



RESEARCH ARTICLE

REVISED The validation of a commercial enzyme-linked immunosorbent assay and the effect of freeze-thaw cycles of serum on the stability of cortisol and testosterone concentrations in Aceh cattle [version 2; peer review: 1 approved, 1 approved with reservations]

Gholib Gholib ¹, Sri Wahyuni², Muslim Akmal³, Muhammad Hasan⁴, Muhammad Agil⁵, Bambang Purwantara⁵

¹Physiology Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Aceh, 23111, Indonesia

²Anatomy Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Aceh, 23111, Indonesia

³Histology Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Aceh, 23111, Indonesia

⁴Clinical Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Aceh, 23111, Indonesia

⁵Department of Veterinary Clinic Reproduction and Pathology, Bogor Agricultural University, Bogor, Jawa Barat, 16680, Indonesia

v2 First published: 30 Jul 2019, 8:1220 (<https://doi.org/10.12688/f1000research.19804.1>)

Latest published: 04 Feb 2020, 8:1220 (<https://doi.org/10.12688/f1000research.19804.2>)

Abstract

Background: To obtain accurate measurements of cortisol (C) and testosterone (T) in Aceh cattle, commercial enzyme-linked immunosorbent assay (ELISA) kits need to be carefully validated. Moreover, repeated freeze-thaw cycles during the storage of the samples may affect the stability of the hormones in the serum. Here, the reliability of C and T concentration measurements in the serum of Aceh cattle, was tested using commercial C and T ELISA kits designed to measure human C and T concentrations. Further, the effect of repeated freeze-thaw cycles on the stability of C and T concentrations in the serum was evaluated.

Methods: Commercial C (Cat. no. EIA-1887) and T (Cat. no. EIA-1559) ELISA kits from DRG Instruments GmbH were validated through an analytical validation test (i.e., parallelism, accuracy, and precision) and a biological validation test (for C: effect of transportation on the C excretion; for T: the concentrations of T between bulls and cows). To test the effects of freeze-thaw cycles, cattle serum was subjected to the following treatments: (i) remained frozen at -20°C (control group); (ii) exposed to freeze-thaw cycles for two, four, six, and eight times (test groups).

Results: Parallelism, accuracy, and precision tests showed that both C and T ELISA kits adequately measured C and T in the serum of Aceh cattle. Concentrations of C post-transportation were significantly higher than pre-transportation ($p < 0.05$). Concentrations of T in bulls were significantly higher than in cows ($p < 0.05$). After four to eight freeze-thaw cycles, C concentrations were significantly lower compared to the control group (all $p < 0.05$). In contrast, T concentrations remained stable (all $p > 0.05$).

Conclusions: Commercial C (EIA-1887) and T (EIA-1559) ELISA kits are

Open Peer Review

Reviewer Status

	Invited Reviewers	
	1	2
version 2 (revision) 04 Feb 2020		
version 1 30 Jul 2019	report	report

1 **Pudji Astuti**, Gadjah Mada University (UGM), Yogyakarta, Indonesia

2 **Annaïs Carbajal** , Autonomous University of Barcelona, Barcelona, Spain

Any reports and responses or comments on the article can be found at the end of the article.

reliable assays for measuring serum C and T, respectively, in Aceh cattle. Repeated freeze-thaw cycles significantly affected the stability of serum C, but did not for T.

Keywords

aceh cattle, enzyme-linked immunosorbent assay, cortisol, testosterone, analytical validation, biological validation, hormone stability, repeated freezing and re-thawing

Corresponding author: Gholib Gholib (gholib@unsyiah.ac.id)

Author roles: **Gholib G:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Writing – Original Draft Preparation, Writing – Review & Editing; **Wahyuni S:** Conceptualization, Funding Acquisition, Investigation, Methodology, Writing – Review & Editing; **Akmal M:** Data Curation, Methodology, Writing – Review & Editing; **Hasan M:** Data Curation, Resources; **Agil M:** Supervision, Writing – Review & Editing; **Purwantara B:** Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This study was funded by The Ministry of Research, Technology and Higher Education, Indonesia [215/SP2H/LT/DPRM/2019]. This paper also supported by USAID through Sustainable Higher Education Research Alliances (SHERA) Program - Center for Collaborative Research Animal Biotechnology and Coral Reef Fisheries (CCR ANBIOCORE).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2020 Gholib G *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Gholib G, Wahyuni S, Akmal M *et al.* **The validation of a commercial enzyme-linked immunosorbent assay and the effect of freeze-thaw cycles of serum on the stability of cortisol and testosterone concentrations in Aceh cattle [version 2; peer review: 1 approved, 1 approved with reservations]** F1000Research 2020, 8:1220 (<https://doi.org/10.12688/f1000research.19804.2>)

First published: 30 Jul 2019, 8:1220 (<https://doi.org/10.12688/f1000research.19804.1>)

REVISED Amendments from Version 1

The major differences between this version (V2) and the previously published version (v1) are that we have divided the objective in three: analytical validation, biological validation, and freeze-thaw cycles and added conclusion related to the biological validation. Moreover, the discussion related to the effect of repeated freeze-thaw cycles on testosterone concentrations was corrected; testosterone concentration is not significantly affected by freeze-thaw cycles. For this version, additional samples were collected to add the number of samples for cortisol and testosterone biological validations. In this version, data were collected from 32 Aceh cattle (16 bulls and 16 cows). In the results section, the detail explanations about parallelism results have also added. Table 2 (Concentration of cortisol and testosterone from serum exposed to repeated freeze-thaw cycles) was deleted. The minor comments and suggestions from the reviewers have been answered and the article has been changed accordingly.

Any further responses from the reviewers can be found at the end of the article

Introduction

Cortisol (C) and testosterone (T) are steroid hormones. Cortisol is produced by the adrenal cortex¹, while T is produced by the Leydig cells in the testes² and also secreted in the adrenal cortex and ovaries in the small amounts³. Cortisol has a key role in physiological stress responses. Accordingly, this hormone is commonly used as an indicator of stress⁴. On the other hand, T plays an important role in male reproduction physiology. Thus, T concentrations can be used to assess male gonadal function². Measuring C and T concentrations can be used to monitor stress and reproduction in order to support the breeding management, because high concentrations of C can suppress the T production².

Steroid hormones can be measured by an enzyme-linked immunosorbent assay (ELISA) technique using various samples (e.g., plasma or serum⁵, urine⁶, feces^{7,8}, saliva^{9,10}, and hair¹¹). This technique is now widely used, as it is simple, rapid, convenient, relatively inexpensive, requires a lower sample volume, and particularly, as it is free of radioisotope waste^{12,13}. Moreover, the availability of commercial ELISA kits makes it easy to apply this technique. However, because many commercial ELISA kits are usually designed for humans, using them for animals must be done with great caution and measurements are only trustworthy after the validation of the assay¹⁴.

In order to reliably measure C and T concentrations in cattle using a human commercial ELISA kit, the ELISA kit needs to be validated analytically, physiologically or biologically^{5,15}. Analytical validation can be performed by examining the specificity (cross-reactions), sensitivity, precision, and parallelism (linearity) of the ELISA kit¹⁵. In addition, it is crucial to perform the biological validation of the assay to examine the ability of the assay to differentiate the variation of the hormone concentration based on the physiological conditions of the

animals¹⁵. The biological validation of T measurements can be achieved by comparing the concentrations of T from individuals of different age (juvenile versus adult), or sex (male versus female) classes⁵. For C measurements, the comparison of C concentrations of the same animal before and after some known stressful events (eg., capture, translocation, transportation, and agonistic interactions) can be used as a biological validation procedure¹⁵.

