Validation of an Enzyme-Linked Immunosorbent Assay Method for Vitellogenin in the Medaka

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An enzyme-linked immunosorbent assay (ELISA) was validated for the measurement of vitellogenin (VTG) in the cyprinid model species, medaka (*Oryzias latipes*). Both polyclonal antibody- and monoclonal antibody-based kits showed very high correlation between VTG values in unknown samples when we used standardized VTG protein. This was confirmed by 5 laboratories which participated in the ring test. The use of one gold standard VTG is essential to measure VTG values using ELISA kits in future OECD ring tests to screen for estrogenic chemicals in the 3 fish species medaka, zebrafish and fathead minnow.

Key words —— medaka, vitellogenin, enzyme-linked immunosorbent assay

INTRODUCTION

Vitellogenin (VTG) is a phospholipoglycoprotein precursor of egg yolk protein which is normally produced by sexually-active females of all oviparous species. The production of VTG is controlled by the interaction of endogenous estrogens with the estrogen receptor (ER). Since males maintain the capacity to produce VTG in response to stimulation by ER agonists, the production of VTG in males and immature females has been successfully exploited as a biomarker of exposure specific for estrogenic compounds in a variety of OECD fish species, notably Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*).[1–9)]

Enzyme-linked immunosorbent assay (ELISA) provides a relatively sensitive and specific measurement technique for assessing VTG concentration in plasma or liver homogenates. ELISA using polyclonal and/or monoclonal VTG antibodies could be useful tools for assessing VTG production in ecotoxicological tests for the endocrine disrupting activity of chemicals.[5,10)] Polyclonal and monoclonal antibodies against medaka VTG, and purified VTG protein from the medaka, have been utilized for the development of an ELISA. The validation of a method for the quantification of VTG is essential to obtain accurate results in all laboratories which will conduct this assay. One reason for the implementation of a standardized VTG assay is the need to perform partial life-cycle and full life-cycle assays which would routinely use VTG measurement as an endpoint. Ideally, both a standard antibody and a standard VTG protein should be broadly available for all laboratories performing life cycle testing.

For screening, as well as in studies of wildlife fish (*e.g.* roach, trout, flounder), the measurement of VTG should be based upon a quantitative, validated method. Consequently, information concerning the intra-assay and inter-assay variability of the method used in a given laboratory to measure VTG should be available.

MATERIALS AND METHODS

Two types of VTG measurement kits are commercially available in Japan. However, these kits differ, as the standard VTG protein provided in each kit differs. Therefore, a well-controlled comparison of these VTG kits will provide useful information if they are to be used to measure VTG production as a biomarker of estrogenic effects in ecotoxicological tests. In the present study, both VTG kits were validated using a standard VTG protein purified by the authors at the National Institute for Environmental Studies (NIES), Tsukuba, Japan. Liver homogenate samples from Japanese medaka exposed to 17β-es-

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tradiol (E_2) for 1 week were then prepared as blind samples. The standard VTG protein and the blind samples were ring-tested by 5 laboratories in Japan. The samples were measured using both commercial VTG measurement kits in each laboratory, using the new standard VTG protein and VTG protein provided in each kit. The measurement results were collected, calculated and statistically evaluated. Furthermore, the VTG in blood and liver prepared from the same fish were compared with each other in the laboratory at NIES.

VTG Antibodies in 2 Types of ELISA Kits ——

Kit A: TransGenic (TransGenic, Inc., Kumamoto):

Fish Preparation: Adult medaka (body weight, *ca*. 250 mg; total length, *ca*. 30 mm) were purchased from a local fish farm (Kumamoto, Japan) and kept for at least two weeks in a 50 l glass tank with dechlorinated tap water at 20–25°C. The dechlorinated tap water was renewed every three days. The male fish were placed under a summer photoperiod (14 hr light : 10 hr dark) and fed a commercial food containing E_2 (Sigma Chemicals, Tokyo, Japan) to cause ascites, as described by Hamazaki *et al*.[11] Ascites fluid was carefully collected from each male and mixed with the same volume of ice-cold preparation buffer: 40 mM ethylenediaminetetraacetic acid (EDTA) and 0.4 mg/ml phenylmethysulfonyfluoride in 20 mM phosphate buffer (pH 6.8) or 20 mM Tris HCl buffer (pH 7.2). The ascites sample was centrifuged at 20000 *g* for 20 min, and the supernatant was used for the purification of VTG.

