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Development and Validation of Enzyme-Linked Immunosorbent Assay (ELISA) Vitellogenin in *Lates calcarifer*

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ABSTRACT
A study was conducted to develop and validate a competitive enzyme-linked immunosorbent assay (ELISA) for detection of plasmatic vtg in Asian sea bass, *Lates calcarifer*. Highly specific polyclonal antibodies against purified vtg (antigen) were employed for quantifying the concentration of plasma vtg. The working ranges of the assay were 31.2 to 1000 ng mL^{-1} with the sensitivity of 6.9 ng mL^{-1}. Antigen concentration of 250 ng mL^{-1} and antibody dilution of 1:1000 were selected as a workable ELISA after several preliminary test. The ELISA demonstrated precision with intra-and inter-assay Coefficient of Variations (CVs) at 90, 80 and 50% of binding were less than 8.4 (n = 9) and 12.1% (n = 5), respectively. Serial plasma dilutions from natural vitellogenic females and E_2 treated were paralleled to the vtg standard curve (purified vtg) as analyzed by ANCOVA ($p<0.05$). No cross-reaction was observed in analyses of male's plasma, indicating non-specific binding. The assay was validated by measuring plasma vtg levels in matured females and males (n = 5) obtained during the reproductive season in captive condition. Female's plasma vtg ranges from 0.9 to 1.54 mg mL^{-1}, while no vtg was detected in males plasma. Our results indicated that vtg levels proposed as an indicator for maturing female Asian sea bass, *L. calcarifer* as well as in female species from genus *Lates*.

Key words: ELISA, *Lates calcarifer,* vitellogenin

INTRODUCTION
Lates calcarifer, commonly known as Asian sea bass or locally called siakap, is a catadramous fish (grows to maturity in fresh or brackish waters and spawns in the sea) in family Latidae of order Perciformes. It is one of the nine *Lates* species from family Latidae which is widely distributed in coastal Australia, Southeast and Eastern Asia and India (Luna, 2008). This species are farmed in cages, salt water ponds as well as fresh water (Webster and Lim, 2002). In recent years, sea bass has gained growing importance in aquaculture both as recreational and commercial fish, with a high and fairly stable price (Luna, 2008). Sea bass production and consumption in Malaysia has increased dramatically over the years with the estimated aquaculture production of 194, 623.11 tonnes. High demand of *L. calcarifer* in aquaculture industry has triggered research scientists studying the reproductive physiology of this species. Susca *et al.* (2001) concluded that

the reproductive physiology of many fish species has been studied by analyzing the vitellogenin (vtg) levels in the blood plasma which is important for broodstock management for spawning purpose in captive condition.

The vtg, classified as phosphoglycolipoprotein is a precursor of egg yolk, synthesized in female oviparous vertebrate in preparation for spawning under the control of natural occurring steroid hormone 17-β estradiol (E_2) (An *et al.*, 2007; Palumbo *et al.*, 2009; Meucci and Arukwe, 2005; Ngamniyom and Panyarachun, 2011). The vtg is transported to the follicle in the ovary via bloodstream, where it is progressively sequestered into developing oocyte and offspring in the reproductive stage (Kordes *et al.*, 2002; Li *et al.*, 2005; Prakash *et al.*, 2007). Its synthesis (vitellogenesis) is controlled by hormone and estradiol is reported as a common hormonal stimulus to trigger the vtg synthesis in many fish species (Nishi *et al.*, 2002). Lomax *et al.* (1998) noted that plasmatic vtg concentration in English sole (*Pleuronectes vetulus*) was dramatically increased in female fish during reproductive period prior to spawning, when estrogen levels were elevated. However, vtg levels were decreased upon spawned out spawners (Methven *et al.*, 1992). Therefore, the measurement of vtg levels in Asian sea bass (*Lates calcarifer*) would provide a useful tool in determining the maturity status of this economically important species in captive and wild condition (Kishida and Specker, 2000). Vtg is normally absent in female blood during non-reproductive period and in male as well as juvenile fish. However, Prakash *et al.* (2007) and Selcer *et al.* (2006) proved that these organisms produced vtg via inducing with synthetic estrogens, 17-β estradiol (E_2) in murrel and Morelet's crocodile (*Crocodylus moreletii*).