Another critical issue for hormone measurements is the repeated freezing and re-thawing of the samples (freeze-thaw cycles) during the storage prior to the analyses¹⁶. Freezing the serum or plasma at -20°C or lower is an ideal storage method¹⁷. However, power outages frequently occur, particularly in developing countries, and they can last for a few hours up to a day. Manuals of commercial ELISA kits always explicitly warn to avoid freeze-thaw cycles, because repeated freezing and re-thawing of the samples may affect the stability of the hormones in the serum or plasma, for example: insulin in rats¹⁸; adrenocorticotrophic hormone in humans¹⁹; and sex hormone-binding globulin (SHBG), progesterone, estrone, estradiol, and dehydroepiandrosterone sulfate (DHEAS) in humans^{20,21}. Conversely, several studies have reported no effect of repeated freeze-thaw cycles on the stability of hormone concentrations, such as progesterone in female dogs (bitches)²² and DHEAS, C, dihydrotestosterone, T, estradiol, and progesterone in humans^{23,24}. These different results suggest that the stability may depend on the number of cycles, duration of cycles, and temperature during the repeated freezing and rethawing, species-specific differences, as well as on the type of hormones measured.

In 2011, the Ministry of Agriculture of Indonesia declared Aceh cattle to be a native Indonesian genome resource. This type of cattle can adapt well to the tropical environment and is important for meat production. Increasing the Aceh cattle population is very important for the fulfillment of the protein requirements of the human population in the Aceh region. In this respect, the measurement of C and T can be used for informing husbandry management and breeding programs for this animal. In preparation for the first study to monitor reproduction and stress physiology of Aceh cattle, the reliability of commercial C and T ELISA kits designed for human serum/plasma was tested to measure C and T concentrations in the serum of Aceh cattle. The objectives of the present study were: First, conducting an analytical validation test to examine the reliability of commercial C (Cat. no. EIA-1887) and T (Cat. no. EIA-1559) ELISA kits that accurately measure C and T concentrations, respectively in Aceh cattle; Second, performing a biological validation test to examine the reliability of commercial C ELISA kit (Cat. no. EIA-1887) to the response a stress event by comparing C concentrations before and after transportation and the reliability of T ELISA kit (Cat. no. EIA-1559) ELISA kits in discriminating the T concentrations from different sexes (bulls versus cows). The hypothesis is that C concentrations should be higher following transportation and the T concentration should be higher in bulls. First, the

stability of C and T concentrations in the serum of Aceh cattle after exposure to several repeated freeze-thaw cycles was evaluated.

Methods

Ethical statement

The Institutional Committee of Animal Ethics of the Faculty of Veterinary Medicine, Universitas Syiah Kuala approved the use of all experimental animals in this study (Ref: 33/KEPH/VI/2019)²⁵ (see *Extended data*). All efforts were made to ameliorate harm to the animals, such as: cattle were placed in the clamp cage to ensure quick, easy and safe collection of the blood sample causing minimal distress; blood samples were collected via the jugular vein without sedation, causing minimal distress. During the study, a member of the study team, accompanied by a veterinarian, observed their behavior for signs of excessive distress or sickness. None of the procedures performed in this study resulted in distress, sickness behavior or weight loss.

Study animals

Samples were collected from 32 adult Aceh cattle consisting of 16 adult males (bulls), and 16 adult females (cows). The age of the cattle ranged from two to five years old, and weighing 150–300 kg. Samples were collected during two weeks in April 2019 and three weeks in November 2019. For the biological validation test of T ELISA kit, all cattle (16 bulls and 16 cows) were used, whereas for the biological validation of C ELISA kit, 16 cattle (8 bulls and 8 cows) were used which is taken from the 32 Aceh cattle above after completion of the biological validation test of testosterone. This sample size was calculated based on Federer's formula: $(t-1)(n-1) \geq 15$, where t is the number of treatments and n is the number of animals. The sample size calculated using this formula is 16. 16 bulls and 10 cows were sampled from a smallholder farmer at Darussalam, Aceh Besar, Aceh Province. These cattle were housed in stables, with each cattle was separated by a wooden partition (individual housing system). Six cows were sampled from a teaching farm (UPT hewan coba), Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh. These cows were housed together in stables (colony housing system). Cattle housing was equipped with feed and water troughs. Feed and water were consumed by the cattle *ad libitum*. Feed given to the cattle is grass at 10% of body weight and 1% of body weight of rice bran and tofu dregs.

Collection and processing of blood samples

Blood samples (around 5 ml per animal) were collected inside on the farm by a veterinarian from the jugular vein using standard operating procedure without sedation. For the biological validation of C, blood samples were collected in the afternoon (16.00 to 18.00), four hours after transportation, while for the biological validation of T, blood samples were collected in the morning (06.00 to 08.00). Afterward, the blood was allowed to clot at room temperature for between 30 minutes and 1 hours. Then, the serum was separated from the red blood cells by centrifugation at 1200xg for 10 minutes at 4°C. After that, the supernatant (serum) was immediately transferred to

polypropylene tubes (Eppendorf Safe-Lock tubes) and stored at -20°C until hormone measurements.

Validation of commercial C and T ELISA kits

To test the capability of the commercial C and T ELISA kits in quantifying concentrations of T and C in the serum of Aceh cattle, the commercial C (Cat. no. EIA-1887) and T (Cat. no. EIA-1559) ELISA kits produced by DRG Instruments GmbH, Germany, were evaluated using analytical and biological validations. The procedure for assay validation was conducted as described by Rangel-Negrin *et al.*¹⁵ and Gholib *et al.*⁵. Briefly, the analytical validation was comprised of parallelism (i.e. running serial dilution of Aceh cattle serum 1:2 to 1:16, assayed together with C and T standards, and comparing the slope of expected dose versus percent bound of diluted Aceh cattle serum with the slope of C and T standards), accuracy (i.e. adding known quantities of hormone to C and T standards and calculating the percentage of recovery), and precision (i.e., measuring some low-quality controls (QC L) and high-quality controls (QC H) in one microplate to calculate intra-assay coefficients of variation (CV) and some QC L and QC H in several microplates to calculate inter-assay CV. The sensitivity was reported as provided by the manufacturer.

For the biological validation of C, we examined the effects of transportation on the C excretion. We used 16 of the Aceh cattle described above (eight bulls and eight cows). Bulls and cows were transported using different road vehicles (open car) around Banda Aceh at 11.00 a.m for an hour (~40 km/hour). During transportation, cattle were secured using a rope around their neck and bulls and cows were transported using different vehicles. For pre-transportation samples, blood samples were collected a day before transporting the cattle at 06.00 to 08.00 to prevent stress. For post-transportation samples, blood samples were collected four hours after transportation at 16.00 to 18.00. Blood samples were then processed as serum as described above. For the biological validation of T, we compared the concentrations of T from 16 bulls and 16 cows. For this purpose, blood samples were collected in the morning at 06.00 to 08.00.

Experiment design of freeze-thaw cycles

To evaluate the stability of C and T concentrations in serum exposed to repeated freeze-thaw cycles, we took seven serum samples (three bulls and four cows) collected from the previous experiment (biological validation of C and T) to be used for the freeze-thaw cycle experiment, in order to avoid several blood sample collections. Each serum sample was then divided into five aliquots and transferred into 1.5 ml micro-tubes (Eppendorf Safe-Lock tubes; total 35 aliquots; 0.2 ml per tube), closed tightly and sealed with parafilm. All aliquots were subsequently stored frozen at -20°C. Later, those aliquots were subjected to the following treatments: (i) aliquots remained frozen at -20°C until the time of hormone analysis as a control group (N=7); and (ii) aliquots were exposed to repeated freeze-thaw cycles for two, four, six, and eight cycles as test groups (N=7 for each group). The serum was thawed for six hours by placing the tube in a room without an air conditioner (mean \pm standard deviation of

temperature $27.6 \pm 0.7^\circ\text{C}$). Afterward, the serum was refrozen for 24 hours prior to re-thawing. After all freeze-thaw cycles were completed, C and T concentrations were measured for all serum samples together.