Purification and Identification of Medaka VTG: VTG was purified from the ascites fluid of males treated with E_2 according to Yamanaka *et al*.[12] Briefly, the ascites sample was subjected to anion exchange chromatography on a POROS 20HQ (Applied Biosystems, Tokyo, Japan) column equilibrated with 20 mM Tris–HCI, at pH 8.0. VTG was eluted using a linear salt gradient (0 to 1.5 M NaCl). VTG-containing fractions were then pooled and treated with aprotinin (10 KIU/ml) and sodium azide (0.02%). Purified samples were divided, mixed with an equal volume of glycerol, and stored at –80°C. When samples were thawed, they were used only once as a standard in the Western blot analysis or ELISA, and then discarded.

The purity of the VTG preparations was analyzed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (PROTEAN, Bio-Rad Laboratories, Tokyo, Japan).[13] Purified VTG samples, the ascites samples from E_2-treated males, and the serum from mature males and females were diluted 50-fold with Laemmli sample buffer, treated with 1% β-mercaptoethanol, and then heated at 90°C for 4 min, respectively. Each 10 μl sample was applied to a separate well of a 7% polyacrylamide gel. Standard marker proteins from 50 to 250 kDa (Bio-Rad Laboratories) were included to estimate the molecular masses of the sample proteins. Samples were run at a constant current of 40 mA. Protein bands were visualized by staining with Coomassie Brilliant Blue.

VTG was identified by N-terminal amino acid sequencing. VTG samples purified by anion exchange chromatography (*ca*. 100 pM) were dialyzed with phosphate-buffered saline (PBS), and then microsequenced by the Edman method using an Applied Biosystems Model 473A protein sequencer with an on-line PTH amino acid analyzer (Applied Biosystems). The resulting sequence was compared with that deduced from the medaka TG cDNA obtained by Murakami *et al*. (unpublished data).

Preparation and Biotin Labeling of Polyclonal Antibodies against VTG: The polyclonal antibody was raised against purified medaka VTG in Japanease white rabbits. Anti-medaka VTG polyclonal anitibody was purified by Protein G affinity chromatography.

Biotinylated antibody was prepared using a biotin labeling kit (Boehringer Mannheim, IN, U.S.A.). A volume of 22.5 μl of the mixture of 10 μl D-biotinoyl-ε-aminocaproic acid N-hydroxy succinimide ester (biotin 7-NHS) solution (20 mg/ml) and 90 μl dimethylsulfoxide (DMSO) was added to 1 ml antibody solution (1 mg/ml PBS),and the mixture was stirred gently for 2 hr at room temperature. The remaining, non-reacted biotin-7-NHS was separated by dialization against PBS overnight at 4°C.

Kit B: EnBio Lab. (EnBio Tec Laboratories Co., Ltd., Tokyo, Japan):

Fish Preparation: Adult medaka (orange-red) (body length, 25–35 mm) were purchased from a local pet shop in Japan and checked daily for a week for signs of illness and maturity. Healthy medaka were kept in a 50 l glass tank with dechlorinated tap water at 25 ± 1°C under a summer photoperiod (14 hr light : 10 hr dark). The dechlorinated tap water was renewed every three days. Fish were fed a commercial food (TetraMin, Warner-Lambert, U.S.A.) twice daily.

Purification and Identification of Medaka VTG: VTG was purified from the ascites fluid of adult females fed food containing 1 mg/g of E_2 (Sigma Chemicals) at 20°C for 10 days, according

to the method previously described.[11] Purification was performed by anion exchange chromatography on a POROS 20HQ (Applied Biosystems) column, as previously described.[12] The purified VTG was confirmed for purity, molecular mass and characterized by comparison with plasma proteins using SDS-PAGE[13] with 7.5% PAGE gels. The concentration of the purified protein was determined using the Lowry method,[14] with bovine serum albumin (BSA) as the standard protein. The purified VTG was used as an antigen for both mouse monoclonal antibodies (mAbs) production and as an assay standard.

