Journal of Crustacean Biology

The Crustacean Society

Journal of
Crustacean Biology

Journal of Crustacean Biology (2017) 1–8. doi:10.1093/jcbiol/rux040

ELISA detection of vitellogenin in the haemolymph of *Penaeus monodon* (Fabricius, 1798) (Caridea: Penaeidae): indirect indicator of female maturity

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(Received 21 March 2016; accepted 17 April 2017)

ABSTRACT

Developing a non-lethal method for analysis of female reproductive maturity will be more accurate and less invasive than assessing maturity through gonad biopsy. Quantification of the expression levels of vitellogenin (Vg) in haemolymph is a vital tool to assess gonadal maturation as Vg is an indirect indicator of maturity. The lack of commercial antibody/ antisera in India for detection of vitellogenin/vitellin (Vg/Vn) levels in haemolypmh of *Penaeus monodon* (Fabricius, 1798) lead to the present study. To develop an assay, Vg/Vn subunits isolated together and purified from ovary of mature female *P. monodon* was used as antigen to raise polyclonal antisera in rabbit. This polyclonal antiserum (anti-PmVg/Vn) was used for developing Vg/Vn enzyme-linked immunosorbent assay (ELISA). Anti-PmVg/Vn demonstrated high specificity in detecting vitellogenin subunits present in haemolymph, vitellin subunits in ovary extract through Western blot analysis, and vitellin-like globules in oocytes by immunostaining/immunohistochemistry. The Vg/Vn ELISA developed through was validated by detecting haemolymph Vg based on gonadosomatic index of immature (stage 1) and sexually mature (stage 2) individuals of *P. monodon*. The specificity of the anti-PmVg/Vn raised was confirmed by detection of recombinant 74 kDa Vg subunit protein of *P. monodon*. The expressed recombinant Vg was also used to validate the ELISA. The results have demonstrated the specificity and sensitivity of anti-PmVg/Vn polyclonal antisera; an efficient means to monitor and transform the technology of induced breeding.

Key Words: gonadosomatic index, haemolymph, polyclonal antisera, Vg gene, Vg/Vn recombinant vitellogenin

INTRODUCTION

Setbacks in shrimp aquaculture industry, particularly in the culture of *Penaeus monodon* (Fabricius, 1798) demand the development of specific pathogen-free broodstock for domestication. This process requires an important step, the identification of the maturation stage of the animal for induced breeding using a non-lethal technique. In crustaceans, the blood-borne lipoprotein present exclusively in females with developing oocytes (Kerr, 1969) was demonstrated immunologically identical to the oocyte vitellin (Vn) and named vitellogenin (Vg). As the lipoprotein precursor of Vn, Vg has been detected in the haemolymph and extra-oocyte tissues of female crustaceans (Meusy & Payen, 1988; Vazquez-Boucard *et al.*, 2002; Avarre *et al.*, 2003). Vitellogenin possesses immunological similarity to Vn, and antibodies prepared against purified Vn often recognise haemolymph Vg in penaeids (Shafir *et al.*, 1992; Chang *et al.*, 1996; Lubzens *et al.*, 1997; Longyant *et al.*, 1999; Garcia-Orozco *et al.*, 2002). As vitellogenesis and ovarian maturation are under hormonal control (Hasegawa & Hirose 1993), detection and estimation of Vg in the haemolymph would provide information on the different stages of female maturity during induced maturation. Therefore, Vg concentration in haemolymph may be used as an indirect indicator of hormonal activity (Longyant *et al.*, 1999). Studies indicate a positive correlation between the levels of Vg in

the haemolymph and a suite of maturation indicators including gonadosomatic index, vitellogenic stages of oocyte development (Jasmani *et al.*, 2000), and Vg mRNA/haemolymph Vg levels, all of which increase as shrimp advance the timeframe for moulting and spawning (Jayasankar *et al.*, 2002). In *P. monodon* Vg has been isolated from the haemolymph (Chang *et al.*, 1994), whereas ovary and hepatopancreas are reported to be the sites of Vg synthesis. Characterization of the *P. monodon* Vg/Vn subunits indicates a large variation in the sizes; classified as large (210, 180, and 150 kDa), medium (110, 105, and 95 kDa), and small (76 and 80 kDa) (Quinitio *et al.*, 1990; Chang *et al.*, 1993; Chen and Chen, 1993, 1994). Gene organization and cloning of the full-length *Vg* cDNA (*P. monodon*) reported by Tiu *et al* (2006) revealed 14 introns and 15 exons coding for a 7.8 kb transcript. The deduced precursor (284 kDa) is similar to the Vg of *Fenneropenaeus merguiensis* (De Man, 1888) and *Penaeus semisulcatus* (De Haan, 1844) with a similarity of 86% and 87%, respectively. Western blot analysis of Vg in the haemolymph of female *P. monodon* at different stages of ovarian development revealed the presence of two prominent Vg subunits of size 74 kDa and 200 kDa respectively (Longyant *et al.*, 2003).

We aimed to: 1) develop polyclonal antisera (anti-PmVg/Vn) raised against antigen Vg/Vn subunits purified together from the ovaries of *P. monodon* and 2) validate the antisera through enzyme-linked immunosorbent assay (ELISA) for quantifying haemolymph Vg. The specificity of the antisera was further assessed by identifying Vn/Vn-like globules in oocytes of *P. monodon* through immunostaining and immunohistochemistry, and confirmed using a recombinant vitellogenin subunit from *P. monodon* expressed in an *E. coli* translation expression system (pET32a+ *E. coli* BL21 (DE3) pLysS).

MATERIALS AND METHODS

Vitellogenin/Vitellin isolation

Vitellogenin/vitellin subunits (Vg/Vn) of *P. monodon* were isolated from ovary extracts by differential centrifugation and the combined subunits were purified by precipitation with increasing concentration of saturated ammonium sulphate (Tsukimura *et al.*, 2000).

Immunization schedule

Two months old New Zealand white rabbits ($N = 6$) were immunised with Vg/Vn protein of female *P. monodon*-purified Vg fraction as per schedule (Table 1).