Several direct and indirect immunoassays were developed for measuring vtg levels, namely Radioimmunoassay RIA (Tao *et al.*, 1993), immunodiffusion (Mananos *et al.*, 1994), alkali-liable phosphate (Periera *et al.*, 1992) and Enzyme Linked Immunosorbent Assay (ELISA). Among these methods, ELISA was highlighted as the most favourable tool used for plasmatic vtg measurement considering its high sensitivity, simple and rapid (Mananos *et al.*, 1994; Lomax *et al.*, 1998; Ebrahimi, 2007; An *et al.*, 2007). Since, then, ELISA was developed and established among several teleost vtg of freshwater and marine fish such as English sole (Lomax *et al.*, 1998), sea bream (Mosconi *et al.*, 1998), Japanese medaka (Nishi *et al.*, 2002), carp (Matsumoto *et al.*, 2002), Feathed minnow (Eidem *et al.*, 2006) and Mossambicus tilapia (Swart and Pool, 2009).

Recently, several ELISA for measuring vtg have been standardized and developed in a commercial available kit. However, the antibody does not display a good cross-reactivity among different species and a new assay is generally needed for specific species. Since, there was no apparent antibody has a binding properties to *L. calcarifer* vtg, polyclonal antibody against purified vtg was developed to study the cross-reactivity in ELISA.

The present study aimed to develop and validate a sensitive and rapid biochemical method capable for quantifying vtg levels in the maturing female's plasma of *L. calcarifer*. The sensitive and accurate measurement of ELISA has a broad application in fisheries and aquaculture industry. The potential ELISA kit developed can help farmers to measure vtg levels of *L. calcarifer* in determining sex and sexual maturity for spawning purpose.

MATERIALS AND METHODS

Fish: Two-year-old juvenile Asian sea bass (*Lates calcarifer*) (initial mean weight 1.5±0.5 kg, length 168±15 cm) were obtained from commercial supplier and transported to the hatchery (Marine Science Station, Port Dickson, Universiti Putra Malaysia, UPM). They were held in 10 tonne tank with aeration and flow through sea water and fed daily with chopped fresh fish.

Preparation of *Lates calcarifer* vtg: Each fish was given a series intraperotoneal (i.p.) injections (2 mg kg^{-1} body weight) of 17-β E_2 (Nacalai tesque, Japan), dissolved in a solution of ethanol and 0.9% NaCl in a ratio of 1:9, v/v (ethanol:0.9% NaCl solution, 1 mL). Three times of injections were given at two days intervals. Three days following the last injection, blood was obtained from caudal vessel with a 5 mL heparinized syringes and directly centrifuged at 12000 rpm for 30 min (4°C). Prior to purification, plasma was stored in -80°C in small aliquots.

Vtg purification: The procedure used for purification of plasmatic vtg was modified from with modifications. One mL of aliquots plasma was purified through gel filtration chromatography using Sepachryl HR-300 column (GE HEealthcare BioScience, Upsalla, Sweeden), eluted with 360 mL 0.05 M Tris-HCl pH 8.0 (Nacalai tesque, Japan). Elution profile was monitored at 280 nm and the fractions around peak were collected as purified *L. calcarifer* vtg. The liquid was further concentrated using Vivaspin centrifuge tube (30 kDa molecular weight cut-off, GE Healthcare BioScience, Upsalla, Sweeden) according to the methods proposed by manufacturer. Finally, the purified vtg concentration was determined by Bradford assay (1976) using Bovine Serum Albumin (BSA) (Sigma Diagnostics, USA) as standard. Freshly purified vtg was used to generate polyclonal antibody and as a standard to develop ELISA. Working solutions of vtg for standard curve were prepared at various concentration of purified vtg (Meucci and Arukwe, 2005).