Production and Labeling of Monoclonal Antibodies against VTG: Balb/c mice were immunized intraperitoneally with 10 μg of the purified-VTG mixed 1 : 1 with complete Freund's adjuvant. Mice were boosted twice at 3-week intervals with 10 μg of an antigen mixed with incomplete Freund's adjuvant. Hybridomas were produced by the standard methods[15] and selected by using the purified VTG. Their subclass was determined by a Mouse Monoclonal Antibody Isotyping kit (Seikagaku-Kougyo, Tokyo, Japan). The mAbs were purified by ammonium sulfate precipitation followed by anion exchange chromatography on a MonoQ HR 10/10 column (Amersham Pharmacia Biotech, Tokyo, Japan).

Purified mAbs were labeled with horseradish peroxidase (HRP, Oriental Yeast, Tokyo, Japan) by the procedures described previously.[15] HRP-labeled antibody solution was divided into aliquots and stored at –80°C until use.

Purification of NIES Standard VTG Protein: VTG was purified from the plasma of adult female medaka fed commercial food (TetraMin) containing 1 mg/g of E_2 (Sigma Chemicals) twice a day for 7 days, according to the method previously described.[11] The E_2-treated fish (n = 20) were put into an ice-cold bath for a few minutes to immobilize them; they were then cut at the ventral midline with a scalpel. The blood was immediately pick up into heparinized micro pipettes (Modulohm, Copenhagen, Denmark), then transferred to 20 μl of 20 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 150 mM NaCl, and 25 KIU/ml aprotinin (Wako, Osaka, Japan). The pooled blood was centrifuged at 8000 rpm (5800 g) at 4°C for 10 min. The supernatant was collected and stored frozen at –80°C until purified. The purification was performed by anion-exchange chromatography on a POROS-HQ column.[12] The purified VTG was confirmed for purity, molecular mass, and characterized by comparison with plasma proteins using SDS-PAGE[13] with 7.5% PAGE gels, and Western blot [Primary: Anti-Medaka vitellogenin IgG, Horseradish Peroxidase conjugate (EnBio), Secondary: Anti-Mouse IgG+A+M Rabbit (Zymed Laboratories Inc., Sanfrancisco, CA, U.S.A.)], according to the method described by Nishi *et al*.[16] Concentration of the purified protein was determined by the density of the VTG band of SDS-PAGE compared with the band density of BSA (BioRad) using NIH Image Software (Scion Image Co., Frederic, Maryland, MD, U.S.A.), as the standard protein.

Examination of VTG Standards Attached in VTG ELISA Kits: The two standard VTG proteins (Kit A standard and Kit B standard) included in the commercially available ELISA kits (Kit A and Kit B) were evaluated using a VTG protein standard newly purified at NIES (NIES standard). The two Kit VTG standards were measured using both ELISA kits at the same time.

Preparation of Medaka Liver and Plasma Samples for the Evaluation of Each Kit: Male fish were exposed to ethinylestradiol (EE_2) (Sigma Chemicals) at nominal concentrations of 320, 100, 31.3, and 9.8 ng/l, and solvent (Dimethyl Sulfoxide, 0.1 g/l) for only 1 week in a continuous flow-through exposure system (flow rate: 18 ml/min/tank). Twelve males per test vessel were exposed to each concentration of EE_2 or the solvent only. Details of the experimental conditions are shown in Table 1. After EE_2 exposure, fish were dissected, and the liver was collected individually. Two livers from fish exposed to the same treatment concentration were pooled and homogenized with 400 μl of 20 mM Tris–HCl at pH 7.5 containing 1 mM EDTA, 150 mM NaCl, and 25 KIU/ml aprotinin (Wako). These samples were centrifuged at 10000 g for 10 min and the supernatant was pooled. Each sample was divided into 5 sets of 200 μl eppendorf tubes as 60 μl aliquots. Thirty samples (six samples per concentration, 5 concentrations including solvent control) and the purified standard VTG protein were sent to five laboratories at –20°C. The 5 laboratories were selected because of their previous experience in the measurement of VTG protein. Subsequently, VTG concentrations of samples were measured simultaneously using the 2 VTG ELISA kits in accordance with the manual of each kit.