C and T concentration measurements

The concentration of C was measured using a commercial C ELISA kit (Cat. no. EIA-1887, DRG Instruments GmbH, Germany). The assay utilizes a monoclonal anti-cortisol antibody and C labeled with horseradish peroxidase as an enzyme conjugate. The measurement of C was conducted following the instructions of the manufacturer (DRG diagnostics). In brief, 20 μl of each standard, control, and samples (serum) in duplicate were dispensed with new disposable tips into appropriate wells on a microplate coated with C monoclonal antibody. After that, each microplate well was filled with 200 μl enzyme conjugate, thoroughly mixed, and then incubated for 60 minutes at room temperature. After incubation, the solution in the wells was briskly shaken out and rinsed out with 350 μl diluted washing solution per well four times. Furthermore, 100 μl substrate solution (tetramethylbenzidine) was added to each well of the microplate, which was then re-incubated for 15–20 minutes at room temperature. After that, the enzymatic reaction was stopped by adding 100 μl of stop solution (0.5 M H_2SO_4) to each well. Finally, absorbance was determined by using an ELISA reader (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories Inc.) at 450 nm. The C concentration was then calculated using the Microplate Manager® 6 Software (Bio-Rad Laboratories Inc.).

The concentration of T was measured using a commercial T ELISA kit (Cat. no. EIA-1559, DRG Instruments GmbH, Germany). The assay utilizes a mouse monoclonal anti-testosterone antibody and T labeled with horseradish peroxidase as an enzyme conjugate. This assay has been previously validated successfully for measuring T concentrations in Kacang goats⁵. Testosterone measurements were conducted following the instructions of the manufacturer (DRG diagnostics) and as described by Gholib *et al.*⁵. In brief, 25 μl of each standard, control, and samples (serum) in duplicate were dispensed with new disposable tips into appropriate wells on a microplate coated with T monoclonal antibody. After that, each microplate well was filled with 200 μl enzyme conjugate, thoroughly mixed, and then incubated for 60 minutes at room temperature. Following this, the microplates were treated and absorbance was measured as described above for C.

Data analysis

Normality distribution of the data was tested using the Shapiro-Wilk test prior to statistical analysis. For the analytical validation, the parallelism between serial dilutions of two selected serum samples and standard curves was determined by a test of equality of two slopes using Logarithmic Regression²⁶. For the biological validation, C data before and after transportation and T data of bulls and cows showed a normal distribution ($p > 0.05$). A paired t-test was used for C, whereas for T, an independent t-test was used. For the freeze-thaw cycles experiments, the proportion of change in C and T concentrations

relative to the control was calculated as $(a_n - x_n/x_n) \times 100$, where a_n is the nth sample value in each freeze-thaw cycles series (two, four, six, eight times) and x_n is the value at time zero (control) of the nth sample^{8,16}. The C data were normally distributed ($p > 0.05$), whereas the T data was not ($p < 0.05$). Therefore, for C, a one-way repeated measures ANOVA followed by *post hoc* analysis using the Bonferroni test was conducted, whereas a Friedman repeated-measures ANOVA on ranks was set up to analyze T concentrations⁸. We used SigmaPlot 11.0 to create graphs and IBM SPSS 20 to carry out the statistical analysis. All statistical tests were two-tailed and the significance level was set at 0.05.

Results

The validity of commercial ELISA kits for measuring C and T in the serum of cattle

The serial dilution of the selected serum (sample one and sample two) showed displacement curves that were parallel to C and T standard curves (Figure 1). From dose response curve, the value of slope on C standard, sample one, and sample two were -20.106, -18.927, and -19.433, respectively. Based on the test of equality of two slopes showed that slope of C standard was parallel or not significantly different with slope of sample one ($t = -0.218$, $p = 0.462$) and slope of sample two ($t = -0.674$, $p = 0.443$; Table 1). Similar results obtained for T which slope of T standard ($b = -21.480$) was parallel with slope of sample one ($b = -17.373$, $t = -0.067$, $p = 0.332$) and slope of sample two ($b = -21.480$, $t = 0.376$, $p = 0.212$; Table 1). The parallelism, dose-response, accuracy, and precision (coefficients of variation/CV of intra-and inter-assay) are presented in Table 1.

The mean (\pm SD) of the C concentrations in the samples post-transportation (34.81 ± 9.22 ng/ml) was significantly higher compared to pre-transportation samples (27.83 ± 9.1 ng/ml; $t = -4.670$, $p = 0.000$; Figure 2A). The mean (\pm SD) of the T concentrations in bulls (4.39 ± 1.41 ng/ml) was significantly higher than the T concentrations in cows (0.63 ± 0.24 ng/ml; $t = 10.552$, $p = 0.000$; Figure 2B). Results on the analytical and biological validations indicated that both C and T ELISA kits were reliable assays for measuring C and T concentrations in Aceh cattle. Raw values of analytical validation results are given in Dataset 1²⁷, whereas raw values of biological validation results are given in Dataset 2 v2²⁸ (see Underlying data).

C and T stabilities after exposure to repeated freeze-thaw cycles

Repeated freeze-thaw cycles in serum significantly affected the stability of C concentrations ($F[4,30] = 11.681$, $p < 0.001$, $N=7$). *Post hoc* analysis showed that after four to eight freeze-thaw cycles, the C concentrations were significantly lower than those of the control group (all $p < 0.05$; Figure 3A). In contrast to the C concentrations, the T concentrations remained stable after exposure to two to eight freeze-thaw cycles ($\chi^2[4] = 7.626$, $p = 0.106$, $N = 7$; Figure 3B). The mean percentage of change in C and T concentrations ranged between 13.43 to 33.94% and 3.55 to 8.33%, respectively, relative to the control group (Figure 3). Raw values of C and