Antibody production: Polyclonal antibody against purified vtg (antigen) were generated in New Zealand white rabbits (body weight 3.5±0.5 kg, n = 4). For the priming, rabbits were immunized intradermal and intramuscularly with purified vtg, 0.05 mg mL^{-1} protein emulsified with Freund's Complete Adjuvant (FCA, Calbiochem, Darmstadt, Germany) in a ratio 1:1 v/v (1 mL). Rabbits were boosted up by three additional injections (0.02 mg mL^{-1} protein) emulsified with Freund's Incomplete Adjuvant (FIA). Blood was collected at weeks 4, 6 and 10 and the serum (anti-vtg) was assayed for reactivity towards vtg by screening ELISA. When antibody titer was sufficient, blood (~10 mL) was withdrawn from ear artery and allowed to clot overnight. Serum was separated by centrifuging the blood at 12000 g, 4°C for 30 min and stored in aliquots. Serum was then used in the development and validation of ELISA as primary antibody.

Development of an Enzyme Linked Immunosorbent Assay (ELISA): A checkerboard titration assay was used to select optimal working concentration of coating vtg and primary antibody for ELISA procedure. Purified vtg (1000 to 31.2 ng mL^{-1}) were prepared by diluting in 0.05 M carbonate buffer, pH 9.6.

Ninety six microtiter plates (Nunc-Immuno Plate MaxiSorp™) were coated with serial dilutions of purified vtg (200 μL per well) and incubated overnight at 4°C. For Non-Specific Binding (NSB), three wells were treated with coating buffer only. The plates were then blocked with blocking solution (1% skim milk in PBST, 300 μL per well) for two hours at 37°C. Following washes, plates were incubated with serial dilutions of primary antibody (1:500 to 1:8000 in blocking buffer, 200 μL per well) and incubated at 37°C for two hours. After four times washing with PBST, each well received 200 μL of secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (prepared in dilution of 1:2000 in blocking buffer) and incubated two hours at 37°C. For substrate development, each well received 100 μL of Tetramethylbenzidine (TMB, Calbiochem, US) and incubated for 30 min. The reaction was stopped by adding 50 μL of 1 M H_2SO_4 to each well. The absorbance of 405 nm was read using microtiter plate reader (Thermoscientific, Finland).

Parameters such as the dilution of polyclonal antibody (anti-vtg), HRP conjugated secondary antibody, incubation time were optimized to develop a sensitive ELISA.

ELISA procedure

Antigen coating: Ninety six-well microtiter plates (Nunc-Immuno Plate MaxiSorp™) with 100 μL of purified vtg (250 ng mL^{-1}, diluted in coating buffer), sealed and incubated overnight at 4°C. For non-specific binding, eight wells were coated with plasma from male samples at the same concentration. The plates were then inverted to empty the contents and washed four times (1 min soak) with 100 μL of PBS-T (Phosphate buffered saline, 0.05% Tween 20, Calbiochem, Darmstadth, Germany) to remove unbound antigen. To reduce non-specific binding, the plates were blocked (200 μL $well^{-1}$) with blocking buffer (1% skim milk in PBST), followed by four washing as described above.

Pre-incubation of samples and standards: Samples and standards were diluted in assay buffer (1% skim milk in PBST). Standards were prepared at initial concentration of 1000 ng mL^{-1} and serially diluted at two fold. The concentrations ranged from 1000 to 31.25 ng mL^{-1}. In parallel, samples of E_2-induced, vitellogenic female and male plasma were diluted in assay buffer in a ratio 1:100 to 1:1000000. Standards and samples were then mixed (1:1, v/v) with primary antibody diluted to 1:1000 in assay buffer. They were pre-incubated for 2 h at 37°C or overnight at 4°C.

Primary antibody incubation: Following pre incubation, standards and samples were dispensed in triplicate (150 μL $well^{-1}$) into the coated plate and incubated for 2 h at 37°C. The plates were then washed as described above.

