International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.9, pp 450-456, 2016

# Level of Cortisol and Catecholamines using Enzyme Linked Immunosorbent Assay (ELISA) and Fourier Transform Infra Red (FTIR) in Dairy Cattle

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**Abstract :** Animal stress is commonly determined by measuring cortisol levels in blood, saliva, feces or urine. Samples used in this study were blood serum from cows that have already known their cortisol levels. Blood samples were taken from the three different times of milking process: one minute (just before milking), 50 minutes after milking, and 100 minutes after milking. The samples were measured at 4000-400cm$^{-1}$ wavelength using MB3000 FTIR. Data absorption band at a wavelength of FTIR was descriptively analyzed using Horizon MB™ FTIR software. The results of FTIR analysis were hydroxyl (-OH) at 3294-3321 cm$^{-1}$, ketone (CO) at 1636 cm$^{-1}$ and the amine (-$NH_2$) at 1551 cm$^{-1}$. A methyl group (-$CH_3$) was absorbed at a low level, thus it cannot get the absorbance peak at 2880 to 2950 cm$^{-1}$ wavelength. The mean of catecholamines levels using ELISA on ± 1 minute before milking, 50 minutes after milking and 100 minutes after milking were 0.0469 ± 0.00546 ng/mL, 0.0467 ± 0.00263 ng/mL and 0.0502 ± 0.00308 ng/mL respectively. Two way ANOVA test results showed that the mean of catecholamines were not significantly different ($p>0.05$) among three milking treatments. Measurement of cortisol and catecholamine levels using ELISA and FTIR showed the same result, and no significant differences were found in ELISA assay and no wavelengths difference were found in FTIR.
**Keyword:** milking stress, FTIR, ELISA, cortisol, catecholamines.

## Introduction

Stress is a non-specific condition of discomfort causing some adverse impacts on animals such as decreased immunity, reproductive failure, decreased carcass weight, and even death[18]. Environmental stressors are not limited to climatic factors, but also from nutrition, housing and every stimulus that demands a response from animals to adapt to new circumstances [14].

Milk is an almost ideal food. It has high nutritive value. It supplies body building proteins, bone forming minerals and health giving vitamins and furnishes energy giving lactose and milk fat. Besides supplying certain essential fatty acid[20]. Stress during milking process is not only violating animal welfare but also negatively affects milk ejection, which increasing the amount of residual milk that could have negative effects on health [23]. Furthermore, the milking process is also increasing risk of udder injury [10,3].

Detection of stress hormones using FTIR (Fourier Transform Infra Red) spectroscopy has previously conducted by other researcher in beef cattle[1]. Until now, FTIR has been applied to studies structural nickel

ferrite[19], to research modification of magnetic nanoparticles with CMC[11], to investigate the spectra in recycled and virgin resin[28], to identify the presence of functional groups in the grown crystal[35], to identify *Nicotiana tabacum* as biodiesel production[9], to know nanostructure of phosphosilicate[21], to analiyse anti bacterial in bioactive coumpound[32], to analyse spectra of antibiotic drug[17] also to estimate quantification of Clonidine hydrochloride in bulk[38].

Researches on cortisol and catecholamine hormones analysis using FTIR and ELISA in dairy cows has not been conducted. There are a number of similar research that have been investigating the mechanism of cortisol and catecholamines with ELISA [27], while FTIR measurements is widely used on human samples.

## Materials and Methods

Blood samples were collected three times: shortly before the portable milking machine was installed (approximately a minute before milking), 50 minutes after milking, and 100 minutes after milking ( Figure 1) . Each blood sample then collected into two venoject tubes without EDTA. To reduce the effects of stress due to blood collection, the blood was taken from coccigeal vein .


**Figure 1. Collected blood sample from minute 1 (before milking), 50 minutes after milking and 100 minutes after milking.**

**ELISA Analysis**.

A commercial kit of TSZ ELISA Bovine catecholamine (Biotang, USA) was used to determine the catecholamine levels. The kit consist of a microplate (96 wells), a bottle of sample buffer (12 ml), a bottle of standards (100 ng), a bottle of biotinylated antibody (6 ml), a bottle of enzyme conjugate (12 ml), a bottle of wash solution (50 ml), a bottle of substrate solution (12 ml), and a bottle of stop solution (12 ml).