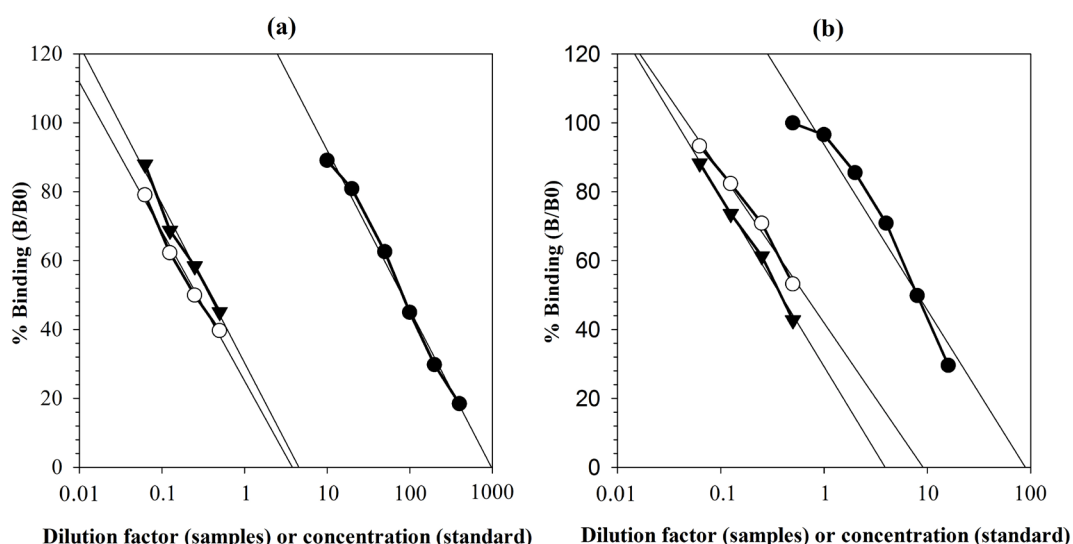


Figure 1. Curves of parallelism test from the serial dilution of tested samples (serum of Aceh cattle) and cortisol and testosterone standards are presented. a) Cortisol (C) enzyme-linked immunosorbent assay (ELISA) kit: sample one (white circle) and sample two (black triangle down) were diluted 1:2 to 1:16 in assay buffer and measured with serial C standards (black circle) ranging from 10 to 400 ng/ml. The curve of sample one, sample two, and C standards produced almost identical R^2 values: 0.987, 0.945, and 0.992, respectively. **b)** Testosterone (T) ELISA kit: sample one (white circle) and sample two (black triangle down) were diluted 1:2 to 1:16 in assay buffer and measured with serial T standards (black circle) ranging from 0.5 to 16 ng/ml. The curve of sample one, sample two, and T standards produced almost identical R^2 -values: 0.964, 0.993, and 0.945, respectively.

Table 1. Results of the analytical validation of the commercial cortisol (DRG, Cat. No. EIA-1887) and testosterone (DRG, Cat. No. EIA-1559) enzyme-linked immunosorbent assay (ELISA) kits.

Measured parameters	Cortisol ELISA kit	Testosterone ELISA kit
Parallelism		
- Standard with sample one	Parallel, $t = -0.218$, $p = 0.833$	Parallel, $t = -0.067$, $p = 0.948$
- Standard with sample two	Parallel, $t = -0.674$, $p = 0.519$	Parallel, $t = 0.376$, $p = 0.716$
Dose response curve		
- Sample one	$y = -18.827 \ln(x) + 25.050$ $R^2 = 0.987$, $p = 0.006$	$y = -17.373 \ln(x) + 43.792$ $R^2 = 0.964$, $p = 0.018$
- Sample two	$y = -19.433 \ln(x) + 30.350$ $R^2 = 0.945$, $p = 0.028$	$y = -21.480 \ln(x) + 29.218$ $R^2 = 0.993$, $p = 0.003$
- Standard	$y = -20.106 \ln(x) + 138.345$ $R^2 = 0.992$, $p = 0.005$	$y = -21.584 \ln(x) + 95.239$ $R^2 = 0.945$, $p = 0.004$
Accuracy \pm SD (%) (N=6)	104.89 \pm 7.09	103.37 \pm 9.62
Coefficient variation (CV) of intra-assay (%)		
- Low quality control (N=6)	8.40	7.32
- High quality control (N=6)	4.86	6.62
Coefficient variation (CV) of inter-assay (%)		
- Low quality control (N=6)	12.45	10.70
- High quality control (N=6)	6.80	8.91
Sensitivity (ng/ml) ^a	2.5	0.083

^a = value in manual protocol of assay from manufacturer

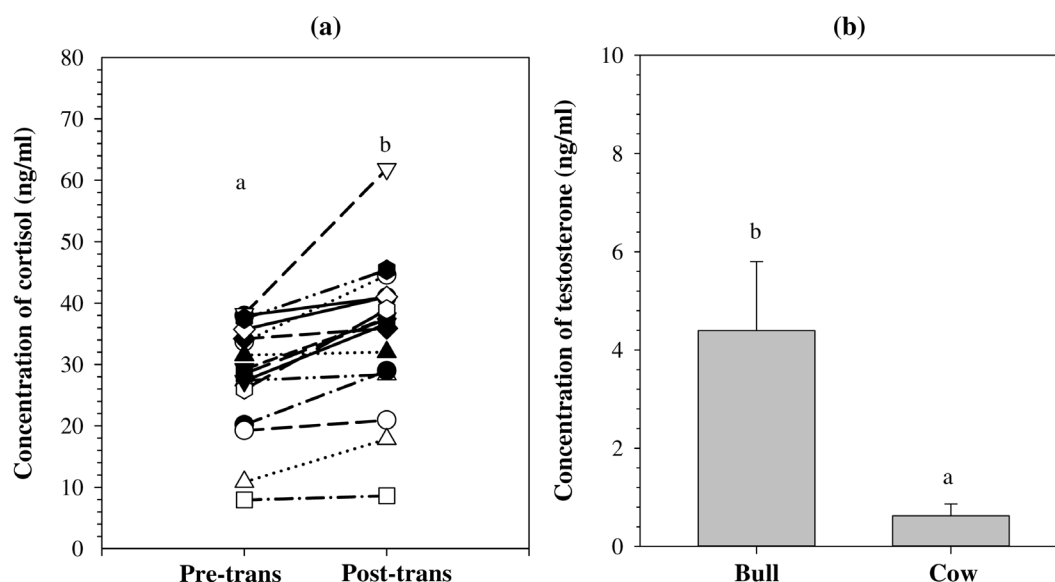


Figure 2. Results of the biological validation of the commercial enzyme-linked immunosorbent assay (ELISA) kits. a) Concentrations of cortisol (C) before (pre-trans) and after (post-trans) transportation measured using a commercial C ELISA kit (DRG, Cat. No. EIA-1887). **b)** Concentrations of testosterone (T) in bulls and cows measured using a commercial T ELISA kit (DRG, Cat. No. EIA-1559). Different superscripts above line symbols and histogram indicate a significant difference between groups ($p < 0.05$).

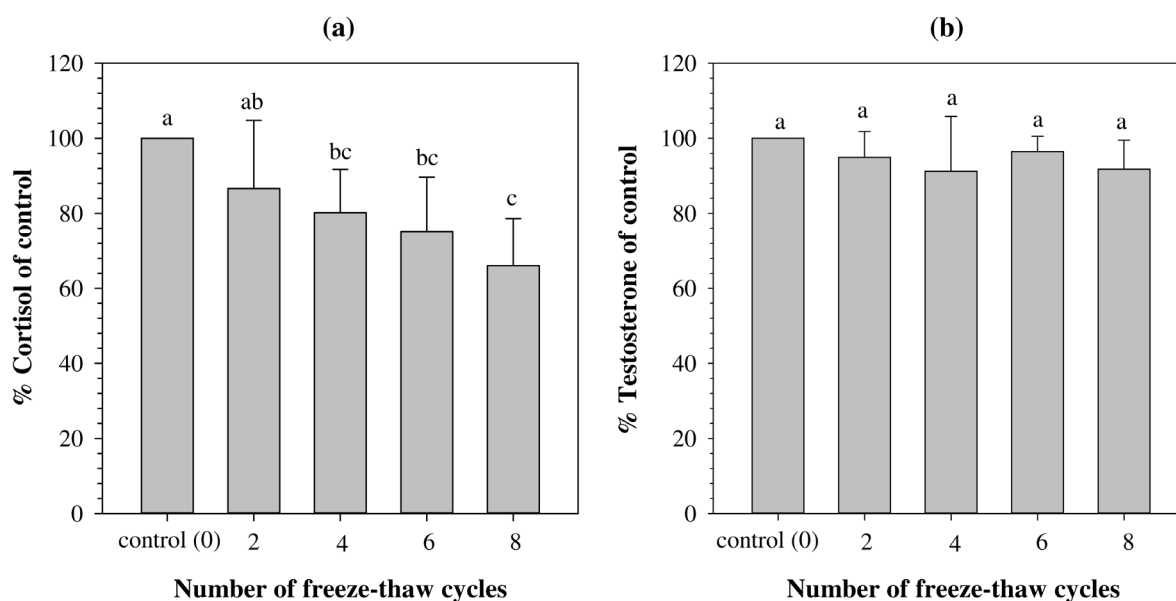


Figure 3. Percentages of hormone concentrations in the serum exposed to repeated freeze-thaw cycles compared to control. a) Cortisol (C). **b)** Testosterone (T). Values represent mean \pm SD relative to control (100%). Different superscripts above histogram indicate a significant difference between groups ($p < 0.05$).