Secondary antibody incubation: Each well received 100 μL of Horse-Reddish Peroxidase conjugated goat anti-rabbit IgG (Nacalai tesque, Japan) diluted in 1:2000 in assay buffer. Plates were incubated for 45 min at 37°C followed by four washing with PBST.

Substrate incubation: For colourization, each well received 100 μL of Tetramethylbenzidine (TMB) substrate solution (Calbiochem, Damstadt, Germany). After 30 min incubation, colour development was stopped by addition of 100 μL of 0.05 M sulphuric acid, H_2SO_4 into each well. The absorbance of 405 nm was read using ELISA microplate reader (Thermo Fisher Scientific, Finland).

Expression of results: The proportion of antibody bound was calculated using the formula Lomax *et al.* (1998):

$$B_i / B_0 (\%) = \frac{(OD - NSB)}{OD_0 - NSB} \times 100$$

where, OD is the absorbance of a sample or standard, OD_0 is the absorbance of zero standards and NSB is non-specific binding value. Binding percentage values were logit transformed according to the formula below:

$$\text{Logit B} = \log_e \left[\frac{B}{100 - B} \right]$$

Parallelisms between the samples were assessed by using analysis of covariance (Lomax *et al.*, 1998). The amount of vtg in plasma samples were calculated by interpolating from linear portion of the plasma dilution curve to the calibration curve.

Standard curve: Standard curve were generated with serial dilutions of purified vtg (31.2, 62.5, 125, 250, 500 and 1000 ng mL^{-1} protein). The primary antibody was diluted 1:1000 in PBST. The vtg concentration in plasma of *L. calcarifer* was calculated based on linear regression from the standard curve.

Assay precision: The precision of the ELISA was assessed through measurement of intra and inter-assay coefficient of variations (CVs) by running the standard curves with different concentration on different days (inter-assay, N = 5). On each plate, every point of standard curve was run in nine replicates (intra-assay). To quantify assay variations, the CV was calculated using the following formula:

$$\frac{SD \times 100}{Mean}$$

Antibody specificity: Serial dilutions of vitellogenic female's plasma and male's plasma (10^{-2} to 10^{-7}) as well as vtg standard were assayed by ELISA to assess parallelism with standard curve. This indicated the specificity of plasma samples to the antibody (anti-vtg).

Validation of ELISA method

Vtg measurement in *L. calcarifer* plasma of adult vitellogenic females and males: Plasmatic vtg concentration for matured females (n = 5, body weight of 6.3±0.5 kg) and male (n = 5, body weight of 4.0±0.5 kg) obtained during the breeding season in captive condition were determined using developed ELISA methods.

RESULTS

Assay characteristics

Antigen: Asian sea bass plasmatic vtg of E_2-treated was purified by gel filtration chromatography using Sepachryl HR-300 column for use as antigen in polyclonal antibody production. The protein concentration of purified vtg was 0.059 mg mL^{-1} as determined by Bradford assay.

Optimal condition of competitive ELISA: A competitive, antibody-capture ELISA was developed for Asian sea bass, *L. calcarifer* vitellogenin utilizing the polyclonal antibody and purified *L. calcarifer* vtg as standard. ELISA checkerboard titration assay of coating vtg and antiserum dilutions were shown in Fig. 1. Six different combinations of vtg concentration (31.2, 62.5, 125, 250, 500 and 1000 ng mL^{-1}) and 5 serial dilutions of antibody (1:500, 1:1000, 1:2000, 1:4000 and 1:8000) were evaluated. The results were summarised in Fig. 2. The combination which yielded an OD near 1 was selected as a workable ELISA (Swart and Pool, 2009). For routine assay conditions, an antiserum dilution of 1:1000 and 250 ng mL^{-1} coating vtg was selected for the use in ELISA.

