

Validation of Commercial ELISA kit for Non-Invasive Measurement of Cortisol Concentrations and the Evaluation of the Sampling Time of Blood and Fecal Sample in Aceh Cattle

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Abstract. Some parameters should be evaluated before the analysis of cortisol hormone using *enzyme-linked immunosorbent assay* (ELISA). The most important one is to test the validity of the ELISA kit itself since most of the commercially available ELISA kit is not designed for animal use. The time of sample collection is also another important parameter needed to be evaluated due to a known diurnal variation of cortisol concentrations. This study aims to validate a commercial ELISA kit and evaluate the sampling time on the diurnal variations of cortisol concentration in blood and feces of Aceh cattle. Blood and fecal samples were collected in the morning and afternoon from 8 Aceh cows aged 2-3 years. A commercial ELISA Kit (Cat. No. EIA-K003-H5, Arbor Assays[®]) was validated using analytical (parallelism, accuracy, and precision/% CV of intra- and inter-assay) and biological validations (by analyzing sample pre- and post-transportation). The results of a parallelism test showed the diluted sample curve from the fecal extract was not significantly different (parallel) to the standard curve of EIA-K003-H5 kits ($p > 0.05$), the accuracy of the assay: $99.76 \pm 3.77\%$, and the % CV of intra- and inter-assay less than 10%. The results from the biological validation test showed that the concentrations of fecal cortisol post-transportation were significantly higher compared to the pre-transportation ($P < 0.05$). Plasma cortisol concentrations collected in the morning were significantly higher (79.34%) compared to afternoon samples ($P < 0.05$). However, cortisol concentrations in feces collected in the morning and afternoon did not show a significant difference ($P > 0.05$). In conclusion, ELISA Kit (EIA-K003-H5, Arbor Assays[®]) is a reliable assay for measuring cortisol in the feces of Aceh cows. Plasma cortisol concentrations in Aceh cows show a diurnal variation which is higher in the morning than afternoon, but the concentration of cortisol in the fecal sample did not.

Keywords: Aceh cattle, non-invasive, validation, sample collection time, ELISA, diurnal variation.

1 Introduction

Global warming has increased the surface temperatures around 0.7°C since the beginning of the 20th century and is expected to rise to 1.8 to 4°C by 2100 [1]. This has a wide impact on the agriculture and livestock sectors particularly due to the effect of heat stress. Heat stress is widely known to affect domestic animals' performance such as suppress the immune system and even causing reproductive health disorders [2]. The direct impact of heat stress on reproduction is the increased incidence of silent estrus, inhibition of hormone secretion, failure of fertilization, decreased conception rates and premature embryonic death [3-4].

Aceh cattle have been declared as native Indonesian beef cattle based on Minister of Agriculture Decree

Number 2907 / Kpts /OT.140/6/2011 2011. The productivity of Aceh cattle is still relatively low. One of the problems is caused by heat stress. To evaluate the levels of stress, it is mostly done through the cortisol measurements in blood samples using an enzyme-linked immunosorbent assay (ELISA) technique [5]. However, these invasive procedures can cause stressed to the animal itself due to the process of handling and restraint and can bias the results due to the stress that arises during sampling [6].

A recently non-invasive technique for monitoring hormone levels through fecal samples has become a preferred tool for field researchers [7-8]. However, there are some precautions in using commercially available ELISA kits for the measurement of fecal hormones. Firstly, most of these kits are intended for blood samples.

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Second, there is a commercial ELISA kit developed for measuring hormones in feces or urine, but it is not specifically intended for animals especially cattle. Therefore, several factors must be considered before conducting hormone analysis, and it is called pre-analytical factors. Pre-analytical factors include the time of sample collections [9], sample preservation and storage time [8], preparation and processing techniques [7], and validation of the ELISA kit used [5,10].