T concentrations are given in Dataset 3²⁹ (see *Underlying data*).

Discussion

In the current study demonstrate that C and T concentrations in the serum of Aceh cattle can be accurately measured using the commercial C and T ELISA kits designed for human C and

T measurements. The results of the parallelism test show that the slope of the diluted sample curves was parallel to the standard curves of commercial C and T ELISA kits. In addition, concentrations of C and T decreased significantly following the dilution levels. Furthermore, these assays also show high accuracy ($\sim 100\%$) and precision ($CV < 10\%$ and $< 15\%$ for intra- and inter-assay, respectively). Therefore, both the commercial

C ELISA kit and the commercial T ELISA kit tested can be used to reliably measure C and T concentrations in the serum of Aceh cattle.

A significant increase in C ~~excretion~~ after transportation was detected by the commercial C ELISA kit tested. This result was predicted because C is the main modulator of physiological stress and it usually increases in response to a stressor³⁰. The commercial T ELISA kit also appeared reliable in its ability to discriminate T concentrations between bulls and cows. T concentrations in bulls were more than five times higher compared to cows. This result was ~~to be~~ expected because T is the major androgen produced by the Leydig cells of the testes³¹, whereas only small amounts are secreted in the adrenal cortex and ovaries of females³.

Our results indicated that the biological validation of commercial C and T ELISA kits can be performed using C measurements in relation to a stressful event and T measurements of different sexes. It is crucial to conduct such ~~as~~ validation to ensure the biological meaningfulness of the analyses^{32,33}. EIA-1887 and EIA-1559 are commercial ELISA kits developed for measuring hormone concentrations, particularly in human serum/plasma. These assays use an antibody highly specific to either C or T. The antigen (C or T) in the serum of Aceh cattle can bind correctly with the antibody (anti-cortisol and anti-testosterone) in these ELISA kits⁵ because the side-chains of the carbon compounds of C (C-21)³⁴ and T(C-19)³ have similar structures in vertebrates which is basic structure of steroid hormones are derived from the cyclopentanoperhydrophenanthrene structure. The validation of commercial ELISA kits for measuring hormones in animals has been successfully conducted in several ~~animals~~ such as progesterone in cattle using human progesterone ELISA kit (Clinpro International Co. LLC, Union City, CA 94587, USA)³⁵, estradiol-17-beta and inhibin A in buffalo using human ELISA kit (For estradiol: Cat No. EIA-2693, DRG Instruments GmbH, Marburg, Germany; For Inhibin A: Inhibin A DSL-10-28100, Diagnostic System Laboratories Inc, Webster, Texas, USA)³⁶, C in horses using Immunotech kit³⁷, and T in Kacang goats using T ELISA kit (Cat No. EIA-1559, DRG Instruments GmbH, Marburg, Germany) developed for human⁵.

In addition to the validation of ELISA kits, the effects of repeated freeze-thaw cycles on the stability of C and T concentrations in serum were tested. Concentrations of C decreased significantly after the exposure to four, six, and eight freeze-thaw cycles, while T concentrations did not. Concentrations of C declined up to 33.94% after eight freeze-thaw cycles, whereas the percentage changes in T concentrations were less than 10% in all test groups. These findings show that T is more resilient to freeze-thaw cycles compared to C. The reason why C concentrations decreased after exposure to repeated freeze-thaw cycles and T concentrations did not, ~~it is~~ is not entirely clear. For C, it is possible, however, that the serum left at high temperatures (27.6±0.7°C) during the six hours of the thawing process might facilitate the increased degradation of C¹⁶. Consequently, less antigen in the serum binds with the antibody of the assay³⁸. Thus, C concentrations in all test groups were lower than the control group.

From a practical point of view, our results suggest that commercial C (EIA-1887) and T (EIA-1559) ELISA kits can accurately measure C and T concentrations, respectively, in the serum of animals such as Aceh cattle. It can be advantageous to use these commercial assays over assays specifically designed for animals because of their relatively low price. On the other hand, repeated freeze-thaw cycles must be considered, especially for the C measurement. Therefore, conditions that may potentially cause repeated freeze-thaw cycles of samples designated for hormone analysis (e.g. frequent power outages) should be avoided. Some efforts can be performed to prevent the possibility of repeated freeze-thaw cycles¹⁶. First, installing a backup generator to supply electricity when there are power outages. Second, filling each freezer with a blanket of ice packs to maintain the temperature inside the freezer during the power outage. Third, averting the use of an aliquot of serum/plasma for several analyses at different times. Fourth, dividing serum into several aliquots when several analyses should be performed from the same sample.

This study has some limitation particularly information regarding the substantive reason for decreasing C concentrations after repeated freeze-thaw cycles is still unclear. To elucidate this reason, the biochemistry of the serum after exposure to freeze thaw-cycles needs to be investigated in future studies. Moreover, it was unclear whether these assays are also reliable for other animals, so they would need a validation test for each animal. Apart from the fact that there are limitations, the results are important to support further studies in Aceh cattle, particularly for maintaining and growing the Aceh cattle population to ensure food security in the Aceh region and Indonesia as a whole.

In conclusion, our study shows that based on the analytical and biological validation tests, commercial C (EIA-1887) and T (EIA-1559) ELISA kits are reliable assays for measuring C and T, in serum of Aceh cattle. Moreover, biological validation showed that fluctuations of C and T in serum of Aceh cattle have a clear biological significance reflecting to the physiological condition of Aceh cattle. Results of the study also demonstrate that more than two repeated freeze-thaw cycles significantly affected the stability of serum C concentrations, but up to eight repeated freeze-thaw cycles did not significantly affect the stability of serum T concentrations.

Data availability

Underlying data

Figshare: Dataset 1. <https://doi.org/10.6084/m9.figshare.8342504.v2>²⁷

This project contains the following underlying data:

- Analytical validation data.csv (raw values of analytical validation results included data of parallelism, accuracy, and precision [coefficients of variation/CV of intra-and inter-assay])

Figshare: Dataset 2 v2. <https://doi.org/10.6084/m9.figshare.11678352>²⁸

This project contains the following underlying data:

- Data of Biological validation.csv (raw values of biological validation results: cortisol concentrations before and after transportation and testosterone concentrations in bulls and cows of Aceh cattle)

Figshare: Dataset 3. <https://doi.org/10.6084/m9.figshare.8342720.v1>²⁹

This project contains the following underlying data:

- Data of freeze-thaw cycles on cortisol and testosterone.csv (raw value of cortisol and testosterone concentration in Aceh cattle serum after exposure repeated freeze-thaw cycles)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC-BY 4.0\)](#).

Extended data

Figshare: Extended data 1. <https://doi.org/10.6084/m9.figshare.8487830.v1>²⁵

This project contains the following extended data:

- Ethical Clearance Approval Gholib *et al.*.pdf (certificate of ethical clearance approval for using animals)

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver \(CC0 1.0 Public domain dedication\)](#).

Acknowledgments

We are grateful to Julia A. Kunz for valuable comments, suggestions, and English corrections during preparing this manuscript.