The standard curve of purified vtg revealed a typical sigmoid pattern with measurable ranges of 31.2 to 1000 ng mL^{-1} (Fig. 3a). For assay detection limit, it was estimated to be 31.2 ng mL^{-1}

Vtg (ng mL^{-1})	Antiserum dolutions				
	1:500	1:1000	1:2000	1:4000	1:8000
1000					
500					
250					
125					
62.5					
31.2					

Fig. 1: Schematic for checkerboard titration. Shaded area resulted in an OD of 0.917 after 30 min color development

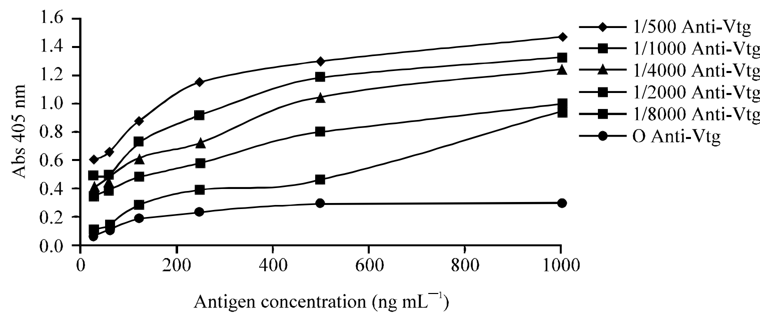

Fig. 2: Optimal assay concentrations for vtg and antiserum. Vtg coating of 250 ng mL^{-1} and antibody dilution of 1:000 was selected for routine condition of ELISA

which corresponds to the vtg concentration of 93.6% antibody binding. The upper and lower limits of ELISA detection were 38 and 93.6%, respectively. The assay characteristics could be modified by changing the pre-incubation and incubation times.

Parallelism of standard curves: In order to test the specificity of the ELISA assay, parallelism of multiple binding curves (vitellogenic female, E_2-treated and control male plasma) was first verified using an analysis of covarians F-test on mean squares to assess similarity of slopes. Standard preparations of various antigens were compared to assess the antiserum (polyclonal antibody) ability to recognize vtg in native form. Figure 4a showed different displacement curves of E_2-treated, vitellogenic females and male plasma dilutions. No significant displacement was found with male plasma. Figure 4b showed the linearization (logit transformation). ANCOVA of E_2-treated and vitellogenic female revealed a good parallelism with the vtg standard curves (Fig. 3b), ($F_{obs} = 0.562 < F_{crit} = 3.178$ for 2,51 df, $p > 0.05$) within the working ranges of the assay, while unexposed male showed little cross reactivity with vtg antibody, showing the specificity of *L. calcarifer* vtg (Shao *et al*., 2005; Prakash *et al*., 2007).

Antibody specificity: Several dilutions of plasma from control male and natural vitellogenic females assayed to confirm the specificity of ELISA. Results showed that, there was no cross-reactivity observed in the male plasma as shown in Fig. 4.

Assay precision: Reproducibility of the assay was confirmed by evaluating inter (N = 5) and intra assay (N = 9). Intra-assay variation was less than 9.4, whereas inter-assay variation was less than