Preparation of Medaka for Comparison between Liver VTG and Plasma VTG: Male medaka fish were exposed to EE_2 at 320, 100, 31.3, and 9.8 ng/l for 1 week, and solvent controls were also prepared. Sixteen males were kept in a test vessel

Table 1. Experimental Condition

Species:	Medaka (*Oryzias latipes*)
Photoperiod:	16 hr light : 8 hr dark
Test type:	Flow-through
Water temperature:	24 ± 2°C
Test chamber size:	6 l
Test solution volume:	5 l
Number of fish per test vessel:	16
Age of test organisms:	Pre-breeding adults (2 months)

Table 2. Characteristics of Two Types of Kits

A Kit	B Kit
direct sandwich ELISA	direct sandwich ELISA
specific polyclonal antibodies to medaka VTG	monoclonal antibodies to medaka VTG
applicable to the determination of hepatic/plasma VTG in medaka	applicable to the determination of hepatic/plasma VTG in medaka
working range: 4–256 ng/ml	working range: 2–100 ng/ml

containing each concentration of EE_2 and controls. After EE_2 exposure, the fish were divided into 2 groups (8 individuals each) for each concentration. Blood was collected from one group and liver was sampled from the other group. Blood was collected in a heparinized microcapillary tube, the volume was measured, and it was then diluted to 1 : 200 with a dilution buffer, 20 mM Tris at pH 7.5 containing 1 mM EDTA, 150 mM NaCl, 1% BSA and 25 KIU/ml aprotinin (Wako). Blood and homogenized liver samples were immediately centrifuged at 10000 *g* for 10 min at 4°C, and the plasma and supernatant were kept frozen at –80°C until use. Subsequently, VTG concentration in plasma or liver samples was measured simultaneously in accordance with the manual of each kit at NIES. At the time of comparison of VTG in plasma and liver, our new standard VTG protein was not available; therefore, VTG was measured using VTG standards attached in each kit.

Statistical Analyses of Coefficient of Variation in the Measurement of Standard Protein: Within-laboratory coefficients of variation (CV's) of VTG values estimated from both types of VTG ELISA kits were calculated from two replicate measurements and averaged for five concentrations. Due to small samples, all the estimated CV's were corrected for bias.[17] For the calculation of within-laboratory CV's, VTG data in the controls were excluded. Statistical significance of kit effect and laboratory effect was tested by nonparametric two-way analysis of variance.[17]

Measurement of VTG in Unknown Samples using NIES VTG Standard: Two standard curves were drawn following the use of each kit. One curve was based on the VTG protein standard provided with each kit. The other curve was based on the use of the NIES VTG protein standard. The VTG measurements were plotted against each other on a log-log plane, and product-moment correlations and coefficient of determinations were calculated. The two different estimates of VTG concentration using the two standards (the standard VTG protein in each kit and NIES VTG standard) were compared with each other in relationship to the VTG measurements by the two types of kits.

Comparison of VTG Concentrations in Plasma and Liver: VTG concentrations in the plasma and liver of male fish exposed to EE_2 were analyzed by nonparametric two-way analysis of variance[17] for statistical significance of kit type effect and EE_2 exposure effect.

RESULTS

Characteristics of the two commercially available VTG ELISA kits for medaka are listed in Table 2. The A kit is based on a polyclonal antibody, whereas the B kit is based on a monoclonal antibody. Both kits contain a standard VTG protein. Working ranges described in each kit's manual are 4 to 250 ng/ml in the A kit and 2 to 100 ng/ml in the

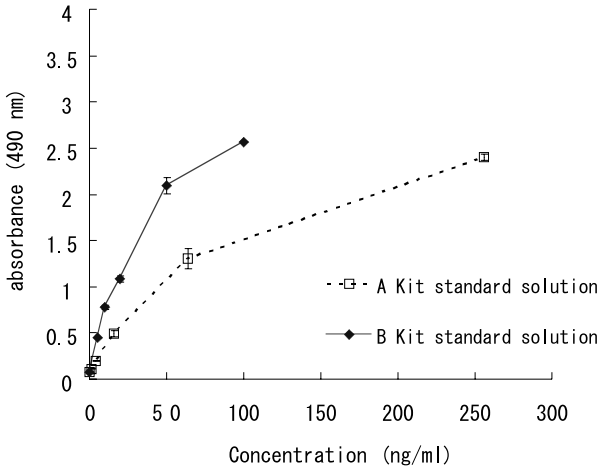

Fig. 1A. VTG Protein Standard in the Two Commercial Kits as Measured by the A Kit
Error bars indicate standard deviations.