References

- Sheriff MJ, Dantzer B, Delehanty B, *et al.*: **Measuring stress in wildlife: techniques for quantifying glucocorticoids.** *Oecologia*. 2011; **166**(4): 869–887. [PubMed Abstract](#) | [Publisher Full Text](#)
- Hardy MP, Gao HB, Dong Q, *et al.*: **Stress hormone and male reproductive function.** *Cell Tissue Res*. 2005; **322**(1): 147–153. [PubMed Abstract](#) | [Publisher Full Text](#)
- Chedrese PJ: **Reproductive Endocrinology: A Molecular approach.** Springer Science + Media Business, New York, USA. 2009. [Publisher Full Text](#)
- Möstl E, Palme R: **Hormones as indicators of stress.** *Dom Anim Endocrinol*. 2002; **23**(1–2): 67–74. [PubMed Abstract](#) | [Publisher Full Text](#)
- Gholib G, Wahyuni S, Kadar OH, *et al.*: **Measurement of serum testosterone in kacang goat by using enzyme-linked immunosorbent assay (elisa) technique: the importance of kit validation.** *J Ked Hewan*. 2016; **10**(1): 32–36. [Publisher Full Text](#)
- Du Z, Keeley T, Janssen T, *et al.*: **Measurement of testosterone and cortisol metabolites and luteinising hormone in captive southern hairy-nosed wombat (*Lasiorhinus latifrons*) urine.** *Gen Comp Endocrinol*. 2017; **250**: 70–79. [PubMed Abstract](#) | [Publisher Full Text](#)
- Nugraha TP, Heistermann M, Agil M, *et al.*: **Validation of a field-friendly extraction and storage method to monitor fecal steroid metabolites in wild orangutans.** *Primates*. 2017; **58**(2): 285–294. [PubMed Abstract](#) | [Publisher Full Text](#)
- Gholib G, Heistermann M, Agil M, *et al.*: **Comparison of fecal preservation and extraction methods for steroid hormone metabolite analysis in wild crested macaques.** *Primates*. 2018; **59**(3): 281–292. [PubMed Abstract](#) | [Publisher Full Text](#)
- Durdiaková J, Fábryová H, Koborová I, *et al.*: **The effects of saliva collection, handling and storage on salivary testosterone measurement.** *Steroids*. 2013; **78**(14): 1325–1331. [PubMed Abstract](#) | [Publisher Full Text](#)
- Bloomer RJ: **Considerations in the Measurement of Testosterone in Saliva and Serum using ELISA Procedures.** *Br J Med Med Res*. 2015; **5**(1): 116–122. [Publisher Full Text](#)
- Stradaoli G, Peric T, Montillo M, *et al.*: **Hair cortisol and testosterone concentrations and semen production of *Bos taurus* bulls.** *Ital J Anim Sci*. 2017; **16**(4): 631–639. [Publisher Full Text](#)
- Dorgan JF, Fears TR, McMahon RP, *et al.*: **Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry.** *Steroids*. 2002; **67**(3–4): 151–158. [PubMed Abstract](#) | [Publisher Full Text](#)
- Sakamoto S, Putalun W, Vimolmangkang S, *et al.*: **Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites.** *J Nat Med*. 2018; **72**(1): 32–42. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kinn Rød AM, Harkstad N, Jellestad FK, *et al.*: **Comparison of commercial ELISA assays for quantification of corticosterone in serum.** *Sci Rep*. 2017; **7**(1): 6748. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Rangel-Negrin A, Flores-Escobar E, Chavira R, *et al.*: **Physiological and analytical validations of fecal steroid hormone measures in black howler monkeys.** *Primates*. 2014; **55**(4): 456–65. [PubMed Abstract](#) | [Publisher Full Text](#)
- Gholib G, Agil M, Supriatna I, *et al.*: **Repeated freeze-thaw cycles but not short-term storage of fecal extracts at ambient temperature influence the stability of steroid metabolite levels in crested macaques.** *J Ked Hewan*. 2017; **11**(2): 78–85. [Publisher Full Text](#)
- Hodges JK, Heistermann M: **Field Endocrinology: Monitoring Hormonal Changes in Free-Ranging Primates.** In: Setchell JM, Curtis DJ (eds) *Field and Laboratory Methods in Primatology: A Practical Guide*. 2nd edn. Cambridge University Press, Cambridge. 2011; 353–370. [Publisher Full Text](#)
- Bielohuby M, Popp S, Bidlingmaier M: **A guide for measurement of circulating metabolic hormones in rodents: Pitfalls during the pre-analytical phase.** *Mol Metab*. 2012; **1**(1–2): 47–60. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Hillebrand JJ, Heijboer AC, Enderit E: **Effects of repeated freeze-thaw cycles on endocrine parameters in plasma and serum.** *Ann Clin Biochem*. 2017; **54**(2): 289–292. [PubMed Abstract](#) | [Publisher Full Text](#)
- Reyna R, Traynor KD, Hines G, *et al.*: **Repeated freezing and thawing does not generally alter assay results for several commonly studied reproductive hormones.** *Fertil Steril*. 2001; **76**(4): 823–825. [PubMed Abstract](#) | [Publisher Full Text](#)
- Comstock GW, Burke AE, Norkus EP, *et al.*: **Effects of Repeated Freeze-Thaw Cycles on Concentrations of Cholesterol, Micronutrients, and Hormones in Human Plasma and Serum.** *Clin Chem*. 2001; **47**(1): 139–142. [PubMed Abstract](#) | [Publisher Full Text](#)
- Tahir MZ, Thoumire S, Raffaelli M, *et al.*: **Effect of blood handling conditions on progesterone assay results obtained by chemiluminescence in the bitch.** *Domest Anim Endocrinol*. 2013; **45**(3): 141–144. [PubMed Abstract](#) | [Publisher Full Text](#)
- Kley HK, Rick W: **The effect of storage and temperature on the analysis of steroids in plasma and blood.** *J Clin Chem Clin Biochem*. 1984; **22**(5): 371–378. [PubMed Abstract](#)
- Bauman JE: **Stability of radioimmunoassayable steroid and protein hormones after repeated freeze-thaw cycles.** *Clin Chem*. 1982; **28**(11): 2336–2337. [PubMed Abstract](#) | [Publisher Full Text](#)
- Gholib G, Wahyuni S, Akmal M, *et al.*: **Extended data 1.figshare.** Figure. 2019. <http://www.doi.org/10.6084/m9.figshare.8487830.v1>
- Zar JH: **Biostatistical Analysis.** Prentice Hall, Upper Saddle River, N.J. 1996.
- Gholib G, Wahyuni S, Akmal M, *et al.*: **Dataset 1. figshare.** Dataset. 2019. <http://www.doi.org/10.6084/m9.figshare.8342504.v2>
- Gholib G, Wahyuni S, Akmal M, *et al.*: **Dataset 2 v2. figshare.** Dataset. 2020. <http://www.doi.org/10.6084/m9.figshare.11678352>
- Gholib G, Wahyuni S, Akmal M, *et al.*: **Dataset 3. figshare.** Dataset. 2019. <http://www.doi.org/10.6084/m9.figshare.8342720.v1>
- Sapolsky RM, Romero LM, Munck AU: **How do glucocorticoids influence stress**

responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev.* 2000; **21**(1): 55–89.