Blood collection and serum preparation

Blood was collected from the marginal ear vein prior to immunization; serum obtained was used as control for ELISA. After immunization, a test bleed was carried out on the 28th day and titre of antiserum was checked. A second booster dose was given and blood was collected on the 7th day from the ear vein. Blood was allowed to clot for 1 h at 37 °C and kept overnight at –20 °C. The next day serum was removed from the clot by centrifugation at 10,000 rpm for 10 min, and preserved at –20 °C. Booster doses of antigen were continued to be given every six weeks.

Determination of antisera titre and construction of standard curve employing ELISA

Polyclonal antisera (anti-PmVg/Vn) were analysed for sensitivity by using ELISA. The antigen Vg/Vn, 100µl (20 µg ml^{-1}), was used to coat the 96-well microtiter plate and incubated overnight at 4 °C. The free sites were blocked by coating the ELISA microtiter plate with 0.1 ml BSA (5% in phosphate-buffered saline (PBS)) for 1 h. Aliquots of different dilutions of antiserum (anti-PmVg/Vn) starting from 1: 200 to 1:819,200 (serially diluted by factor of two) was added and incubated for 1 h. Triplicates were kept for each dilution. Serum from the animal prior to immunization and PBS were added instead of antiserum in the control wells. An aliquot of 0.1 ml mouse antirabbit IgG peroxidase conjugate (1: 2,000 dilution) in 3% BSA/PBS was added and incubated for 1 h. The microtiter plate was washed thrice between each step with PBST (phosphate–buffered saline pH 7.2 with Tween 20 (0.01%)). TMB substrate (0.1 ml; 3, 3', 5, 5'-tetramethylbenzidine base (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 10 min. The reaction was stopped by adding 50 µl 1 M H_2SO_4 after the development of a blue colour. The yellow colour that developed was measured at 450 nm using ELISA/Microplate reader (Tecan Infinite M200, Untersbergstrasse, Salzburg, Austria). The measured absorbance was compared with the control serum and PBS.

ELISA was performed for constructing standard curve, and trend-line analysis. Different concentrations (100 µl) of antigen (Vg/Vn) ranging from 5 x 10^{-5} to 50 ng ml^{-1} were used to coat the microtiter plate and incubated overnight at 4 °C. When using recombinant Vg as antigen (100 µl), the concentrations ranging from < 5 µg to 100 µg was coated in a microtiter plate and incubated overnight. For antisera concentration optimization, 50 µg of recombinant Vg was coated and detected using the antisera dilutions starting from 1: 3,200 to 1:51,200. ELISA was performed as previously described. The trend line fixed between the points was used for obtaining value of R.

Specificity of Vg/Vn polyclonal antisera: Western blot and immunostaining

Western blot was performed following Fabbretti *et al.* (1995) with modifications to verify the specificity of the Vg/Vn polyclonal antisera raised in rabbit. The ovary extract of immature (stage 1, ovary not visible through exoskeleton) and sexually mature (stage 2 early maturing, ovary visible through exoskeleton) shrimp (20 µg), haemolymph of male and female shrimp (20 µg each) were separated in 12% SDS-PAGE. The resolved protein was transferred to nitrocellulose membrane for Western blot analysis. The nitrocellulose membrane was blocked with 5% skimmed milk in PBST, pH 7.2 (phosphate buffered saline + Tween 20 (0.1%)) for 1.5 h, incubated overnight at 4 °C with antibody raised against the Vg/Vn (anti-PmVg/Vn), diluted (1:25,600) in 5% skimmed milk-PBST, followed by three washes

Table 1. Immunization schedule representing immunization regimes used in rabbit experiment. Rabbits ($N = 6$) were immunised four times with 2.31, 0.5, 0.5, and 0.5 mg of Vg/Vn antigen followed by first test bleed on the 28th day. Booster doses continued every six weeks and test bled on the seventh day to test the titre. The immunization was carried out for a period of 289 days. FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant

	Day of immunization	Quantity of antigen (ml)	Quantity of adjuvant (ml)	Route of injection	Dosage (ml)
1	0	1 (2.31mg)	1 FCA	Intradermal	2
2	7	0.5 (1.16mg)	0.5 FIA	Intramuscular	1
3	14	0.5 (1.16mg)	0.5 FIA	Intramuscular	1
4	21	0.5 (1.16mg)	0.5 FIA	Intramuscular	1

with PBST (5–10 min each). The membrane was incubated in the secondary antibody, anti-rabbit IgG HRP conjugated (1: 1,000 dilution) (Sigma, USA) in 5% skimmed milk-PBST for 1 h with gentle agitation at 25 °C. The membrane was washed twice with PBST at a time interval of 10 min. An aliquot of 4-chloronaphthol in 30% H_2O_2 was added to the membrane and incubated for 10 min. Purple colour development was observed and the membrane was washed with distilled water to stop the reaction. The colour developed was documented using Gel Documentation system (Gel Doc™ XR+ imaging system; Bio-Rad, Irvine, CA, USA).

For immunostaining, the slide was coated with poly-l-lysine (0.01%). Developing ovary was used as the sample. The oocytes separated in PBS were sedimented on to a slide coated with poly-l-lysine using a cytocentrifuge. The stage 2 ovary was fixed in Davidson's fixative (24 hr) for immunohistochemistry. The tissue was embedded in paraffin following conventional methods. Sections (5 µm) were mounted onto the slide and dried at 25 °C overnight. The tissue sections were dewaxed in xylene (twice for 10 min) and dehydrated through a series of ethanol dilutions (70%, 95%, 100%,). The free sites for immunostaining and immunohistochemistry were blocked with BSA (3% BSA in PBS) for 1 h in a humidified chamber. Primary antiserum (anti-PmVg/Vn in 1:51,200 dilutions in 3% BSA-PBS) was added and incubated for 1.5 h and washed with PBS/Tween 20. The secondary antibody, anti-rabbit IgG HRP conjugate (Sigma, USA) (1: 1,000 dilution) in 3% BSA-PBS, was added and incubated for 1 h in a humidified chamber. The slide was washed with PBS/Tween 20. The substrate, diaminobenzidine (DAB), was added and incubated for 20 min, washed with PBS and counter stained using haemotoxylin for 30 sec. The slides were washed with PBS and observed under bright-field microscope (Olympus, Hamburg, Germany and Leica, Mannheim, Germany). The sections were washed with PBS/Tween20 (0.01%) at intervals after completing the incubations.

