MEASUREMENT OF SERUM TESTOSTERONE IN KACANG GOAT BY USING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TECHNIQUE: THE IMPORTANCE OF KIT VALIDATION

Pengukuran Testosteron Serum Kambing Kacang dengan Teknik Enzyme-Linked Immunosorbent Assay (ELISA): Pentingnya Validasi Kit

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ABSTRACT

This study was conducted to validate a commercial testosterone enzyme-linked immunosorbent assay (ELISA) kits (DRG EIA-1559) in analytic and biological manner for measuring serum testosterone concentrations in kacang goats. This study used 18 healthy kacang goats, six bucks (>2 years), six kids (<6 months), and six does (>2 years). Blood samples were collected from jugular vein and prepared as serum. Two validation tests were performed, an analytical validation comprises a parallelism, accuracy, precision and sensitivity and a biological validation by comparing testosterone concentration from bucks, kids, and does. Testosterone concentrations were measured using ELISA technique. Data of analytical validation were analyzed descriptively and test of equality of slope was performed to see the parallelism between samples and standard curves. Analysis of variance (ANOVA) was used for biological validation data. Results of parallelism showed that sample curve was parallel to the standard curve. Accuracy, precision (% CV of intra-and inter-assay) and sensitivity of the assay were 99.65±4.27%, <10%, <15% and 0.083 ng/ml, respectively. Results of biological validation showed that the assay used were accurately measured testosterone which testosterone concentrations in bucks were significantly higher compared to kids and does (P<0.05). In conclusion, a commercial testosterone ELISA kits (DRG EIA-1559) is a reliable assay for measuring serum testosterone concentration in kacang goats.

Key words: analytical and biological validations, ELISA, testosterone, kacang goat

ABSTRAK

Penelitian ini bertujuan melakukan validasi terhadap kit enzyme-linked immunosorbent assay (ELISA) testosteron komersial (DRG EIA-1559) secara analitik dan biologis untuk mengukur konsentrasi testosteron serum kambing kacang. Dalam penelitian ini digunakan 18 ekor kambing kacang yang sehat terdiri atas 6 ekor jantan dewasa (>2 tahun), 6 ekor jantan anak (<6 bulan), dan 6 ekor betina dewasa (>2 tahun). Sampel darah diambil dari vena jugularis dan dipreparasi menjadi serum. Uji validasi yang dilakukan terdiri atas validasi analitik (pararellism, akurasi, presisi dan sensitivitas) dan validasi biologis dengan membandingkan konsentrasi testosteron pada kambing jantan dewasa, anak, dan betina dewasa. Konsentrasi testosteron diukur dengan teknik ELISA. Data hasil uji validasi analitik dianalisis secara deskriptif dan uji kesamaan slope (test of equality of slopes) dan analisis sidik ragam (ANAVA) untuk data hasil validasi biologis. Hasil uji pararellism menunjukkan bahwa kurva sampel sejajar dengan kurva standar. Akurasi, presisi (% CV intra- dan inter-assay), dan sensitivitas berturut-turut adalah 99,65±4,27%; <15%; <10% dan 0,083 ng/ml. Hasil uji validasi biologis menunjukkan bahwa kit ELISA yang digunakan mampu mengukur testosteron dengan tepat dengan konsentrasi testosteron kambing jantan dewasa lebih tinggi dibandingkan anak kambing jantan dan betina dewasa (P<0,05). Kesimpulan dari penelitian ini adalah kit ELISA testosteron komersial (DRG EIA-1559) memiliki validitas yang baik untuk digunakan dalam mengukur konsentrasi testosteron pada serum kambing kacang.

Kata kunci: validasi analitik dan biologis, ELISA, testosteron, kambing kacang

INTRODUCTION

One of the major breeds of local goat in Indonesia is kacang goat with local name kambing kacang. Kacang goat is relatively small with a compact body frame, have erected ears and a short horn in both sexes (Sodiq and Sumaryadi, 2002). The body weight of an adult male is approximately 20-30 kg, whereas adult females are around 15-20 kg (Davendra and Burns, 1994; Mirdhayati et al., 2014). Average litter size of kacang goat is 2.06 kids per birth (Sodiq and Sumaryadi, 2002). Kacang goats can adapt well to the tropical environment with poor feeding condition. Therefore, this local goat had a good potency as resources of meat production. To improve its potency, it can be done through a selection of superior male goats. For this purpose, a study concerning with the male endocrine (e.g., testosterone) can be used as an indicator of a superior male goat.