**FTIR Analysis**.

The FTIR spectrum for serum specimens were recorded in 400-4000 $cm^{-1}$ frequency range using MB3000 FTIR spectrophotometer. Wavelengths produced were analyzed using Horizon MB™ FTIR software. The MB3000 FTIR spectrophotometer equipped with DTGS detector which cooled with air and thallium bromide crystal window (transparent IR).

**Calculation and statistical analysis**.

The data of absorption band at FTIR wavelength were analyzed descriptively with Horizon MB™ FTIR software. Quantitative confirmation test was conducted by measuring the levels of cortisol and catecholamines with ELISA. The differences among three groups were analyzed by two way Analysis of Variance (ANOVA), if there were any significant differences ($p < 0.05$) Duncan test would be conducted [5].

## Result and Discussion

Catecholamines (epinephrine and norepinephrine) and its metabolites have been used to evaluate several types of animal behaviors, neuroendocrine disorders and stress conditions, from the point of physiology and pathology as a neurotransmitter or a hormone [4]. Serum samples used in this study have already measured its cortisol level in the previous study (Tabel 1), while the catecholamine levels detected using ELISA in this study were 0.04-0.058 ng/ml. The mean and standard deviation of catecholamines shortly before milking, 50 minutes after milking, and 100 minutes after milking were 0.0469 ± 0.00546 ng/ml, 0.0467 ± 0.00263 ng/ml, and 0.0502 ± 0.00308 ng/ml respectively (Table 2).

**Tabel 1. The mean and standard deviation of cortisol levels in the blood serum Frisien types Holstein dairy cows 1 minute before milking, 50 minutes after milking and 100 minutes after milking (27).**

| Treatment | Cortisol level (ng/ml) (27) |
|---|---|
| 1 minute before milking | 7,27 ± 4,64 |
| 50 minutes after milking | 7,42 ± 5,17 |
| 100 minutes after milking | 4,94 ± 1,76 |

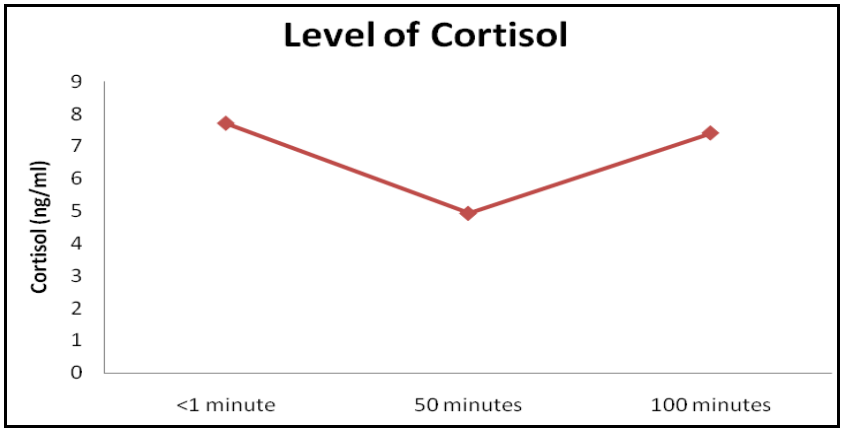


**Figure 2. Cortisol level in the blood serum Frisien Holstein dairy cow**

The result of catecholamine measurements using ELISA were still considered as normal and were not much different compared to the results obtained by other researcher, i.e. 0.05 ng/ml [16]. Another study conducted by Lay et. al. [15] stated that catecholamine levels in dairy cows were not affected by lactation process. This founding was supported by Pesce et. al. [25], who found that adrenal gland demodulation in mice that were lactating did not alter the normal levels of oxytocin, but significantly increases the feeding induction which further stimulates the release of oxytocin, given that the action of adrenal catecholamines are inhibited in central neurohypophysis.