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from the ovary of secondary vitellogenic *P. monodon* using commercially available TriReagent (Sigma, USA). The total RNA was subjected to DNase treatment by adding 0.2U of RNase free DNase 1 (New England Biolabs, Hitchin, UK) to 1 µg RNA and incubated at 37 °C for 10 min followed by inactivation at 75 °C for 10 min. RNA concentration and quality were determined spectrophotometrically (Hitachi, Model U-2800, Tokyo, Japan) by measuring absorbance (260/280 nm). An aliquot of 5 µg RNA was reverse transcribed for first-strand cDNA synthesis. The 20 µl reaction mix (New England Biolabs, UK and Sigma, USA) contained M-MuLV reverse transcriptase (200 U), RNase inhibitor (8 U), dNTP mix (1 mM), RTase buffer (1X), Oligo $(dT)_{12}$ primer (40 pmoles), and $MgCl_2$ (2 mM). The cDNA was synthesised by incubating the reaction mix at 42 °C for 1 h followed by inactivation at 65 °C for 20 min.

PCR amplification, cloning, and sequencing of the Vg gene fragment

Gene specific primers were designed using Primer 3 Software (http://bioinfo.ut.ee/primer3-0.4.0/) for the amplification of vitellogenin partial gene fragment based on the protein and cDNA sequence information available at NCBI GenBank for *P. monodon* vitellogenin precursor molecule (GenBank accession no. ABB89953.1 and DQ288843.1). PCR was conducted in a 25 µl reaction volume containing *Taq* DNA polymerase buffer (1X), dNTP mix (0.25mM), PmVg.29-forward (5' ATGACCACCTCAACCCTCCT 3') and PmVg.2248- reverse primers (5' GCCAAAGAAGTCCGAGATGA 3') (10 pmol μl^{-1} each), *Taq* DNA polymerase (New England Biolabs, UK) (0.5 U) and 1 µl cDNA template. The PCR was carried out in a Thermal cycler (Eppendorf, Hamburg, Germany) with an initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 58 °C for 45 s, and extension at 72 °C for 40 s followed by final extension at 72 °C for 10 min. The amplified PCR products were resolved by 1% agarose gel electrophoresis. The PCR product of Vg was cloned into pGEM-T easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Three clones were randomly picked and sequenced on Applied Biosystems ABI 3730x1DNA analyser at SciGenom (Kochi, India).

DNA sequences obtained for Vg were edited and assembled using the Gene Tool software (Layon, 2000). Homology search was performed using the NCBI nucleotide BLAST (Altschul *et al.*, 1997). The ORF translation tool was used to generate the amino acid composition of DNA sequences (Bjellqvist *et al.*, 1993, 1994). Calculation of theoretical MW of the deduced protein was done using ExPASy Proteomics (http://us.expasy.org/tools/pi_tool.html) (Gasteiger *et al.*, 2005). Functional analysis was carried out using multiple sequence alignment of protein sequences constructed using Cluster3 (http://bonsai.hgc.jp/~mdehoon/software/cluster/) (de Hoon *et al.*, 2006).

Expression cloning of Vg gene fragment

The Vg gene fragment (~2.221 kb) was amplified for recombinant expression using Pfu DNA polymerase and a new set of primers with EcoR I and Xho I restriction enzyme overhangs. The resulting PCR product and the expression vector pET32a+ were digested with enzymes EcoR I and Xho I, purified and ligated to make the Pm*Vg*+pET32a+construct.

Expression of thioredoxin-fused Vg protein using PmVg+pET32a+vector construct

The Pm*Vg*+pET32a+vector construct was initially transformed into *E. coli* DH5α and it was confirmed by sequencing that the gene was in correct frame for protein expression. *Escherichia coli* BL21 (DE3) pLysS competent cells (Novagen, Madison, WI, USA) were transformed with Pm*Vg*+pET32a+construct and the transformants were selected on LB/ampicillin (100 µg ml^{-1}) plates. Bacterial cells from single colonies were grown in 3 ml LB/ampicillin (100 µg ml^{-1}) medium and incubated at 37 °C in a shaker incubator at 250 rpm until an absorbance at 600 nm of 0.5 was obtained. The entire 3 ml culture was added to 100 ml LB/ampicillin (100 µg ml^{-1}) medium and further incubated at 37 °C for 2 h until the absorbance at 600 nm between 0.5–0.8 was attained. Thereafter, IPTG was added to a concentration of 1 mM to induce recombinant protein expression. The culture was incubated for an additional 3.5–4 h at 22 °C after which bacterial cells were harvested by centrifugation. The cells were lysed by sonication in two lysis buffers, buffer 1 (50 mM KH_2PO_4 at pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole) and buffer 2 (31.25 mM Tris buffer pH 6.8, 25% Glycerol, 10% SDS) to separate the soluble and the insoluble fractions. The fractions were subjected to 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The negative control included uninduced *E. coli* BL21 (DE3) pLysS with Pm*Vg*+pET32a+vector.

Specificity of Vg/Vn polyclonal antisera using expressed recombinant Vg protein

Western blot was performed as previously described (Fabbretti *et al.*, 1995) with modifications to verify the specificity of the Vg/Vn polyclonal antisera raised in rabbit. The induced and uninduced fractions (10 µg) of *E. coli* BL21 (DE3) pLysS with Pm*Vg*+pET32a+vector were separated in 12% SDS-PAGE and the resolved protein was transferred to nitrocellulose membrane

for Western blot analysis using semi-dry transfer Ecl blotter (Amersham Biosciences, Piscataway, NJ, USA).