Testosterone is the major androgen hormone in males and has a critical role in the process of spermatogenesis (sperm production) and expression of sexual behavior (Chedrese, 2009). Therefore, measurement of testosterone level can serve as the basis for the monitoring of male gonadal status. Measurement of testosterone can be performed by

using the enzyme-linked immunosorbent assay (ELISA) technique (Heistermann, 2010). The ELISA is relatively new technique and has several advantages compared to radioimmunoassay (RIA) technique particularly free of radioisotope waste (Hodges and Heistermann, 2011). Testosterone can be measured from plasma or serum using commercially available testosterone ELISA kit (Polat et al., 2011). However, these ELISA kits must be carefully validated analytically and biologically in species of interest (Todini et al., 2007; Todini et al., 2010; Bielohuby et al., 2012). This is due to many of these ELISA kits are designed for human hormone measurements.

Analytical validation comprises of specificity (cross-reactions), sensitivity, accuracy, precision, and parallelism of the dose-response relationship for the standard of the assay (Möstl et al., 2005). Additionally, it is also essential to determine whether the assay detects biologically meaningful changes in hormone levels (Möstl et al., 2005; Heistermann, 2010). In this respect, for testosterone measurement, it can be performed by comparing the concentration of testosterone from the different gonadal status of kacang goats (e.g. bucks, kids, does). Thus, this ELISA kit can be evaluated whether it can discriminate the variation of the testosterone concentration based on the physiological condition of kacang goats.

Several previous studies have been reported that commercial human ELISA kits can be used for measuring hormones in animals such as testosterone in rat (*Rattus norvegicus*) (Siregar et al., 2014), and estrogen and progesterone in aceh cattle (Amiruddin et al., 2013). However, data concerning the ELISA validation of these animals including kacang goat is still absent. The goal of this study was therefore to perform an analytical validation to evaluate that a commercial testosterone ELISA kit (DRG EIA-1559) can accurately measure testosterone levels in kacang goats, and to examine the reliability of a commercial testosterone ELISA kit (DRG EIA-1559) in discriminating gonadal status by comparing testosterone concentration from different reproductive stages (e.g., bucks, kids, and does).

MATERIALS AND METHODS

In total 18 healthy kacang goats comprises six bucks (>2 years), six kids (<6 months), and six does (>2 years or had previously giving birth) were used. Blood from those goats was collected and prepared as a serum. Serum was then used to validate a commercial testosterone ELISA kit (DRG EIA-1559) produced by DRG Instruments GmbH, Germany. The concentration of testosterone was measured using an ELISA technique.

Blood Collection and Preparation

In total 3 ml of blood was collected from jugular vein without anticoagulant. Blood was allowed to clot at ambient temperature. Serum was separated from the whole blood by centrifugation at 1200x g, 4° C for 10 minutes. Serum was then decanted into micro tubes and stored at -20° C until the time of analysis.

Analytical Validation

To examine the capability and precision of a commercial testosterone ELISA kits (DRG EIA-1559) for the quantifying concentration of testosterone in kacang goat, an analytical validation was performed. The procedure of this validation was adopted from previous studies (Pettitt et al., 2007; Rangel-Negrin et al., 2014). The analytical validation comprises of parallelism test, accuracy, and precision. Additionally, the sensitivity, specificity, and standard range were reported as provided by the manufacturer.

Parallelism Test

One serum from a buck was diluted (1:2 to 1:16) using assay buffer. Diluted serum was then assayed together with testosterone standard (serial dilution of testosterone standard 0.2-16 ng/ml). Afterward test of the equality of slope was performed following Pettitt et al. (2007) and Zar (1996) to compare the slope of expected dose versus percent bound of diluted serum with the slope of the standard dilutions.

Accuracy

One of testosterone standards with a known concentration of 4 ng/ml ("expected concentration") was taken as a control sample. The standard then assayed six times in a microtitreplates and concentration of testosterone was then measured as "observed concentration". Percentage of assay accuracy was calculated by using formula [(A/B)*100], where A is the observed concentration and B is the expected concentration.

Precision

Two quality-control standards (a low-quality control at c.a. 30% binding, and a high-quality control at c.a. 70 % binding) were used to measure intra- and inter-assay coefficients of variation (CVs). The quality control was assayed six times in a microtitreplates and testosterone concentrations were then measured. The % CVs was calculated by using formula [(SD/mean)*100].

Biological Validation

A biological validation was conducted to examine whether the commercially available testosterone ELISA kits (DRG EIA-1559) is sensitive enough to detect biologically meaningful differences in testosterone concentration from different reproductive stages. In total 18 serum from six bucks, six kids, and six does were used. The serum was then measured for testosterone concentrations using ELISA technique.

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Hormone Analysis

Hormone analysis was performed following the instruction from the manufacturer. In brief, duplicate 25 µl aliquots of serum were assayed along with 25 µl aliquots standard (dose range 0.2-16 ng/ml) and control

on microtitreplates coated with a monoclonal mouse antibody of testosterone. Afterwards, 200 µl enzyme conjugate was added to each well and the mixture incubated for 60 minutes at room temperature. Following incubation, the plates were washed four times with washing solution and blotted dry. After that, 200 µl substrate solution (tetramethylbenzidine) was then added to each well. The plates re-incubated for 15-20 minutes at room temperature. The enzyme reaction was stopped with 100 µl 0.5 M H_2SO_4 in each well. Finally, absorbance was measured at 450 nm on an automatic plate reader (ELISA reader). The testosterone was calculated automatically using MPM6 program.