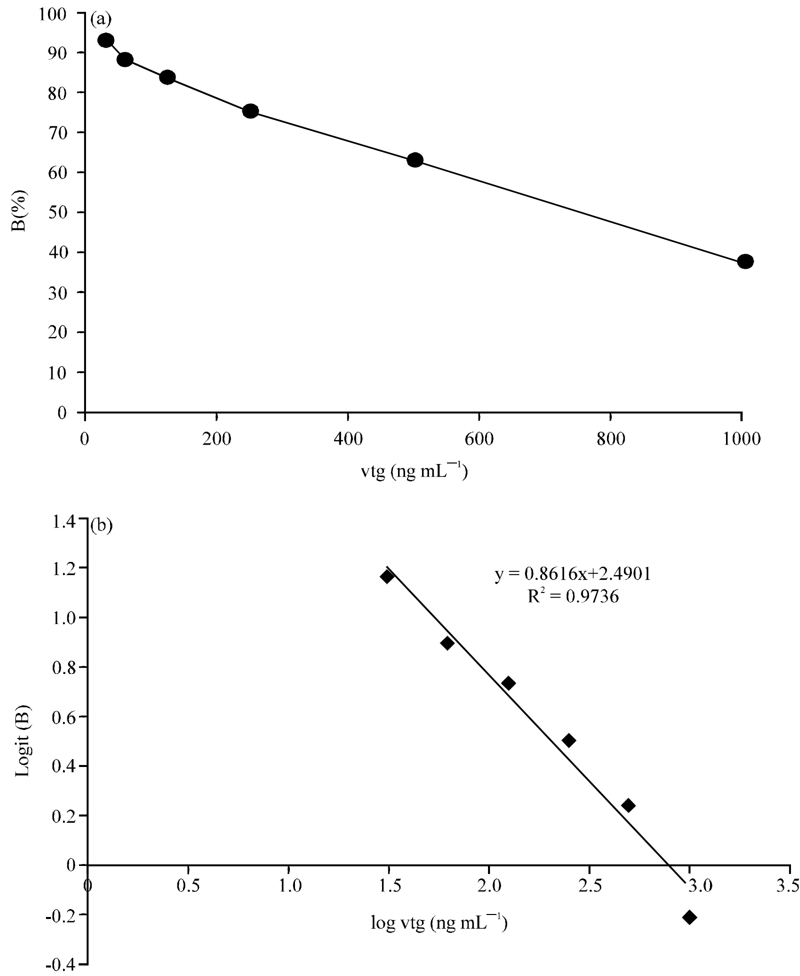

Fig. 3(a-b): Binding displacement curve and linearization of vtg standard used in competitive ELISA. (a) Representative binding (B%) of vtg standards ranging from 31.2 to 1000 ng mL^{-1} (b) Logit transformation of the binding curve (s = -0.8616, R^2 = 0.9736). Each point represents the mean of triplicate determinations

Table 1: Characteristics of *Lates calcarifer* vtg competitive ELISA

	Binding		
	Percentage of maximum		
	90	80	50
CV intra-assay (%)	7.6	7.1	8.4
CV inter-assay (%)	7.6	10.6	12.1

For routine measurement 250 ng mL^{-1} of antigen concentration and 1:1000 of anti-serum dilution was selected. CV = Coefficient of variation

7.5 for 90% binding and less than 12.1 for 80 and 50% binding (Table 1). These ELISA performance characteristics were generally similar to those reported from other fish species (Korsgaard and Pedersen, 1998; Fenske *et al*., 2001; Kordes *et al*., 2002; Shao *et al*., 2005). The