Validating maturity in female P. monodon with anti-PmVg/Vn antisera

Haemolypmh (100–200 µl) from immature and mature shrimp ($N = 3$) was collected and used as antigen for validating ELISA as previously described. The body weight was taken prior to sacrificing specimens for the dissection of the ovary in order to calculate the gonadosomatic index (GSI) of the immature (stage 1) and mature (stages 2 and 3) female *P. monodon*.

Statistical analysis

The data analysis was performed by curve fitting and trend line analysis to obtain $R^2 = 1$. The assay performances were evaluated by monitoring the uniformity in colour development in the presence and absence of antigen as well by calculating the percentage coefficient of variation (% CV) of triplicate and quadruplicate determinations. The graphical representations were considered significant at $P < 0.05$. The statistical analyses were performed with the SPSS 11.5 software package.

RESULTS

The sensitivity of the polyclonal antiserum (anti-PmVg/Vn) raised was analysed by ELISA; the absorbance value that reciprocates 1.0–2.0 units was selected based on the anti-PmVg//Vn polyclonal antisera concentration optimization assay. The optimization assay specified that titres from 1:204,800 to 1:51,200 yield absorbance between 1.11–2.0 OD, against purified Vg/Vn (Fig. 1a, b). Based on curve fitting and trend line analysis, the antisera titre of 1:51,200, which yielded absorbance of 1.93 was chosen for all routine assays (ELISA, Western blot and immunostaining). The ELISA was validated further by curve fitting and trend line analysis using higher and lower concentrations of Vg/Vn and recombinant Vg as antigen. The standard curve analysis (Fig. 2a) was observed to be linear over the range of 5 x 10^{-3} to 50 ng ml^{-1} (correlation coefficient (r) = 1). ELISA results based on standard curve revealed the trend line with $R^2 = 0.9999$ using Vg/Vn as antigen. The inter and intra-assay coefficients (% CV) were calculated to validate the ELISA for precision and repeatability. The inter-assay % CV obtained was 7.72. For intra-assay % CV the Vg concentrations were measured in quadruplicates for 32 samples. The intra-assay % CV was 6.77. Using recombinant Vg as antigen, the standard curve analysis was found to be linear over the range of above 20 µg. The trend-line analysis revealed $R^2 = 0.9979$ (Fig. 2b). The antisera optimization using recombinant Vg revealed $R^2 = 0.9436$ with linear range from 1: 3,200 to 1:51,200 antisera dilutions (Fig. 2c). The inter and intra-assay coefficient of variations (% CV) obtained using recombinant Vg as antigen was 5.45 and 7.89.

The specificity of the anti-PmVg/Vn antisera was confirmed by Western blot analysis. The analysis of ovarian extracts (20 µg) from immature (stage 1) (GSI = 0.858) and sexually mature (stage 2) (GSI = 4.32 and GSI = 4.982) detected the major and minor subunits of Vg/Vn. The anti-PmVg/Vn detected 210, 180, 150, 95, and 76 kDa subunits in the ovary extract, which was similar to that of the Vg/Vn antigen. The anti-PmVg/Vn also detected possible Vg subunits in female haemolymph of immature and mature *P. monodon* in comparison to no subunit detection in male haemolymph. The subunits detected in female haemolymph (20 µg) ranged between ~45, 74, 83, and 200 kDa to negative band detection in the haemolymph of male shrimp (Fig. 3). Further immunostaining validated the specificity of the anti-PmVg/Vn polyclonal antiserum. Anti-PmVg/Vn antisera immunostained Vn-like oocytes in extra-oocyte location within the theca of the ovary (Fig. 4A) and Vg/Vn-like globules in ovarian extract (Fig. 4B) in comparison to negative control (absence of antiserum) (Fig. 4C). Immunohistochemistry revealed vitellogenic cells in ovarian section (stage 2) compared to negative control (absence of antiserum) (Fig. 5).

To check the specificity of the polyclonal antisera (anti-PmVg/Vn), vitellogenin subunit was cloned and expressed in pET32a+