Data Analysis

Data of accuracy and precision were analyzed descriptively, whereas data of parallelism was analyzed using the test of equality of slope to see the parallelism between samples and standard curves (Zar, 1996; Pettitt et al., 2007). The concentration of testosterone from biological validation was analyzed using one-way analysis of variance (ANOVA). All statistical tests were two-tailed and statistical significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Analytical Validation

Prior to hormone analysis, an assay must be analytically validated for each given species under investigation. This includes certain criteria, such as parallelism, accuracy, precision, specificity (cross-reactions), and sensitivity (Möstl et al., 2005). The results showed that serial dilution of serum kacang goat was significantly decreased following to the dilution level (D-R curve: $r^2 = 0.977$, $p = 0.012$ Fig. 1). Moreover, the slope of the serial dilution of serum testosterone was also parallel to the respective standard curves ($t = -0.52$, $p = 0.9502$, Table 1). Those results were indicating that antigen testosterone in serum kacang goat, can bound correctly with the antibody of this assay.

Parallelism is one of the analytical validation tests which is performed to determine if the assay is actually what it should be measuring. Additionally, it can also show what dilution of sample to use for the assay. It is a critically needed to perform this test to evaluate the optimal dilution factor for samples. For this, serum of kacang goats should be run at 1:2 dilution based on the binding linear range at 30-70% (Table 1). However, for

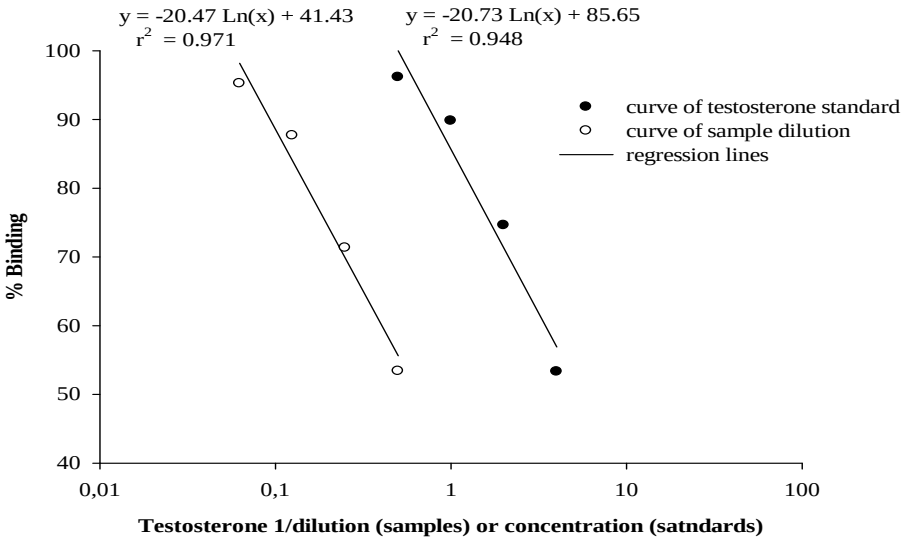

Figure 1. Serial dilution results from a buck kacang goat serum are presented. The sample (open symbol) was diluted 1:2, 1:4, 1:8 and 1:16 in assay buffer and tested for binding with the testosterone conjugate antibody in parallel with serially diluted standard (closed symbol). Regression equations derived from linear portion of the curves produced almost similar r^2 value for samples and standard (0.971 and 0.948, respectively)

Table 1. Analytical validation of a commercial testosterone ELISA kit (DRG EIA-1559)

Parameters measured	Result
Parallelism	YES; t= -0.52 , p= 0.9502
Dose-respone curve (N)	y= -94.94x + 99.5 (4) r^2= 0.977 , p= 0.012
Accuracy ± SD % (N)	99.65±4.27% (6)
Intra-assay CV (%)	
Low control (N)	5.8% (6)
High control (N)	4.6% (6)
Inter-assay CV (%)	
Low control (N)	8.7% (6)
High control (N)	6.3% (6)
Sensitivity	0.083 ng/ml
Standard range	0.2-16 ng/ml
Dilution of samples	1:2 (50 µl serum + 50 µl assay buffer)

the special cases of kacang goats (having lower/higher concentration of testosterone), it must be tested first. Parallelism is sometimes given as a parameter of specificity of an immunoassay (Möstl et al., 2005). However, parallelism test is not a marker of specificity of the assay, but is a proof of a dose–response relationship (Möstl et al., 2005). The specificity of antibodies (cross-reactivity) of this assay as described by manufacturer is testosterone 100%, 5α-dihydrotestosterone 0.8%, androstenedione 0.9%, 11β-hydroxyestosterone 3.3%, 17α-methyltestosterone 0.1%, 19-nortestosterone 3.3%, and < 0.1% for epitestosterone, oestradiol, progesterone, cortisol, oestrone, and danazol.