The time of sample collection must be considered. This is due to the diurnal pattern of hormone secretion (i.e., cortisol) called the circadian rhythm. It may cause differences in cortisol secretion in the morning and afternoon. Pawluski *et al.* [11] reported that the highest plasma cortisol concentrations in horses are in the morning (28.5 ± 1.4 ng/ml), while the lowest concentration was reached in the afternoon (15.2 ± 1.8 ng/ml). A study In brown spider monkeys, morning concentrations of fecal samples are higher (203 ± 10 ng/g feces) compared to the afternoon samples (83 ± 13 ng/g feces) [12]. However, on some species such as gorillas (morning: 114.1 ± 52.0 ng/g feces, afternoon: 121.5 ± 67.9 ng/g feces) [13], and howler monkeys (morning: 287 ± 80 ng/g feces, afternoon: 284 ± 23 ng/g feces) [12], there are no differences between the concentration of fecal cortisol in the morning and the afternoon. Therefore, it is necessary to evaluate the sampling time in each species.

Measurement of cortisol hormone concentration using ELISA methods requires a cortisol ELISA kit. It can use a commercially available ELISA kit or a custom home assay kit that made specifically for the intended purpose. When a commercial ELISA kit is preferred, it is very important to conduct a validation test on the kit. This is especially when the ELISA kit used is not specifically designed for the intended animal or the type of sample used (e.g., fecal samples). Validation tests include analytical validation and biological/physiological validation. Both tests aim to validate the reliability of the ELISA kit used in measuring the concentration of the intended hormone [5].

The present study was conducted to 1) examine the validity of commercially available cortisol ELISA kits (Cat. No.EIA-K003-H5, Arbor Assays®) to measure cortisol hormone in fecal samples of Aceh cattle, and 2) evaluate the effect of sampling time on diurnal variation of cortisol concentrations in blood and fecal samples of Aceh cattle.

2 Materials and Methods

2.1 Study animals

Blood and fecal samples were collected from eight female Aceh cattle (cow), aged 2-3 years, from a teaching farm (UPT hewan coba), Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh. These cows were housed together in stables using a colony housing system. Cattle housing was equipped with feed and water troughs.

2.2 Collection and preparation of blood samples

Blood samples were collected in the morning (06.00 to 08.00) and the afternoon (12.00 to 14.00) from the jugular vein. A 3 ml blood samples were then placed into a tube containing Ethylenediaminetetraacetic acid (EDTA) to prevent blood clots. Blood samples were then centrifuged at 1200xg for 10 minutes at 4°C. The plasma obtained was stored in a freezer of -20°C until hormone measurements.

2.3 Collection, preparation, and extraction of fecal samples

Fecal samples were collected in the morning at 06.00 to 08.00 and afternoon at 12.00 to 13.00. Approximately, 10 g of fecal samples are collected immediately after defecation and dried using an oven [7]. Fecal samples were placed in the oven at 50°C for 3 days until the fecal sample was dried evenly. The dried feces was then pulverized to get the fecal powder. ~0.5 g fecal powder was put into a tube containing 3 ml of 80 % methanol. The mixture was then extracted using a multitube vortexer for 10 minutes. Afterward, the mixture was centrifugated for 10 minutes at 3000 rpm and the supernatant (fecal extract) was then decanted into 2 ml microtube. 0.5 ml of fecal extract is aliquoted in a tube that is covered with the perforated parafilm and then dried in an oven at 37°C for 2 days until the solvent disappears. Afterward, 0.5 ml of assay buffer is added into the tube and then vortexed until homogeneous and stored at -20°C in freezer until hormone analysis.

2.4 ELISA kit validation

Validation of a commercial cortisol ELISA Kit (EIA-K003-H5, Arbor Assays®) is conducted through analytical and biological validation as described by Gholib *et al.* [5]. The analytical validation consists of a parallelism test, accuracy, and precision (% coefficient variation (CV) of intra- and inter-assay), while the sensitivity was reported as written in the ELISA Kit manual (product protocol). Biological validation was performed by comparing cortisol concentrations before and after transport. Fecal samples were collected from three cows before and after being transported.