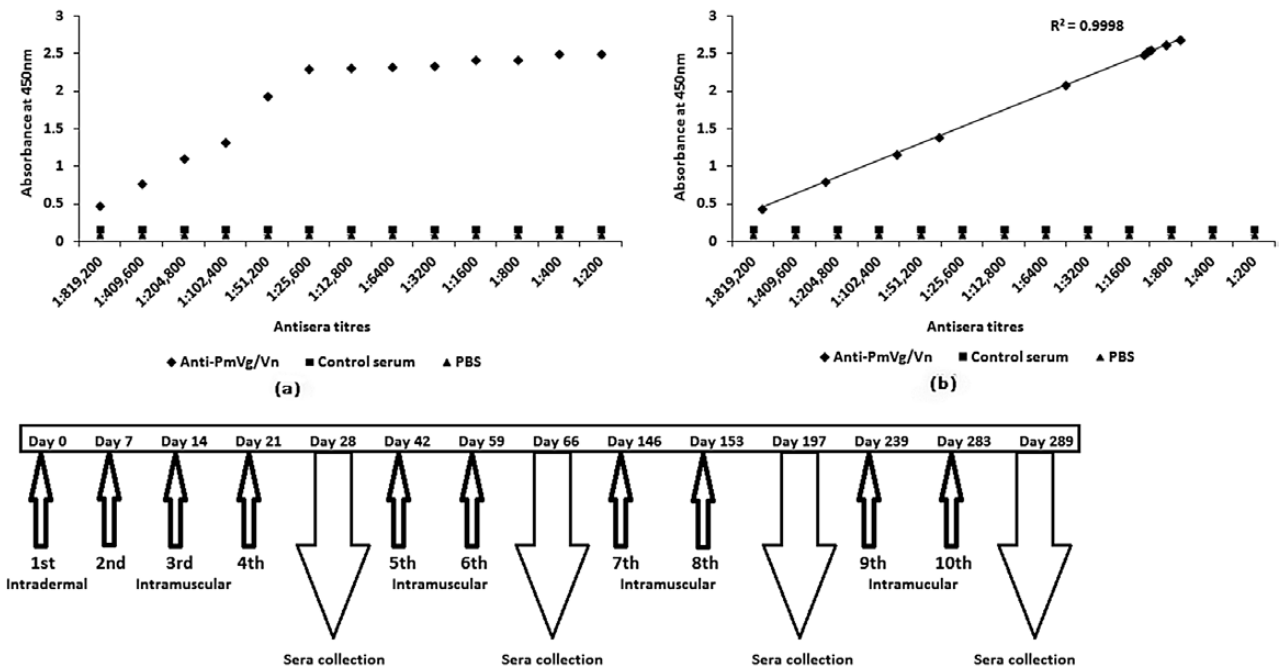

Figure 1. ELISA validation performed using polyclonal antisera anti-PmVg/Vn (following a dilution series of factor 2) and anti-rabbit IgG-HRP conjugate (1: 1,000 dilution) to identify the titre best for carrying out all assays, with inset figure below depicting days of injections and sera collection, the sera tested on the final day of test bleed (day 289) (**a**) and trend-line analysis/curve fitting to obtain an R^2 value is represented in figure (**b**).

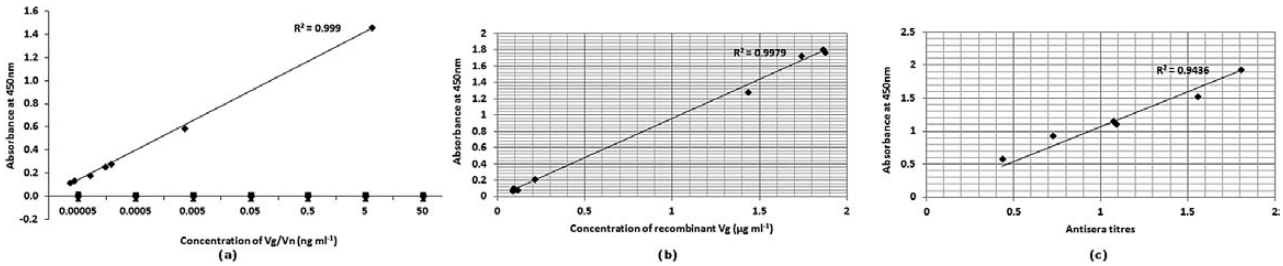

Figure 2. **(2a)** ELISA validation performed using polyclonal antisera anti-PmVg/Vn (1:51,200 dilution) and anti-rabbit IgG HRP conjugate (1: 1,000 dilution) to confirm the sensitivity of the antisera raised against purified Vg/Vn protein at concentrations ranging from $5x10^{-5}$ to 50 ng ml^{-1} in comparison with Vg/Vn (20 µg ml^{-1}) (no antiserum), BSA (20 µg ml^{-1}) + antiserum and vibrio (1OD) + antiserum. The trend line obtained between the points, show an R^2 = 0.999 value. The ELISA validation was performed using recombinant Vg with varying recombinant Vg concentrations **(2b)** and with varying antisera dilutions **(2c).** The validation was confirmed by calculating the inter- and intra-assay coefficient of variability (% CV).

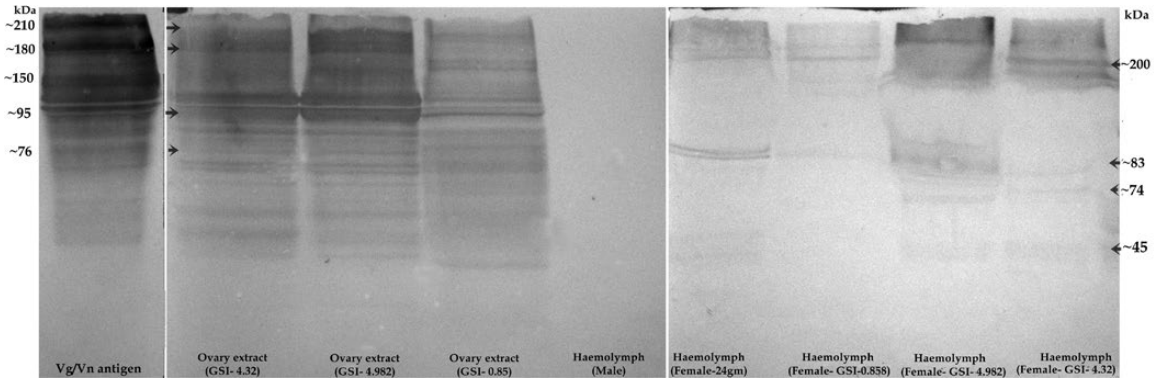

Figure 3. Western analysis to assess the specificity in detecting Vg/Vn subunits present in Vg/Vn antigen, ovary extracts based on the GSI, haemolymph of male shrimp, haemolymph of female shrimp in immature stage, GSI = 0.858, GSI = 4.982, GSI = 4.32 of *P. monodon* using anti-PmVg/Vn polyclonal antisera (1:51,200 dilution).

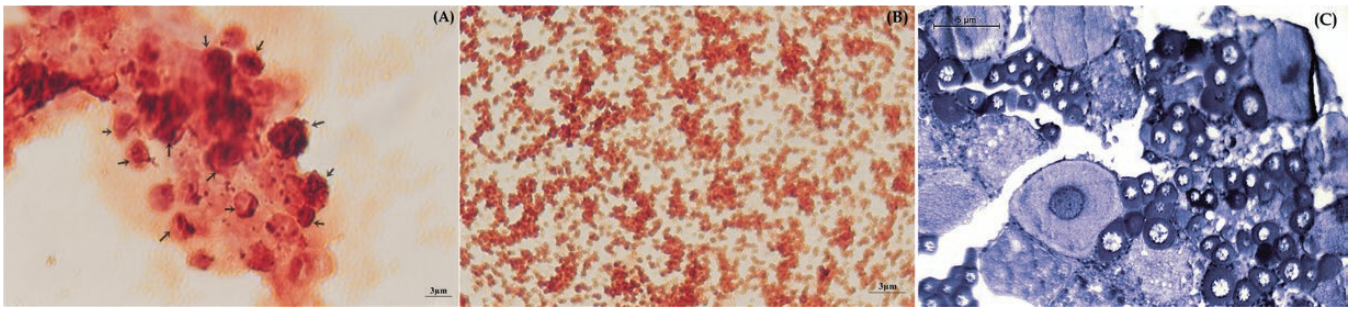

Figure 4. Specificity of anti-PmVg/Vn antisera confirmed by immunostaining of vitellin-like oocytes in an extra-oocyte location within the theca of the ovary (**A**); vitellin-like globules observed in ovary extract (**B**) in comparison with negative control (without antisera) (**C**).