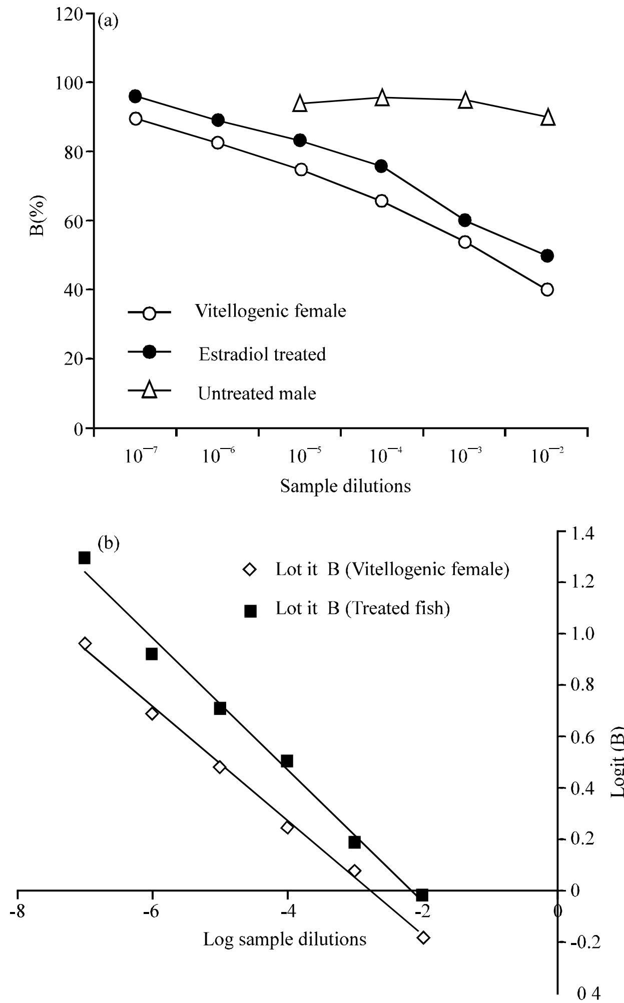

Fig. 4(a-b): Bindig displacement curves from serial dilutions of plasma vitellogenic females and E2-treated juvenile L. calcarifer. (a) Binding curves (B%). (b) Linearization of the plasma binding curves by logit transformation

CVs for assay reproducibility is <15%. ELISA sensitivity, defined as imprecision of measuring of zero dose was 6.9 ng mL^{-1}. The value was derived by plotting the graph of relationship between vtg dose (ng mL^{-1}) versus SD of versus vtg concentration (Sherry *et al*., 1999).

Assay validation

Vtg measurement in plasma of matured females and males in captive condition: ELISA was validated by measuring vtg in plasma of natural vitellogenic females and matured males *L. calcarifer* (Fig. 6) using vtg standard curve (Fig. 5) which ranged from 31.2 to 1000 ng mL^{-1} (R^2 = 0.979, y = 0.0008x+0.3614). Vtg concentrations of matured female samples ranged from 0.9 to 1.54 mg mL^{-1}. There was no vtg detected in any of the male plasma (Fig. 6). Results showed that vtg levels in natural vitellogenic females were higher, indicating that the fish were ready to undergo spawning.

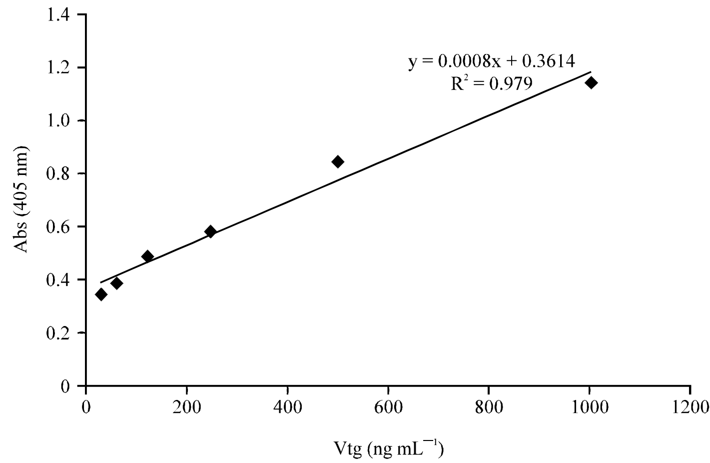

Fig. 5: Vtg standard curves of the ELISA

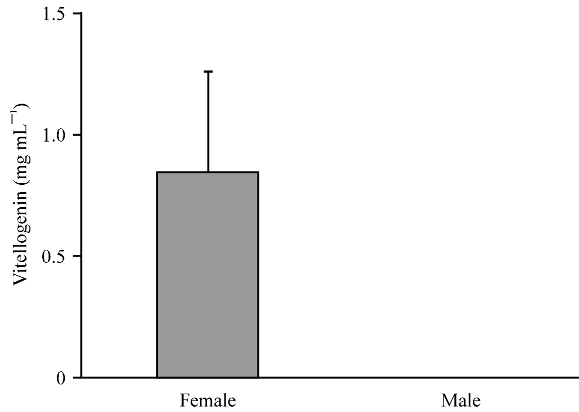

Fig. 6: Vtg concentration for the plasma of vitellofenic females and matured males in captive condition. (Bars represent the Mean±0.4 standard error). The vtg was not detected in plasma samples of males (n = 5)