2.5 Cortisol measurements

The measurement of cortisol concentrations in plasma samples was conducted using a commercial cortisol ELISA kit produced by DRG Instrument GmbH, Germany (Cat. No. EIA-1887). This assay has been validated for Aceh cattle [5]. A total of 20 µL of solution standards and 20 µL samples were filled into microplate wells. Furthermore, 200 µL of the enzyme-conjugate was added to each well except for the blank well, covered with cling film and homogenized for 10 seconds by slow shaking. The microplate was then incubated at room temperature for 60 minutes and then washed four times

using 300 µL of washing solution on each well. Subsequently, a 200 µL substrate solution (TMB) was added to each well and covered with the cling film and then re-incubated for 20 minutes at room temperature. A total of 100 µL stop solution (0.5 M H₂SO₄) was added to each well to stop the enzymatic reaction. The absorbance was determined by using an ELISA reader (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories Inc.) at 450 nm. The concentration of cortisol was then calculated using the Microplate Manager ® 6 Software (Bio-Rad Laboratories Inc.).

Measurement of cortisol concentration in fecal samples was conducted using a commercial cortisol ELISA kit produced by Arbor Assays® (Cat. No. EIA-K003-H5) 50 µL of standard and samples were filled into the microplate well. Moreover, 75 µL of assay buffer was filled into the non-specific binding (NSB) wells and 50 µL of assay buffer into the maximum binding (B0 or Zero standard) well. Afterward, 25 µL of the DetectX® cortisol conjugate was added to each well using a repeater pipet. 25 µL of the DetectX® cortisol antibody was then added to each well, except the NSB wells. The microplate was then covered with the plate sealer, homogenized for 10 seconds and then incubated at room temperature for 1 hour. After that, the microplate was washed with 300 µL of washing solution four times. Afterward, 100 µL of TMB substrate solution was added to each well and cover with the sealer plate and then re-incubate for 30 minutes at room temperature. Finally, 50 µL stop solution (0.5 M H₂SO₄) was added to each well to stop the enzymatic reaction. The absorbance was determined by using an ELISA reader (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories Inc.) at 450 nm. The concentration of cortisol was then calculated using the Microplate Manager ® 6 Software (Bio-Rad Laboratories Inc.).

2.6 Data analysis

The *parallelism* test data were analyzed using the test of equality of slope between the sample curve and the standard curve [14]. The precision data is calculated based on the % CV of intra- and inter-assay of the standard control with the formula [(standard deviation/average) x 100]. The accuracy data is calculated % *recovery* using the formula [(measured concentration / known concentration) x 100]. Data on biological validation and sample collection times were analyzed using the T-test.

3 Results and Discussion

3.1 ELISA Kit Validation

Diluted fecal samples (1:10 to 1:160) showed a decrease in cortisol concentrations in line with increased levels of dilution (Table 1). Moreover, the serial dilutions of fecal samples (pre- and post-transportation) generated curves that were parallel to the cortisol standard curve (Figure 1, Table 1). The slopes of the pre- and post-transportation gave a value that was similar (not significantly different) to the slope of the cortisol standard curve ($p > 0.05$). The

accuracy of EIA-K003-H5 kit was $99,76 \pm 3.77\%$. Intra-assay (within microplate) coefficient of variation (% CV) was 4.65%, and 3.72% for Low-Quality Control (QC Low) and High-Quality Control (QC-high), respectively. Whereas % CV of inter-assay (between microplate) was 0.94%, and 4.48% for QC Low and QC high, respectively. The sensitivity of the EIA-K003-H5 kit was 27.6 pg/mL, while the limit of detection was 45.5 pg/mL. These results indicate that EIA-K003-H5 kit is an analytically reliable assay for measuring cortisol concentrations in feces of Aceh cows.

Table 1. Fecal cortisol concentrations after serial diluted and measured using EIA-K003-H5 kit.

Dilution levels	The concentration of fecal cortisol measured (ng/mL)	
	Pre-transportation sample	Post-transportation sample
1:10	48.05	69.20
1:20	21.80	31.80
1:40	11.43	17.44
1:80	7.71	9.79
1:160	4.31	4.12

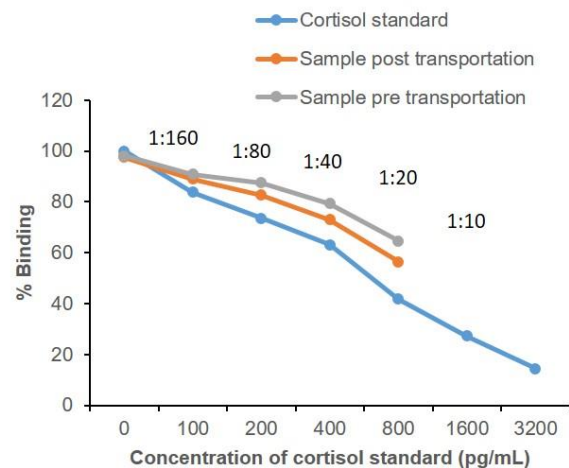


Figure 1. Curves of parallelism from cortisol standard and diluted samples of pre-transportation and post-transportation.