vector. Initially, the Pm-*Vg* cDNA was synthesised using the mRNA isolated from *P. monodon* ovary. The product obtained (~2.221 kb) was identified, cloned into pGEM-T easy-vector and sequenced. The sequence BLAST search in NCBI database revealed 99% identity to vitellogenin of *P. monodon*. The confirmed vitellogenin PCR product (Pm-*Vg*) was further cloned into translation expression vector pET32a+, which was designated Pm-*Vg*+pET32a+. The Pm-*Vg*+pET32a+ translation expression vector was used successfully for the production of recombinant Pm-Vg protein (Pm-*Vg* 94 kDa (74 + 20 kDa)) in the *E. coli* BL21 (DE3) pLysS system with IPTG induction (Fig. 6a). The original size of the Pm-*Vg* subunit was 74 kDa with additional 20 kDa of thioredoxin (trx) from the vector, conferring the recombinant Pm-*Vg* size of 94 kDa. The specific band of 94 kDa was detected from induced *E. coli* BL21 (DE3) pLysS transformed with Pm-*Vg*+pET32a+ expression vector in comparison with uninduced (Fig. 6b). Western blot analysis of the expressed recombinant Pm-*Vg* (10 µg) with anti-PmVg/Vn polyclonal antisera demonstrated specific band of 94 kDa size. This confirmed the identity and specificity of the anti-PmVg/Vn polyclonal antisera.

The anti-Pm-Vg/Vn antisera developed was validated by monitoring the Vg/Vn in the haemolymph of immature and mature female *P. monodon* in relation to GSI. The least concentration (21.03 ng ml^{-1}) of Vg/Vn was observed in the animal of stage 1 (immature) ovary, while maximum (32.24 ng ml^{-1}) was observed in stage 2. The graphic representation of haemolymph Vg/Vn concentration depicts a steady rise in the Vg/Vn concentrations with increasing GSI (Fig. 7).

DISCUSSION

Vg/Vn was isolated and purified from the ovary of *P. monodon*, which ensured the presence of both Vg and Vn protein subunits.

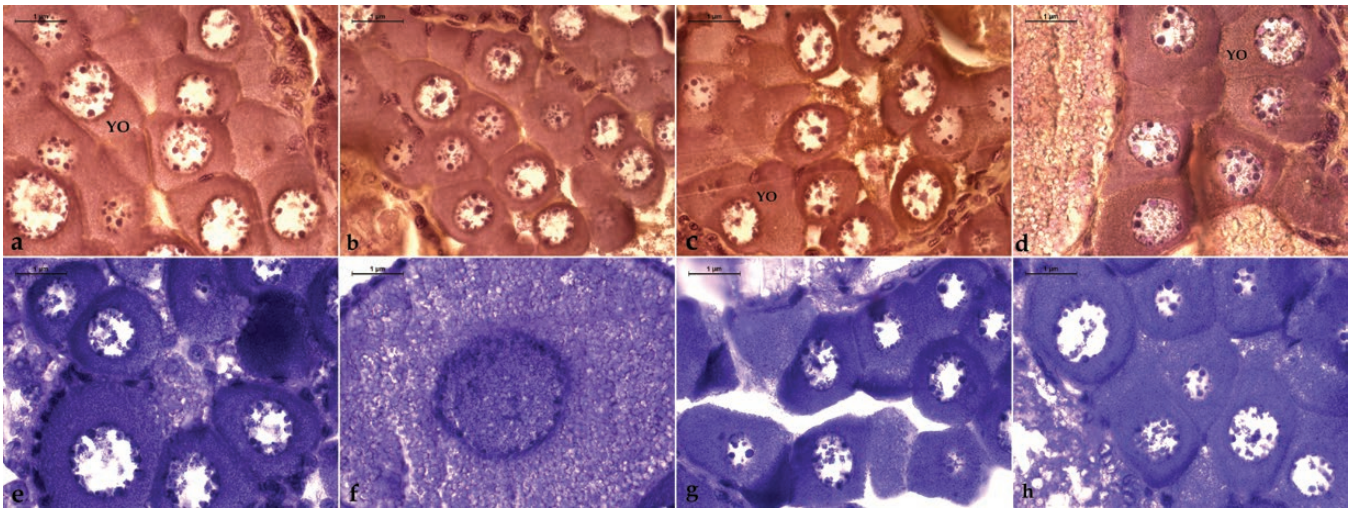

Figure 5. Immunohistochemistry showing immunoreactivity in ovarian cells of matured *P. monodon* at stage 2 (**a–d**) compared with control section (without primary antisera) (**e–h**) using anti-PmVg/Vn; YO, yolk oocytes.