[PubMed Abstract](#) | [Publisher Full Text](#)

31. Swerdloff RS, Wang C, Sinha-Hikim AP: **Hypothalamic–pituitary–gonadal axis in men.** In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT (eds) *Hormones, brain, and behavior*. 2nd edn. Academic Press, San Diego. 2009; Part V: 2357–2395.
[Publisher Full Text](#)
32. Hodges K, Brown J, Heistermann M: **Endocrine monitoring of reproduction and stress.** In: Kleiman DG, Thompson KV, Kirk Baer C (eds) *Wild mammals in captivity: principles and techniques for zoo management*. The University of Chicago Press, Chicago. 2010; 447–468.
[Reference Source](#)
33. Heistermann M, Palme R, Ganswindt A: **Comparison of different enzyme-immunoassays for assessment of adrenocortical activity in primates based on fecal analysis.** *Am J Primatol.* 2006; **68**(3): 257–273.
[PubMed Abstract](#) | [Publisher Full Text](#)
34. Möstl E, Messmann S, Bagu E, *et al.*: **Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock.** *Zentralbl Veterinärmed A.* 1999; **46**(10): 621–631.
[PubMed Abstract](#) | [Publisher Full Text](#)
35. Bayemi PH, Nsongka VM, Perera BMAO, *et al.*: **Validation of a human progesterone enzyme immunoassay (EIA) kit for use on serum of cattle in Cameroon.** *Trop Anim Health Prod.* 2007; **39**(5): 335–338.
[PubMed Abstract](#) | [Publisher Full Text](#)
36. Todini S, Terzano GM, Malfatti A: **Validation of ELISA kits for determination of Inhibin-A and Estradiol-17-beta concentrations in Buffalo plasma.** *Ital J Anim Sci.* 2007; **6**(sup2): 585–588.
[Publisher Full Text](#)
37. Pawluski J, Jegu P, Henry S, *et al.*: **Low plasma cortisol and fecal cortisol metabolite measures as indicators of compromised welfare in domestic horses (*Equus caballus*).** *PLoS One.* 2017; **12**(9): e0182257.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
38. Pappano DJ, Roberts EK, Beehner JC: **Testing extraction and storage parameters for a fecal hormone method.** *Am J Primatol.* 2010; **72**(11): 934–941.
[PubMed Abstract](#) | [Publisher Full Text](#)

Open Peer Review

Current Peer Review Status:



Version 2

Reviewer Report 05 February 2020

<https://doi.org/10.5256/f1000research.24459.r59514>

© 2020 Astuti P. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Pudji Astuti

Department of Veterinary Medicine, Gadjah Mada University (UGM), Yogyakarta, Indonesia

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 16 October 2019

<https://doi.org/10.5256/f1000research.21725.r54435>

© 2019 Carbajal A. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Annaïs Carbajal 

Department of Animal Health and Anatomy, Autonomous University of Barcelona, Barcelona, Spain

The authors validated an EIA for testosterone and cortisol detection in serum of Aceh cattle. They also performed the biological validation of both hormones in serum. And finally, they evaluated the effect of freeze-thaw cycles on the stability of these hormones in serum.

Although the technique they present is not new, the validation the authors perform are imperative for the proper hormone interpretation in every matrix, and thus this should be done for each species, matrix and assay of interest. The study has a robust experimental design despite using a low number of samples in some experiments. Results they present are very useful and may provide guidance for future studies to

perform appropriate validations of their methods.

My major concern is why the authors discuss that T is affected by the freeze-thaw cycles if results do not indicate the same. Additionally, I would suggest dividing the aims in three: analytical validation, biological validation and freeze-thaw cycles, as they may provide three different results and conclusions. I have also added some minor comments which can be found in the [pdf document](#) that I hope they will be useful for the authors.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Endocrinology and animal stress

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Jan 2020

Gholib Gholib, Universitas Syiah Kuala, Banda Aceh, Indonesia

Response to Reviewer 2.

First, we would like to thanks to the reviewer 2 for reviewing our paper and gave approved with reservations . Her comments and suggestions have helped us to further improve the paper. As you will see in the response-to-reviewer below, we have answered all points raised and have changed the paper accordingly. We very much hope that the paper is now finally approved.

1. New sentence should not start with an abbreviation. This needs to be rectified in the whole text

Response: All abbreviation in new sentences have been changed throughout text.

2. T is also produced by the ovaries and the adrenals

Response: Yes, you are right, T is also produced by the ovaries and the adrenals. An explanation has been added in revised paper

3. Could also be due to species-specific differences? **Response: Yes, it can be influence by species-specific differences, Thank you very much for the revision. It has been added to the revised paper.**

4. The aims of the study are not fully clear here. As three different results are discussed, I would recommend dividing the aims in 3 clear objectives: Analytical validation, biological validation and freeze-thaw cycles

Response: Thank you very much for your suggestion. Now, The aims were divided into three objectives. The objective of the present study were: First, conducting an analytical validation test to examine the reliability of commercial C (Cat. no. EIA-1887) and T (Cat. no. EIA-1559) ELISA kits that accurately measure C and T concentrations, respectively in Aceh cattle; Second, performing a biological validation test to examine the reliability of commercial C ELISA kit (Cat. no. EIA-1887) to the response a stress event by comparing C concentrations before and after transportation and the reliability of T ELISA kit (Cat. no. EIA-1559) ELISA kits in discriminating the T concentrations from different sexes (bulls versus cows). The hypothesis is that C concentrations should be higher following transportation and the T concentration should be higher in bulls. Finally, the stability of C and T concentrations in the serum of Aceh cattle after exposure to several repeated freeze-thaw cycles was evaluated. For details, please see in the revised paper.

5. This is not totally true since only 5 individuals are used for the C validation.

Response: Thank you for your correction. Thank you for your correction. *We have collected additional samples (10 bulls and 10 cows) to increase the number of samples. Now, in total 32 Aceh cattle were used: 16 bulls and 16 cows. For the testosterone validation test, all cattle (16 bulls and 16 cows) were used. For cortisol validation test, 16 cattle (8 bulls and 8 cows) were used which is taken from 32 Aceh cattle above. The explanation has been added in the revised paper.*

6. Information is lacking here. For example, why did you collect samples at 16h and 6h? This information should go to the subsection "biological validation" - experimental design.

Response: For biological validation of C, Samples were collected at 16 h, four hours after the trip to monitor the increase in Cortisol after the transportation. For biological validation of T, Samples were collected in the morning (6h-8h) because in some speceies including cattle, there is diurnal variation of T in blood which concentration of T is higher in the morning compared to afternoon. This explanation has been added to the subsection of biological validation.

7. Hypothesis are written in the introduction

Response: Hypothesis has been deleted in this section and moved to the subsection "introduction".

8. Why serum was collected a day before and not immediately before transport? Please, specify the time of day when samples were collected. **Response: Sample was collected a day before to prevent the stress in cattle and sometimes blood collection in cattle needs some times. More explanation has been added in the revised paper**

9. This is mean and standard deviation? Please, specify

Response: Thank you very much for your correction, it has been added in the revise

paper.

10. Ref 24 extended data 1 shows: Certificate of ethical clearance approval for using animals for study on monitoring reproduction and stress in aceh cattle Data on the test of equality is not provided. Here the reference should be ref 26.

Indicate the name of the statistical model used to "test of equality of two slopes"

Response: Model used for the test of equality of two slopes is Logarithmic regression.

Thank you for your correction, the reference No. was also changed.

11. More information is needed to interpret the data. Parallelism: indicate which test was made in order to obtain results of t and p

Dose response curve: is this a different test or they are results from the parallelism test? Do the equations provide extra information with regard to the parallelism test results (t and p)?

Response: Thank you for your suggestion. For the parallelism, a test of equality of two slopes was used to compare between standard slope (C and T) and slope of sample one and sample two obtained from the regression equations. From the equations {dose response curve) containing value of slope for standard , sample one and two. Based on the results, we compared the standard slope (C and T) with the slope of sample one and sample to see whether they are parallel to standard slope based on the the pvalue and t value. Since the pValue >0.05, it is indicated that these slopes are similar/parallel with the standard slope (not significant different). More explanation has been added in the revised paper

12. Table 2 and Figure 3 provide the same information. If percentages were used to perform statistics, I suggest removing Table 2.