The mean \pm SD of the fecal cortisol concentrations before transportation (pre-transportation) and after transportation (post-transportation) was 80.09 ± 36.95 ng/mL, and 120.01 ± 52.95 ng/mL, respectively. The fecal cortisol concentrations post-transportation were significantly higher (33.27%) compared to pre-transportation samples (Figure 2). These results indicate that EIA-K003-H5 kit is a biologically reliable assay for measuring cortisol concentrations in feces of Aceh cows. This is because the biological validation showed a clear biological significance before and after transportation reflecting the physiological condition of the animals [5].

3.2 Plasma Cortisol Concentrations of Aceh Cows

Cortisol concentrations in plasma of Aceh cows collected in the morning and afternoon were presented in Table 2.

The mean (\pm SD) of plasma cortisol concentration in the morning and the afternoon was 39.24 ± 24.43 ng/ml and 21.88 ± 13.18 ng/ml, respectively. Concentrations of cortisol concentration in plasma collected in the morning were significantly higher at 79.34% compared to the afternoon ($p < 0.05$). This result indicates that cortisol concentrations in plasma of Aceh cows show a diurnal variation.

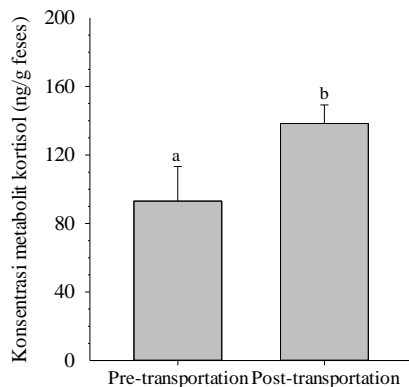


Figure 2. The mean \pm SD of fecal cortisol concentration before transportation (pre-transportation) and after transportation (post-transportation). Different superscripts above histogram indicate a significant difference between group ($p < 0.05$)

Table 2. Plasma cortisol concentrations of Aceh cows collected in the morning and afternoon.

Samples	Sample collection time	
	Morning	Afternoon
	----- ng/ml -----	
1	19.15	16.98
2	32.32	19.15
3	13.77	2.75
4	26.73	15.94
5	22.88	11,11
6	72.45	31.91
7	76.38	40.57
8	52.45	36.65
Mean\pmSD	39.24 \pm 24.43^a	21.88 \pm 13.18^b

Note: ^{a,b}Different superscripts showed significant differences ($p < 0.05$).

According to Cauter *et al.* [15], determine the diurnal variation of cortisol in plasma can be carried out in a basal condition, a condition when exogenous challenge can be controlled. It is important because the variation of cortisol secretion can be observed without the influence of external factors. For diurnal animals such as cattle, plasma cortisol concentrations will increase in the morning in response to the presence of a signal of stimulation from the body (endogenous stimulatory signal) which will eventually trigger the circadian pacemaker that is in the suprachiasmatic nuclei of the

hypothalamus. Furthermore, the hypothalamus-pituitary-adrenal (HPA) axis will respond by releasing corticotropin-releasing hormone (CRH) from the paraventricular nucleus from the hypothalamus to the anterior pituitary that will increase the release of an *adrenocorticotropic-releasing hormone* (ACTH) into the bloodstream. ACTH secretion will further trigger the adrenal cortex to release glucocorticoid hormones in the form of cortisol, corticosterone, or a combination of both [16]. In the afternoon and evening, plasma cortisol concentrations will decrease as a reflex of the *recovery* process from HPA to *endogenous signals* [15].