Accuracy and precision are other important parameters to consider when doing hormone analysis especially using commercially available ELISA kit that it is not specifically designed for animals. The mean percentage of accuracy for this assay was 99.65±4.27% whereas, intra-and inter-assay coefficients of variation (% CV) of high and low value quality controls for the assay precision were below 10% and 15% respectively. Those results indicating that the accuracy and precision of assay were acceptable for measuring serum testosterone in this species (Bayemi et al., 2007; Heisterman, 2010; Bielohuby et al., 2012). Both accuracy and precision reflect how close a measurement is to the actual value. Accuracy refers to the degree to which the measured concentration corresponds to the true concentration. It can be used to test for potential interference caused by interfearing substances contained with in the biological sample that may interfere a binding of antigen and antibody (Goymann, 2005; Gholib et al., 2014). Based on accuracy results indicating that there were no substances in the serum that interfered with the binding of the assay antibody. Together with dose-response, parallelism, accuracy, and precision (Table 1) suggest that this assay was analytically reliable for serum testosterone measurements in this species.

Biological Validation

It is difficult to select the best-suited assay to assess male gonadal activity only based on the analytical validation. To achieve that, analytical validation must be equipped with a biological validation as well (Gholib et al., 2014). Through a biological validation, it can be examined if the assay is used for measuring a hormone profile that accurately reflects the physiological event of interest. In accordance with this, we found that testosterone concentration in bucks was higher compared to kids and does (317.5% and 614.8%, respectively). The mean (± SD) of testosterone concentration in bucks, kids, and does was 7.25±1.45 ng/ml, 1.62±0.72 ng/ml, 0.95±0.77 ng/ml, respectively. Based on the results ANOVA showed that testosterone concentration in bucks was significantly higher compared to kids and does ($P<0.05$). Variation of testosterone concentrations was indicating that the assay capable to discriminate testosterone concentration that appropriate with their reproductive status which meaningful biologically. On the other hand, a singnificant higher of testosterone concentration in bucks may reflect to gonadal activities. After puberty, the hypothalamus can produce more and more GnRH and thus initiated the pituitary to release more and more LH which will stimulate the Leydig cells to produce testosterone (Cheldrese, 2009).

The principle of test of this ELISA kit is based on the competitive binding system, called a single competitive assay (Heisterman, 2010; Bielohuby et al., 2012). The principle test is endogenous testosterone in samples (serum/plasma) that filled into the microtitreplates together with a testosterone horseradish pereroxidase conjugate compete for binding to the testosterone antibody coated in microtitreplates. After incubation the unbound conjugate is washed off. Substrate solution was then added and the color intensity developed which is reverse proportional to the concentration of testosterone in samples.

Validation of human commercial ELISA kit was reported by Todini et al. (2007). They had validated two ELISA kits, estradiol (DRG, EIA-2693) and inhibin-A (DSL-10-28100, Diagnostic Systems Laboratories Inc., USA) for measuring the concentration of 17β estradiol and inhibin-A in plasma buffalo. Results showed that both of the ELISA KIT used are capable and have a good validity for measuring both hormones. Other validation tests were also been reported by other researchers to measure progesterone in cattle (Bayemi et al., 2007), and thyroid hormone in donkey (Todini et al., 2010).

Testosterone concentrations of bucks measured in this study were higher compared to 2-year-old white goats, 4.30±0.47 ng/ml (Polat et al., 2011) and etawah crossbreed goats, 6.82±4.18 ng/ml (Rachmawati et al., 2013). However, it was lower compared with kejobong goats (12.00±6.56 ng/ml), and bligon (9.23±4.73 ng/ml) (Rachmawati et al., 2013). Differences of testosterone concentration among them can be caused by differences in age, sexes, and season. Another thing to be concerned when using commercially available ELISA KIT is a different brand and even different batch may be yielding a different hormone value of the same samples. This might be the results of different antibody used, a different set of the standard used and different chemical reagent (between different brands) which can result in different hormone value. Therefore, for hormonal analysis using ELISA KIT it is better to stay with one manufacturer brand and within the same production batch if possible particularly when doing same samples. Moreover, direct comparison of results (absolute hormone value) between the different brand of the assay should be avoided (Heistermann, 2010).

CONCLUSION

Based on the results, a commercially available testosterone ELISA kits (DRG EIA-1559) was a reliable assay for measuring serum testosterone concentration in kacang goats.

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