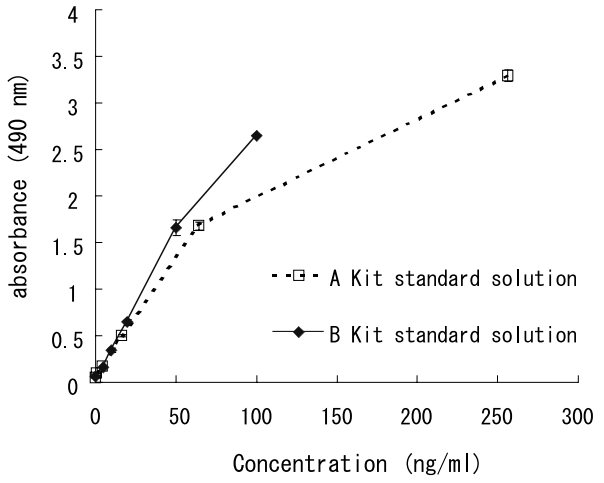

Fig. 1B. VTG Protein Standard in the Two Commercial Kits was Measured Using the B Kit
Error bars indicate standard deviations.

B kit.

Comparison with Standard Proteins in Two Commercial Kits

Standard VTG protein supplied with each kit was measured using either the A kit (Fig. 1A) or the B kit (Fig. 1B). Protein concentrations as seen in the (X-axis) were used based on the method suggested by each kit. The standard curves generated by each kit using the two different VTG standards gave different slopes. The differences in the standard curves generated by the two kits could be based on either the characteristics of the antibody and/or differences in VTG concentrations in each kit.

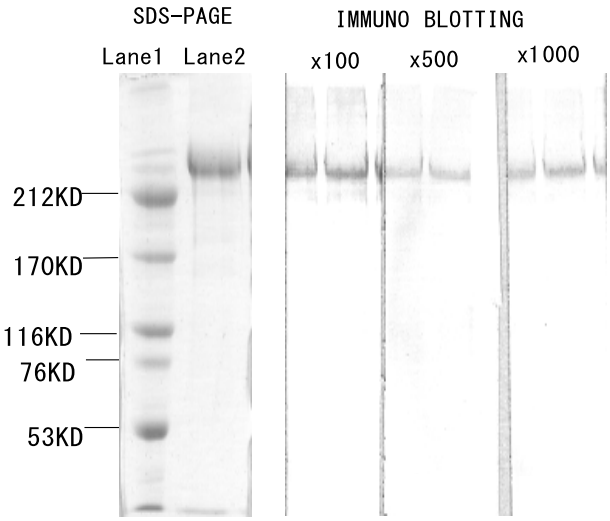

Fig. 2. SDS-PAGE and Immuno-Blotting of the Purified VTG Protein from Ascites of E_2-Exposed Medaka
Lane 1: Molecular marker proteins. Lane 2: Purified VTG protein. X100: Primary antibody (100 μg/ml) diluted 1 : 100. X500: Primary antibody (100 μg/ml) diluted 1 : 500. X1000: Primary antibody (100 μg/ml) diluted 1 : 1000.

Western Blot of NIES Standard VTG

SDS-PAGE of newly purified VTG protein is shown in Fig. 2. Western blot of this purified VTG using an antibody against VTG (Anti-Medaka vitellogenin IgG, Horseradish Peroxidase conjugate, EnBio) showed a single band of almost 200 kDa (Fig. 2). The primary antibody was diluted with TBS/Tween20/BSA. Anti-Mouse IgG+A+M Rabbit (Zymed) was used as secondary antibody diluted at 1 : 1000 with TBS/Tween20/BSA.