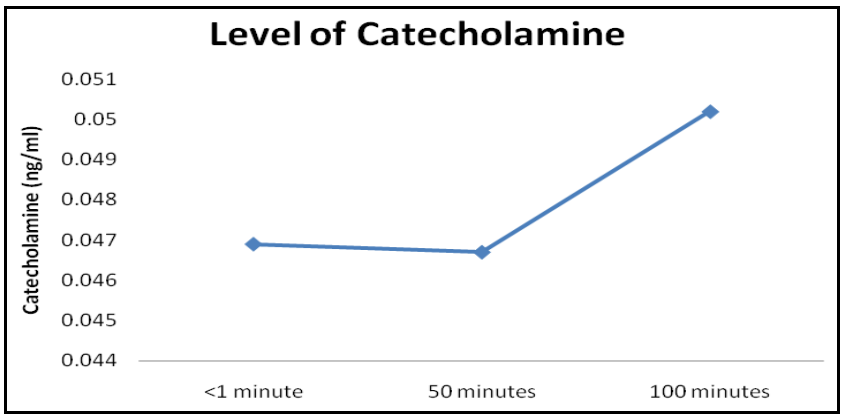


**Figure 3. Cathecolamine level in the blood serum Frisien Holstein dairy cow**

**Tabel 2. The mean and standard deviation of catecholamine levels in the blood serum Frisien Holstein dairy cows 1 minute before milking, 50 minutes after milking and 100 minutes after milking**

| Treatment | Cathecolamine level ng/ml |
|---|---|
| 1 minute before milking | 0,0469 ± 0,00546 |
| 50 minutes after milking | 0,0467 ± 0,00263 |
| 100 minutes after milking | 0,0502 ± 0,00308 |

The result of two way ANOVA test showed that the difference of mean in three milking treatments: shortly before milking, 50 minutes, and 100 minutes after milking were not significant ($p > 0.05$). Similar results were also obtained on cortisol level measurements that have been conducted previously by Titisari [36], who found that there were no differences on cortisol levels shortly before milking, 50 minutes after milking, and 100 minutes after milking. These foundings showed that catecholamines and cortisol had a same pattern, so that measuring catecholamine or cortisol levels could be a good indicator of stress response in animals.

**The measurement of stress levels using FTIR**

Stress level was measured by determining the wavelength and evaluating the absorption level of functional groups composing cortisol and catecholamines on FTIR at 400-4000 $cm^{-1}$ wavelength (Figure 5 and figure 6). Serum spectrum contains several absorption bands in mid-IR region (600-4000 $cm^{-1}$) which is typical for biological samples [24].

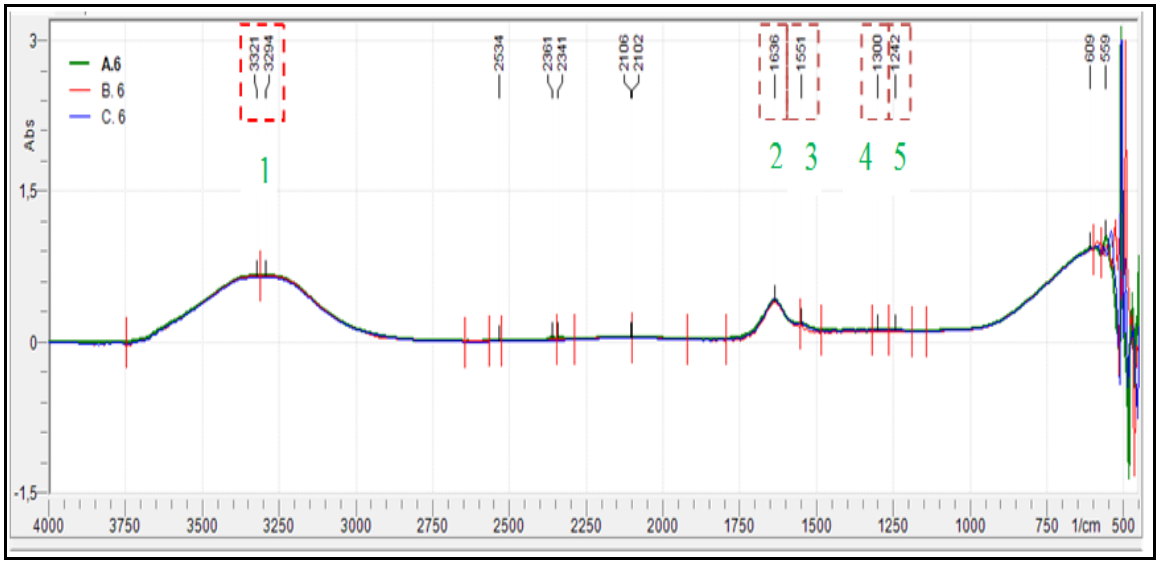


**Figure 5. Results and rate of absorption wavelength component of serum cortisol and catecholamines in the time before treatment, 50 minutes after treatment and 100 minutes after treatment.**