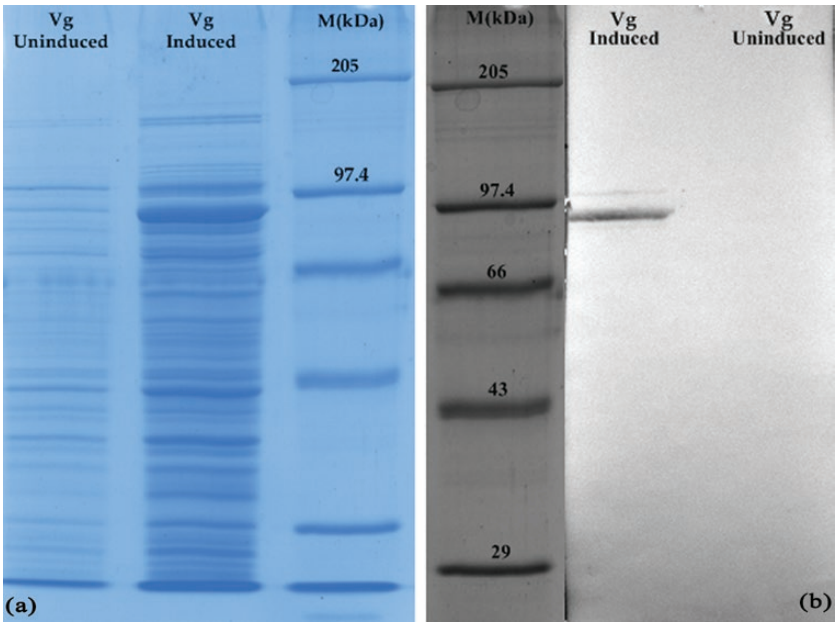

Figure 6. (**a**) SDS-PAGE analysis of recombinant Pm-*Vg* protein (Pm*Vg*-94 kDa (74 + 20 kDa) expressed in IPTG induced *E. coli* BL21 (DE3) pLysS system compared with uninduced Pm*Vg* by Coomasie staining and (**b**) Western blot analysis of the total protein extracted from *E. coli* BL21 (DE3) pLysS bacterial cells transformed with *Vg* expression vector in comparison with uninduced Pm-*Vg* using polyclonal antisera (anti-PmVg/Vn; 1:51,200 dilution).

This purified Vg/Vn subunits from ovary was used as antigen to raise polyclonal antisera in rabbits. Based on the hypothesis that haemolymph Vg is definitely not the precursor of Vn in penaeid shrimps (Avarre *et al.*, 2003), we used the combined Vg/Vn protein subunits for generating polyclonal antisera. In previous studies that applied antibody/antisera as an immunological tool to measure haemolymph vitellogenin levels in *Macrobrachium rosenbergii* (De Man, 1879) (Chen *et al.*, 1998), *P. monodon* (Fabricius, 1798) (Longyant *et al.*, 1999), *Sicyonia ingentis* (Burkenroad, 1938) (Tsukimura *et al.*, 2000), *Pandalus hypsinotus* (Brandt, 1851) (Okumura *et al.*, 2004), and *Litopenaeus merguiensis* (De Man, 1888) (Auttarat *et al.*, 2006), the protocol was to extract Vg/Vn from ovarian tissue. The isolation of Vg/Vn from ovary in our study ensured the presence of all the major Vg and Vn subunits.

The anti-Vt/Vg antiserum/antibody raised against Vt/Vg isolated from vitellogenic ovaries were used to develop an ELISA for monitoring changes in haemolymph levels of yolk protein precursor vitellogenin (Chen *et al.*, 1998; Longyant *et al.*, 1999; Tsukimura *et al.*, 2000; Okumura *et al.*, 2004; Auttarat *et al.*, 2006). Here, we used purified Vg/Vn subunits (combined) isolated from ovary to raise polyclonal antisera in rabbit. Through ELISA, anti-PmVg/Vn was demonstrated to be sensitive to detect $5x10^{-3}$ ng ml^{-1} of purified Vg/Vn. The sensitivity is found much higher compared to the ones reported in earlier investigations, where it was 125–1,200 ng in *Penaeus vannamei* (Boone, 1931) (Quackenbush, 1986), 8.2–1,050 ng ml^{-1} in *Macrobrachium nipponense* (De Haan, 1849) (Okumura *et al.*, 1992), and 0.1 µg in *M. rosenbergii* (see Chang & Shih, 1995). The detection limit obtained was 10 ng ml^{-1} in *Callinectes sapidus* (Rathbun, 1896) (Lee & Walker, 1995) and 0.05–50 µg ml^{-1} in *M. rosenbergii*, both using monoclonal antibodies. With the aid of Vn monoclonal antibody specific to *P. monodon*, the detection range of antilipovitellin immunoreactive protein was 0.25–200 µg ml^{-1}(Longyant *et al.*, 1999) and 25 ng ml^{-1} (Vincent *et al.*, 2001). The ELISA in our study was more sensitive ($5x10^{-3}$

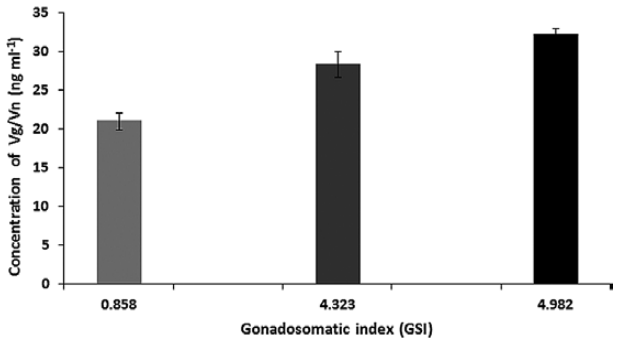

Figure 7. Validation of maturity by the analysis of the haemolymph Vg levels in mature female *P. monodon* ($N = 3$) based on gonadosomatic index (GSI) using anti-PmVg/Vn ELISA (1:51,200 dilution).

ng ml^{-1}) in comparison to the sensitivity (0.5 ng/ml) of sandwich Time Resolved-Fluoroimmuno Assay (TR-FIA) developed for the protandric shrimp *Pandalus hypsinotus* (Okumura *et al.*, 2004). The inter-assay % CV is 7.72, 5.45, and 7.89; the acceptable inter-assay % CV should be less than 15. The intra-assay % CV obtained was 6.77, and the acceptable value should be less than 10. The scores obtained express the precision and repeatability of the assay.