Ring Test of VTG Measurement of NIES-Produced Liver and Plasma Samples

The NIES VTG standard protein and the 30 plasma samples of unknown concentration from EE_2 exposed male medaka were delivered to 5 laboratories in Japan. They measured VTG levels in the 30 samples in duplicate using both kits with the NIES standard VTG protein. The within-laboratory CV's of the NIES standard VTG generated, following the use of both kits, are illustrated in Fig. 3. A statistically significant kit effect ($p < 0.05$) was detected, indicating lower CV's for the B kit than the A kit (Fig. 3).

The 30 unknown samples were analyzed by the 5 laboratories. The generated values were compared using VTG protein standard supplied with the kit (Fig. 4) or with the NIES standard VTG protein (Fig. 5). The coefficient of determination (R^2) from

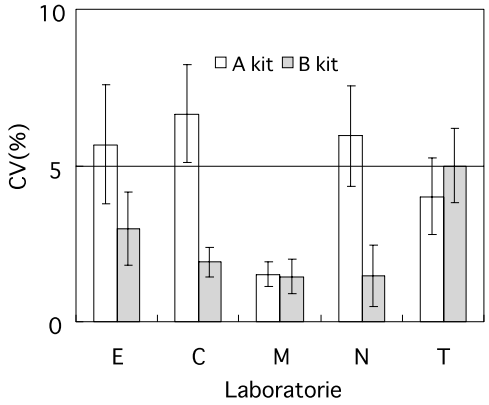

Fig. 3. Comparison of Within-Laboratory Coefficients of Variation (CV's) of VTG Measurements by the Two Types of ELISA Kits among Five Laboratories

Error bars indicate standard errors.

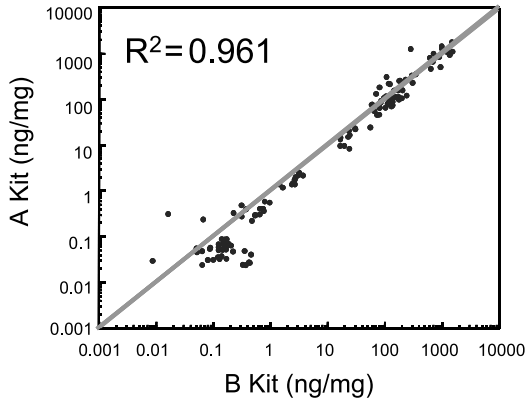

Fig. 5. Comparison of Two Types of VTG ELISA Kits Using the Gold Standard VTG

Data presented represents that obtained from the analysis of 30 unknown samples analyzed in 5 laboratories.

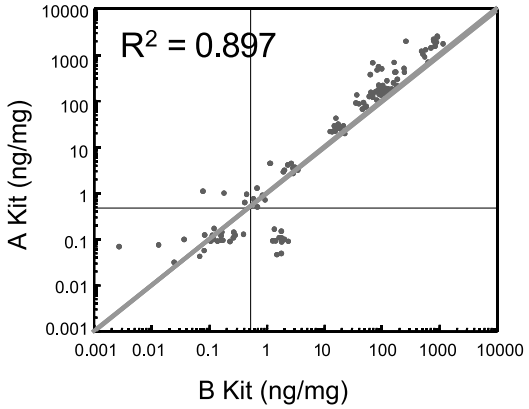

Fig. 4. Comparison of Two Types of VTG ELISA Kits Using the VTG Standard Provided with the Kit

Data presented represents that obtained from the analysis of 30 unknown samples analyzed in 5 laboratories.

