohydrate on growth, protein and energy utilization, body composition, and plasma titres of growth hormone and insulin-like growth factor-1 in non-transgenic and growth hormone transgenic coho salmon, *Oncorhynchus kisutch* (Walbaum). *Aquaculture*, *286*(1), 127–137. https://doi.org/10.1016/j.aquaculture.2008.08.036

Mahavidyalaya, P., Aurangabad, D., & Deshmukh, D. (2020). *Our Heritage One Day Interdisciplinary National Conference on "Arts, Sculpture and Architecture in Marathwada" Study on Haematological Parameters in Fresh Water Fishes Catla Catla and Labeo Rohita From Paithan Maharastra*.

Mao, Q., Wu, W., Huang, L., Yi, G., Jia, D., Chen, Q., Chen, H., & Wei, T. (2020). Insect Bacterial Symbiont-Mediated Vitellogenin Uptake into Oocytes To Support Egg Development. *MBio*, *11*(6). https://doi.org/10.1128/mBio.01142-20

Muhammad, N. A., Christianus, A., Daud, S. K., Saad, C. R., Harmin, S., & Ina-Salwany, M. Y. (2011). Estrogen-Induced Vitellogenin in *Tor tambroides* (Bleeker, 1854): Purification, Characterization and ELISA Development. *Journal of Fisheries and Aquatic Science*, *6*, 700–714. https://doi.org/10.3923/jfas.2011.700.714

Othman, R. (2019). Vitellogenin Characterization for Development of Enzyme-Linked Immunosorbent Assay (ELISA) and Annual Vitellogenic Cycle of *Hemibagrus nemurus* Valenciennes. (Unpublished PhD's thesis). Universiti Selangor, Malaysia.

Othman, R., Harmin, S. A., & Yasin, I.-S. M. (2015). Protein identification of vitellogenin in river catfish (*Hemibagrus nemurus*). *Bioremediation Science and Technology Research*, *3*(1), 1–5.

Pamungkas, W., Jusadi, D., Jr, M. Z., Setiawati, M., Supriyono, E., & Imron, I. (2019). *Induction of ovarian rematuration in striped catfish (Pangasianodon hypophthalmus) using pregnant mare serum gonadotropin hormone in out-of spawning season*. *12*(3), 10.

Pamungkas, W., Jusadi, D., Junior, M. Z., Setiawati, M., Supriyono, E., & Imron, I. (2019). Profile of 17ß-estradiol, vitellogenin, and egg diameter during gonad maturation process of striped catfish *Pangasianodon hypophthalmus*. *Jurnal Akuakultur Indonesia*, *18*(2), 152–161. https://doi.org/10.19027/jai.18.2.152-161

Park, H. G., Lee, K. S., Kim, B. Y., Yoon, H. J., Choi, Y. S., Lee, K. Y., Wan, H., Li, J., & Jin, B. R. (2018). Honeybee (Apis cerana) vitellogenin acts as an antimicrobial and antioxidant agent in the body and venom. *Developmental & Comparative Immunology*, *85*, 51–60. https://doi.org/10.1016/j.dci.2018.04.001

Rawung, L. D., & Saruan, J. (2020). Effectiveness of Curcumin Supplementation in feed on liver productivity and reproductive performance of female carp *Cyprinus carpio* L.: Effectiveness of Curcumin Supplementation in feed on liver productivity and reproductive performance of female carp *Cyprinus carpio* L. *Indonesian Biodiversity Journal*, *1*(1), 22–33.

Reading, B., Sullivan, C., & Schilling, J. (2017). *Vitellogenesis in Fishes*.

In: Reference module in Life Sciences. Elsevier.https://doi.org/10.1016/B978-0-12-

809633-8.03076-4

Sanchis, P. J., Nocillado, J., Cheetham, N., Miller, A., Powell, D., Wang, T., & Elizur, A. (2020). Development and validation of a competitive hybrid ELISA for *Seriola lalandi* Vitellogenin. *Aquaculture Research*, *51*(6), 2205–2215. https://doi.org/10.1111/are.14565

Soliman, G., Abdelaziz, M., Eissa, A. E., Elias, N., & Moustafa, M. (2020). Impacts of natural and experimental phenol pollution on the reproductive performance, vitellogenin synthesis and pathological alterations of male *Oreochromis niloticus*. *Egyptian Journal of Aquatic Biology and Fisheries*, *24*(4), 479–495. https://doi.org/10.21608/ejabf.2020.101713

Sun, W., Li, L., Li, H., Zhou, K., Li, W., & Wang, Q. (2020). Vitellogenin receptor expression in ovaries controls innate immunity in the Chinese mitten crab (*Eriocheir sinensis)* by regulating vitellogenin accumulation in the hemolymph. *Fish & Shellfish Immunology*, *107*, 480–489. https://doi.org/10.1016/j.fsi.2020.09.007

Swart, J. C., & Pool, E. J. (2009). The Development and Validation of a Quantitative ELISA for in vivo and in vitro Synthesized Vitellogenin from Mossambicus tilapia (*Oreochromis mossambicus*). *Journal of Immunoassay and Immunochemistry*, *30*(2), 208–223. https://doi.org/10.1080/15321810902782905

Thanomsit, C., Saowakoon, K., Wattanakornsiri, A., Khongchareonporn, N., Nanuam, J., Prasatkaew, W., & Nanthanawat, P. (2020). Acetylcholinesterase (AChE) polyclonal antibody from hybrid catfish (*C. macrocephalus* × *C. gariepinus*): Specification, sensitivity and cross reactivity. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *237*, 108837. https://doi.org/10.1016/j.cbpc.2020.108837

Wang, Y., Li, Y., Chen, Q., & Liu, Z. (2019). Long-term exposure of xenoestrogens with environmental relevant concentrations disrupted spermatogenesis of zebrafish through altering sex hormone balance, stimulating germ cell proliferation, meiosis and enhancing apoptosis. *Environmental Pollution*, *244*, 486–494. https://doi.org/10.1016/j.envpol.2018.10.079

Watts, M., Pankhurst, N. W., Pryce, A., & Sun, B. (2003). Vitellogenin isolation, purification and antigenic cross-reactivity in three teleost species. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *134*(3), 467–476. https://doi.org/10.1016/S1096-4959(02)00288-9

Zhang, D., Xiong, H., Mennigen, J. A., Popesku, J. T., Marlatt, V. L., Martyniuk, C. J., Crump, K., Cossins, A. R., Xia, X., & Trudeau, V. L. (2009). Defining Global Neuroendocrine Gene Expression Patterns Associated with Reproductive Seasonality in Fish. *PLOS ONE*, *4*(6), e5816. https://doi.org/10.1371/journal.pone.0005816

Zou, M.-M., Wang, Q., Chu, L.-N., Vasseur, L., Zhai, Y.-L., Qin, Y.-D., He, W.-Y., Yang, G., Zhou, Y.-Y., Peng, L., & You, M.-S. (2020). CRISPR/Cas9-induced vitellogenin knockout lead to incomplete embryonic development in *Plutella xylostella*. *Insect Biochemistry and Molecular Biology*, *123*, 103406. https://doi.org/10.1016/j.ibmb.2020.103406