Anti-PmVg/Vn conferred positive reaction with the haemolymph of female and ovarian extracts; however, no cross reactivity was detected with the haemolymph of male *P. monodon*. These observations agreed with the results of the study conducted using monoclonal antibodies (Mabs) of Vn and Vg of *P. monodon*. The Mabs bound with ovarian extract proteins and with female haemolymph, but not with male haemolymph (Longyant *et al.*, 1999). In the Western blot results the anti-PmVg/Vn detected 45, 74, 83, and 200 kDa protein subunits in female haemolymph, while in the crude ovarian extracts 210, 180, 150, 95 and 76 kDa Vg/Vn subunits were detected. No band or smear could be detected in the male haemolymph. This unequivocally suggests that the anti-PmVg/Vn developed was similar in competency to the Mabs of vitellogenin and Vn of *P. monodon* (Longyant *et al.*, 1999). Using anti-PmVg/Vn various major subunits of 'Vn' and 'vitellogenin' in female haemolymph and ovarian extract of *P. monodon* could be successfully detected. The detection of Vg/Vn subunit (sizes 210, 180, 150, 95, and 76 kDa) in the present study agree with those of Vg/Vn subunit detection of *P. semisulcatus* (Avarre *et al.*, 2003), *P. hypsinotus* (Okumura *et al.*, 2004), *L. merguiensis* (Auttarat *et al.*, 2006), and *M. rosenbergii* (Chen *et al.*, 1998), with marginal variations in the Mw of the subunits.

The specificity of anti-PmVg/Vn antisera was validated by the detection of Vn-like globules (extra-oocyte) and Vn globules (intra oocyte) in the oocytes of *P. monodon* by immunostaining. The Vn-like globules observed were consistent with electron microscopic observation of exogenous vitellogenic oocytes of *P. hypsinotus* using anti-vitellin antiserum (Okumura *et al.*, 2004). Mature oocytes observed in the immunostained vitellogenic ovarian tissue were characterised by germinal vesicle breakdown and the invisibility of nucleus at the centre of the cytoplasm. The detection of yolk globules confirmed the accumulation of Vn in the oocyte and maturity of the oocytes. This is suggestive of specificity of anti-PmVg/Vn antiserum of *P. monodon* being specific to Vn in ovarian tissue hitherto not revealed in other studies related to Vg/Vn of *P. monodon*.

With the confirmation that anti-Pm-Vg/Vn antisera were capable of detecting Vg and Vn, haemolymph vitellogenin levels of mature female *P. monodon* were validated based on their GSI. Medina *et al.* (1996) found that the GSI of pond-reared *Penaues kerathurus* (Forskål, 1775) was not highly variable based on the body weight. A 20.5 ± 2.3 g (BW) individual of this species had a GSI (%) of 5.5 ± 0.8, whereas an individual of 22.6 ± 2.0 g had 3.7 ± 0.9, and an 18.4 ± 1.0 g individual had a GSI of 5.2 ± 0.7. The GSI of our specimens was likewise not concordant with body weight. A stage 1 (immature) individual with a body weight of 79.2 ± 0.26 g had a GSI of 0.858 ± 0.019, whereas a stage 2 individual of 96.47 ± 0.25 g had a GSI of 4.98 ± 0.002 and another stage 2 individual of 124.17 ± 0.15 g had a GSI of 4.32 ± 0.023. The validation experiment in the present study is therefore purely based on the GSI of *P. monodon*. The concentration of haemolymph vitellogenin increased steadily with increasing GSI of mature females, with a positive correlation of 1. The maximum rise of 32.23 ± 0.82 ng ml^{-1} was observed in a 4.98 ± 0.002 mature female. These levels of haemolymph vitellogenin are usually observed in mature females with active stage ovaries. Our results concur with results obtained in haemolymph vitellogenin levels of female *P. semisulcatus* (Shafir *et al.*, 1992) and *P. hypsinotus* (Okumura *et al.*, 2004). Based on the above results on specificity, sensitivity, and validation, female maturity in *P. monodon* can be successfully detected in haemolymph using anti-PmVg/Vn without gonad biopsy for assessing maturity.

Immunological confirmation of anti-PmVg/Vn polyclonal antisera using a recombinant vitellogenin protein (94 kDa-74 kDa +20 kDa trx) expressed *in vitro* has hitherto not been reported in *P. monodon*. The recombinant vitellogenin protein subunit (94kDa) was detected by Western blot, which verified that anti-PmVg/Vn polyclonal antiserum was specific in detecting the vitellogenin subunit. The most important result of our study is the confirmation of the specificity of the anti-PmVg/Vn antisera in detecting the Vg/Vn subunit (74 kDa), as the early and later stage of shrimp maturation are prominently marked with the stable incidence of this 74 kDa vitellogenin subunit in the haemolymph of the shrimp.

The isolation from ovary generates the possibility of obtaining major and minor subunits of Vg/Vn, thus the antisera developed could detect similar subunits *de novo* from haemolymph and ovary of *P. monodon*. Vital information generated in the study is the confirmation of the anti-PmVg/Vn antisera, which could detect a recombinant vitellogenin subunit of *P. monodon* expressed in *E. coli* expression system. The sensitivity and specificity of the anti-PmVg/Vn antisera is therefore comparable to any other Vg/Vn polyclonal/monoclonal antibody developed in detecting the Vg levels in haemolymph and mature oocytes in the ovarian tissues of *P. monodon*. The antisera can be used as a successful tool in detecting and quantifying Vg in haemolymph of *P. monodon* during maturation, which could help in monitoring maturation of the shrimp in breeding and domestication programmes without gonad biopsy. The recombinant vitellogenin subunit of *P. monodon* expressed in *E. coli* expression system is a good candidate antigen for generating a monoclonal antibody to detect and quantify Vg levels in haemolymph in order to follow the gonadal maturation of *P. monodon*.

ACKNOWLEDGMENTS

This work was carried out with the financial support from Department of Biotechnology (DBT), Government of India under the project 'Development and application of CMG family recombinant hormones, their antagonists and RNAi technique for induced maturation and spawning of *Penaeus monodon* (File number BT/PR5721/AAQ/03/238/2005). The first author thanks DBT and Cochin University of Science and Technology for a fellowship. We thank the anonymous reviewers for reviewing the manuscript. The authors have no conflict of interest with regard to the work conducted or for the submission of the manuscript. Funding was provided by Department of Biotechnology (DBT), Government of India (file number BT/PR5721/AAQ/03/238/2005).

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