Response: Thank you for your suggestion, the Table 2 has been deleted from the revised paper

13. Reviewer 2: used to reliably measure C and T concentrations in serum of Aceh cattle

Response: Thank you, the specific assay used in each study has been added in the revised paper.

14. Reviewer 2: Please specify the matrix you are talking about

Response: Thank you, the specific assay used in each study has been added in the revised paper.

"The validation of commercial ELISA kits for measuring hormones in animals has been successfully conducted in several animals, such as progesterone in cattle using human progesterone ELISA kit (Clinpro International Co. LLC, Union City, CA 94587, USA) , estradiol-17-beta and inhibin A in buffalo using human ELISA kit (For estradiol: Cat No. EIA-2693, DRG Instruments GmbH, Marburg, Germany; For Inhibin A: Inhibin A DSL-10-28100, Diagnostic System Laboratories Inc, Webster, Texas, US), C in horses using Immunotech kit , and T in Kacang goats using T ELISA kit (Cat No. EIA-1559, DRG Instruments GmbH, Marburg, Germany) developed for human."

15. Your results suggest the opposite regarding the T levels Your results suggest the opposite regarding the T levels

Response: Thank you for your correction, Although, T was decreased less 10%, but this is not significant. You are right, T is not significantly affected by freeze thaw cycles. The explanation has been improved.

16. Consider shortening the sentence or dividing the information in 2-3 shorter sentences. This way, it will be easier for the reader to understand.

Response: Thank you very much for your suggestion. The sentence has been divided into 4 shorter sentences.

17. Reviewer 2: Same as before. If the T concentrations remained stable, why do you say that T concentrations decreased?**Response: Thank you, it has been corrected in the revised paper.**

18. Reviewer 2: An important conclusion is lacking: The biological validation suggests that fluctuations of C and T in serum of Aceh cattle have a clear biological significance.

Response: Thank you for your suggestion. An additional conclusion has been added in the revised paper.

"In conclusion, our study shows that based on the analytical and biological validation tests, commercial C (EIA-1887) and T (EIA-1559) ELISA kits are reliable assays for measuring C and T, in serum of Aceh cattle. Moreover, biological validation showed that fluctuations of C and T in serum of Aceh cattle have a clear biological significance reflecting to the physiological condition of Aceh cattle. Results of the study also demonstrate that more than two repeated freeze-thaw cycles significantly affected the stability of serum C concentrations, but up to eight repeated freeze-thaw cycles did not significantly affect the stability of serum T concentrations."

Competing Interests: The authors declare that they have no competing interests

Reviewer Report 03 September 2019

<https://doi.org/10.5256/f1000research.21725.r52712>

© 2019 Astuti P. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Pudji Astuti

Department of Veterinary Medicine, Gadjah Mada University (UGM), Yogyakarta, Indonesia

1. The details comment of the paper are attached (Please see [attachment](#)).
2. Overall the content are complete including the validation test and the Biological validation are conducted.
3. The theory of similarity base chemical formula namely cyclopentano perhydro phenanthrene both of human and animal should be added.
4. The information standard of parallelism should be added.

5. Although the number of samples is less than specified (based on the Frederer formula), the results can still be accounted for because the amount of 12 samples is still very sufficient considering the large number of replications.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 23 Jan 2020

Gholib Gholib, Universitas Syiah Kuala, Banda Aceh, Indonesia

Response to Reviewer 1.

First, we would like to thanks to the reviewer 1 for approved our paper. Her comments and suggestions have helped us to further improve the paper. As you will see in the response-to-reviewer below, we have answered all points raised and have changed the paper accordingly.

1. Do not use personal pronouns. Please don't use we, I etc

Response: It has been changed throughout the text.

2. Choose the most 5 important words

Response: **In the guideline, it was mentioned that we can use until 8 keywords. We still keep the keywords because using more keywords will help people to find our paper.**

3. Do not use abbreviation word on the beginning of the line

Response: It has been changed throughout the text.

4. Is there any correlation between C and T in this case?

Response: **Yes there is a correlation between cortisol and testosterone in some animals from previous studies. From the previous study, high levels of cortisol can suppress the**

testosterone production.

An additional sentence and references have been added in the revised paper

5. Due to the same base form of steroid (cyclopentanoperhydrophenantrend), we can use the same kit for animal as in human.

Response: ***Thank you for your suggestion, This statement has been mentioned in the discussion section.***

6. Do not use personal pronouns

Response: ***It has been changed throughout texts***

7. Should be done by veterinarians

Response: ***During the study, our team was accompanied by a veterinarian during data collection. Blood samples were performed by veterinarians. This statement has been written in the paper (ethical statement, collection and processing of blood samples)***

8. How to validate the data, because the samples are less than it should be?

Response: ***We have added the number of samples. Now, 32 Aceh cattle were used: 16 bulls and 16 cows. For the testosterone validation test, all cattle (16 bulls and 16 cows) were used. For cortisol validation test, 16 cattle (8 bulls and 8 cows) were used which is taken from 32 Aceh cattle above. The explanation has been added in the revised paper***

9. Please add some information of the feed and the volume per head.

Response: ***Feed given to the cattle is grass at 10% of body weight and 1% of body weight of rice bran and tofu dregs. The explanation has been added in the revised paper***

10. until the assay process are conducted

Response: ***Thank you for your suggestion, we have added some words in revised paper***

11. Please add sign of significant on testosterone

Response: ***Superskrip “ a” has been added to the testosterone data in the Figure 3, because they showed not significant different. Table 2. has been deleted from the revised paper.***

12. Due to the same base form, the kit also can be used in animals as in human. Please add this explanation

Response: ***The explanation has been added in the discussion section, paragraph 3rd in revised paper***

13. Please explain about gradient slope that is used in the research as standard. Is there prerequisite to determine parallelism standart?

Response: ***To determine whether curve of serial dilution samples was parallel with the curve of hormone standard (C and T), the slope of the serial dilution samples curve must be compare with the slope of the standard curve. For this, we can do a statistical analysis to compare the value of the slope from dilution samples curve and standard curev. We can determine that the both slopes (sample curve and standard curve) were parallel when there was not significant different between slope of dilution sample curve with standard curve. From our results, Slope of Sample one and sample two were not significant different with the slope of the standard curve ($p>0.05$). It is indicated that both slope are***

similar.

14. *Why biology validation should be done? Is parallelism test based on dilution can be confirmed? In the real condition, does the treatment should be validated by biology validation? Does parallelism test not enough?*

Response: To test the reliability of the assay, there are several validations should be done namely: analytical validation, physiological validation, and biological validation.

Parallelism is one part of the analytical validation. This test aimed to evaluate whether antigen (hormone) can bind with the antibody in the assay accurately and find the optimal dilution if needed. However, parallelism test is not a marker of specificity of the assay, but a

proof of a dose-response relationship (Möstl *et al.*, 2005). Therefore, it is not possible to select

the best-suited assays to assess physiological stress and reproduction only based on the parallelism results. To achieve that, it is important to perform a biological validation or physiological validation. For physiological validation of stress hormone, a widely used method is an adrenocorticotrophic hormone (ACTH) challenge test by injecting animals with ACTH (for review see Tauma & Palme 2005) and by injecting radiolabeled testosterone [4(C)-14C testosterone] for testosterone validation (Möhle *et al.* 2002).

Competing Interests: The authors declare that they have no competing interests

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research