DISCUSSION

The present study described development and validation of competitive ELISA utilizing polyclonal antibody for quantifying vtg concentration in *L. calcarifer*, as a measure of their maturational status. ELISA was reported as the most optimum immunological methods proposed for detecting plasma vtg as it combined reasonable sensitivity and speed (Heppel *et al*., 1999). Purified vtg was used as a standard to generate the assay. The assay was used to determine plasmatic vtg levels in females and males of *L. calcarifer* taken during the breeding season in captive condition. Jackson and Sullivan (1995) observed that ELISA assay enabled fishery biologist to identify gender and maturational status of individual fish broodstock in striped bass, white perch and white bass.

Purified *L. calcarifer* vtg was used to generate standard curve in this assay. Constructed ELISA produced a linear standard curve of vtg concentration. The result revealed that the vtg ELISA was dose dependent and sensitive enough to be used for detecting plasmatic vtg levels. Similar finding

was also noted by Selcer *et al.* (2006) for vtg measurement in Morelet's crocodile. The developed assay was sensitive enough (6.9 ng mL^{-1}) to study the vtg patterns in female *L. calcarifer* during the reproductive season. The lowest sensitivity of ELISA assay was 2.0 to 3.0 ng mL^{-1} in *Danio rerio* Hamilton-Buchanan. Sherry *et al.* (1999) suggested that the sensitivity of the assay can be improved by optimizing the incubation periods of primary and secondary antibody as well as adjusting the anti-vtg or coated vtg-ratios.

For assay validation, vtg levels were evaluated in matured females and males in captivity. Results demonstrated that there was no vtg detected in males compared to matured female. This is in agreement with finding of Chang *et al.* (1995) which also did not detected any vtg levels in males protandrous black porgy, *Acanthopagrus schlegeli*. The vtg concentration found in females *L. calcarifer* are comparable to those observed in other fish species as noted by Selcer *et al.* (2006). High vtg levels in pre spawning and spawning seasons might be correlated with sex reversal in certain fish species (Chang et al. (1995).

Accuracy, precision, sensitivity and specificity determine reliability of assay which ensures concentration is valid and provide identical result with the true (Prakash *et al.*, 2007). The repeated measurements of vtg standard demonstrated precision with inter and intra-assay coefficient of variations (Cvs) falls within the acceptable ranges as reported in several other findings (Mananos *et al.*, 1994; Mosconi *et al.*, 1998; Bulukin *et al.*, 2007; Swart and Pool, 2009). High degree of homogeneity plates were used to avoid inconsistencies in the reproducibility and accuracy of the developed assay. The sensitivity of the assay (<10 ng mL^{-1}) was almost equal to that ELISA reported form other studies (Mosconi *et al.*, 1998; Korsgaard and Pedersen, 1998; Fenske *et al.*, 2001; Shao *et al.*, 2005). In this study, the result demonstrated that polyclonal antibody did not cross-react with any plasma protein in non-estrogenised *L. calcarifer*, thus, confirmed that the protein, vtg are female specific. Parallelism curves of vtg standard, natural vitellogenic females and E_2-treated demonstrated that the response of plasma dilutions were proportional to the adsorbed antigen concentration. The covariance (ANCOVA) did not analyze the significant difference in slopes ($p>0.05$), demonstrating the specificity of antibody.

Meucci and Arukwe (2005) concluded that vtg acts as sex determinant in dimorphism fish species such as groupers. Hence, it can be applied in Asian sea bass species, since, this species also considered as dimorphism fish. Information about vtg was useful for screening fish maturity status (Kishida and Specker, 2000).

CONCLUSION

The ELISA developed in the present study resulted in acceptable parameters of specificity, precision and sensitivity which are needed for measuring *L. calcarifer* vtg. The availability of the assay capable of measuring vtg in plasma of naturally vitellogenic and estradiol treated juvenile in captive condition. It also facilitates further research on regulatory mechanisms of vitellogenesis and reproductive physiology of *L. calcarifer*.

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