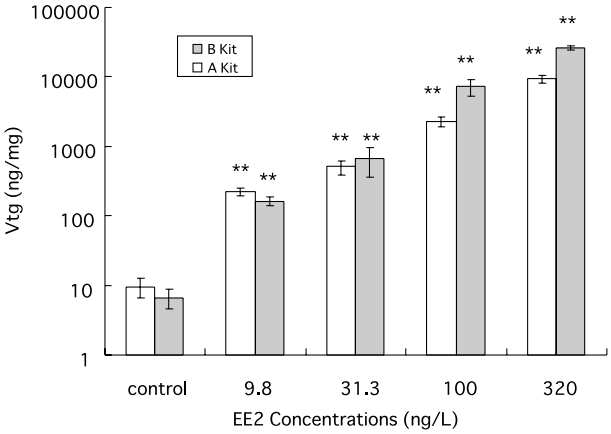

Fig. 6. VTG in Plasma of EE_2-Exposed Males Using Two Types of VTG ELISA Kits with the NIES VTG Standard Protein

Error bars indicate standard errors. $^{**}p < 0.01$ *vs.* controls.

the correlation using VTG standard supplied with the kits or the NIES standard was 0.897 and 0.961, respectively. Thus, both VTG ELISA kits generated similar values using the NIES standard VTG protein.

Measurement of VTG in Plasma and Liver

The concentration of VTG in plasma and liver of EE_2-exposed male medaka was measured using both types of kits with the NIES standard VTG; data are presented in Figs. 6 and 7, respectively. There was no difference in the VTG values generated by the kits. Statistically significant elevations of VTG in plasma and liver were found at 9.8 ng/l EE_2 exposure. The concentration of VTG measured in plasma was larger than that in liver (Figs. 6 and 7); however, VTG values from both materials showed the same tendency.

DISCUSSION

Two types of ELISA kits for the measurement of medaka VTG are commercially available. One is based on a polyclonal antibody whereas the other is based on a monoclonal antibody. In the process of establishing a test applicable for screening estrogenic chemicals in the OECD, a validated measurement method for VTG in fish is needed. The aim of this study, therefore, is to validate both available ELISA kits for medaka VTG and to standardize the mea-

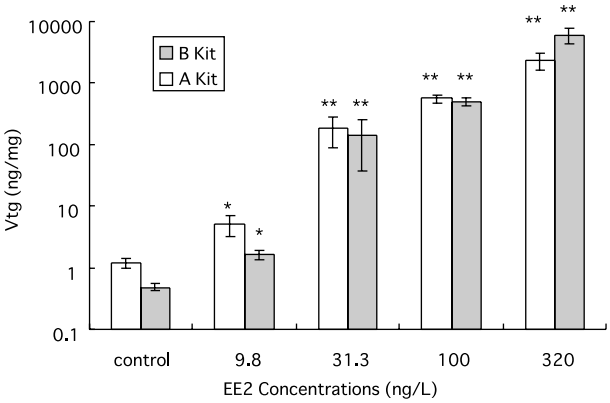

Fig. 7. VTG in Liver of EE_2-Exposed Males Using Two Types of VTG ELISA Kits with the NIES VTG Standard Protein.
$^*p < 0.05$, $^{**}p < 0.01$ *vs.* controls.

surement method using these kits.

Even with an ELISA system, small technique variations by a researcher can result in variation in the results. But as far as our results are concerned, within-laboratory CV's were not significantly different among laboratories. On the other hand, within-laboratory CV's of the B kit were significantly lower than those of the A kit. The process outlined in the B kit requires one step less than that described in the A kit. This difference might be reflected in the significant difference in within-laboratory CV's (Fig. 3).

A ring test measuring VTG concentration in 30 unknown samples using both ELISA kits with the NIES standard VTG protein was conducted, with 5 Japanese laboratories participating. The results revealed that each laboratory showed very low within-laboratory CV's and a very high correlation among the values generated using ELISA kits. A greater correlation was obtained using a single standard VTG protein newly purified by NIES. Both VTG ELISA kits gave almost the same VTG values when the NIES standard VTG protein was used. These results revealed that the small differences in the two VTG ELISA kits might originate from differences in the VTG standard supplied with each kit. Thus, we strongly recommend using one standard VTG protein — a gold standard — in future studies examining VTG when screening estrogenic chemicals using fish models.

We also compared blood samples and liver samples. Medaka are small, therefore, liver homogenate is easier to obtain than blood collection, which is difficult in fish this size. In the present study, VTG in plasma showed higher values than those in the liver. VTG induction by EE_2 could be detected in both the plasma and liver of male medaka, though the absolute value was less in the liver. As VTG values from both materials showed the same tendency, we recommend using liver homogenate for the measurement of VTG values in medaka.

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