The results of this study were similar to the previous studies that have reported diurnal variation of cortisol concentrations in plasma such as in horses [11]. In dogs, it was reported that cortisol concentrations were high at 10.00 and 13.00 (46.5 ± 17.0 nmol/L) and decreased to reach concentrations of 38.9 ± 16.0 nmol/L at 19:00 and 22:00 [17]. However, in contrast to Aceh cattle, horses and dogs, cortisol concentrations in rats are also showing diurnal variations, but the highest concentrations can be seen at 20.00 (13.98 ± 0.54 ng/ml) and the lowest concentrations in the morning at 8:00 (7.85 ± 0.35 ng/ml [18]. This due to the rat is a nocturnal animal that active during the night, whereas Aceh's cattle, horses and dogs are diurnal animals that are active during the day.

The variation in cortisol concentrations in plasma of Aceh cow collected in the morning and afternoon indicates the importance of evaluating the time of collection of blood samples. This is because the differences in sample collection time will give a different result. Therefore, to monitor stress in Aceh cows through the measurement of cortisol from blood, the time of sample collection must be uniformed to avoid the effects of diurnal variation.

3.3 Cortisol Concentration in Feces

Different results were seen in the fecal cortisol concentrations. The concentration of fecal cortisol collected in the morning and afternoon was relatively similar (Table 3). The mean (\pm SD) of fecal cortisol concentration in the morning and afternoon was 50.91 ± 15.27 ng/g feces, and 47.59 ± 15.78 ng/g feces, respectively. The differences in cortisol concentrations in feces collected in the morning and afternoon were only about 6.98% and it was not significantly ($p > 0.05$). This result indicates that cortisol concentrations in feces of Aceh cow did not display a diurnal variation.

According to Heistermann *et al.* [19], diurnal variations often seen in blood or urine but less likely to occur in the fecal samples, especially in large animals. This is presumably because the measured hormones in the fecal are hormones that resulted from the metabolism which requires a long process until the time of the metabolites excreted in the feces. Therefore, hormone metabolites from feces are accumulations of metabolic products released in the feces [20].

According to Palme [21], after steroid hormones are produced by the endocrine glands and circulated through the blood circulation system, the hormones are metabolized and inactivated in target organs, liver, and

kidneys which then released by the body through the digestive tract, urinary tract, salivary glands, and udder glands. This inactivation is needed to stop and control the activity of hormones in target organs and body physiology [22]. In fecal samples, steroid metabolites are excreted between 30 minutes to several days depending on the species, activity and even season [21]. However, on large mammals, the process generally ranged from one to two days [13].

Table 3. Fecal cortisol concentrations in Aceh cows collected in the morning and afternoon

Samples	Sample collection time	
	Morning	Afternoon
	----- ng / g feces -----	
1	55.28	42,611
2	56.21	21.72
3	41.09	40.64
4	68.08	71.82
5	27.28	38.62
6	33.07	44.97
7	66.25	58.1
8	60.03	62.28
Mean±SD	50.91 ± 15.27^a	47.59 ± 15.78^a

Note: ^aThe same superscript showed no significant difference (p>0.05)

The results are consistent with the previous studies in other large animals such as the gorilla, *Gorilla gorilla gorilla* [13], orangutan, *Pongo* sp. [23], Rhino, *Diceros bicornis* [24], baboons, *Papio Cynocephalus cynocephalus* [25]. However, in fecal samples, diurnal variation of the fecal cortisol is often seen in the small animals e.g., in *marmoset monkeys*, *Callithrix jacchus* [26], snow rabbits, *Lepus americanus* [27], mice [28], and spider monkeys, *Ateles hybridus* [12]. This is because the time required to metabolize the hormones in small animals are generally shorter [29].

Considering the time of the fecal sample collection did not affect the concentration of fecal cortisol in Aceh cow, the sampling time of fecal samples to monitor stress hormones can be conducted in the morning, afternoon or throughout the day. This is an advantage and a practical value for researchers in the field when using fecal samples for the analysis of the cortisol.

4 Conclusion

A commercial cortisol ELISA kit(EIA-K003-H5) is a reliable assay to be used for measuring cortisol concentrations in feces of Aceh cow. There is a diurnal variation of the plasma cortisol concentration in which plasma cortisol concentrations were higher in the afternoon than in the afternoon. But, fecal cortisol concentrations did not.

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