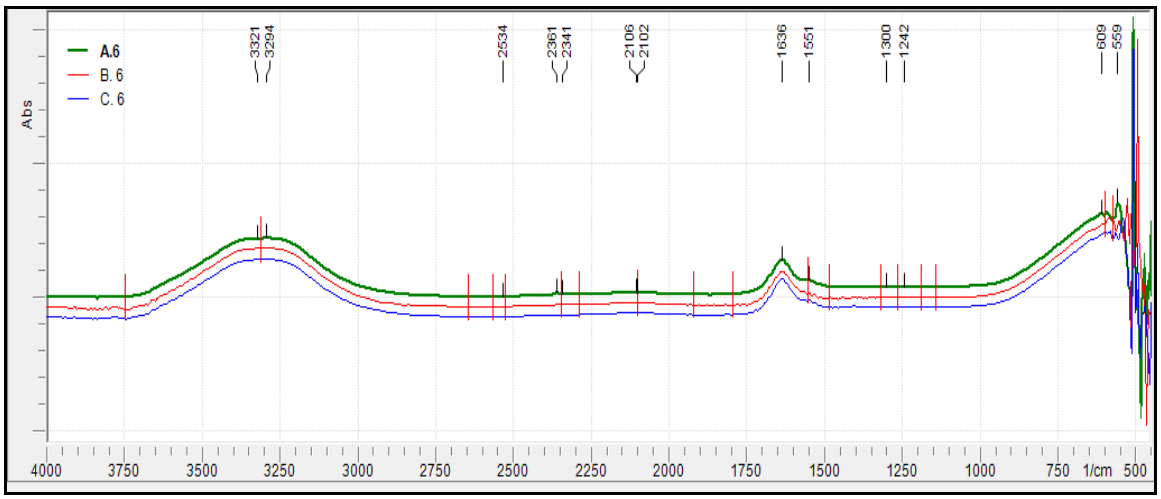


**Figure 6. Three-wavelength spectral separation, no visible difference from the third treatment**

The peak of absorption bands was at 3321$cm^{-1}$ and 3294 $cm^{-1}$ wavelength (number 1), 1636$cm^{-1}$ (number 2), 1551 $cm^{-1}$ (number 3), 1300 $cm^{-1}$ (number 4) and 1242 $cm^{-1}$ (number 5). Each of those wavelength was an absorption area for υO-H (3570-3120 $cm^{-1}$), δC=O (1720-1600 $cm^{-1}$), δNH2 (1600-1480 $cm^{-1}$), υC-O and υC-O-C (1300-900 $cm^{-1}$) and υC-O (1270-1200 $cm^{-1}$). The possibility of functional groups in number 1 and 2 were hydroxyl and ketone which are the constituent group of the cortisol hormone, while the functional group

at number 3 was amide group II, number 4 was the lactate group, and number 5 was a long chain fatty acid group.

The absorption rate in all three milking treatments showed that there was no peak for methyl ($CH_3$) and methylene group ($CH_2$). The absence of the peak of methyl signifies that $CH_3$ and $CH_2$ groups in the sample was absorbed at a low level absorbance. If infrared light was passed through organic compounds, then there were several frequencies absorbed and several other forwarded or transmitted without being absorbed. The absorption of light by molecules depends on the structure of the molecules. In the energy absorbing molecules, there were changes in vibration energy and rotational energy level. At room temperature, molecules of the organic compound are stable, each bond has a frequency that is characteristic for the stretching vibration and bending vibration where infrared light can be absorbed at these frequencies. The stretch energy of a bond is greater than its bending energy so that the stretch absorption of a bond appeared at a higher frequency in the infrared spectrum compared to the bending absorption from the same bond. Infrared spectrum graphics were created from the percentage of absorption (absorbance) against its characteristics frequency. The shape of light spectrum from organic compounds is closely related to transitions among electronic energy levels [26].

The role of cortisol in helping the body to cope with stress is likely related to its metabolic effects. Cortisol increases blood glucose levels by utilizing protein and fat deposits. It is a logical assumption that the glucose, amino acids, and fatty acids deposits are always available to use anytime when needed, for example in a stress condition [8].

The 3600-3000 $cm^{-1}$ region is the primary and secondary amine absorption band (NH, NHR) and also OH, while the peak at 3371$cm^{-1}$ was placed for VNH or VOH [13]. Amino acids are utilized in other processes beside growth, such as if the animal is under the effect of stress. Injury, infection, and stress increase the need of amino acids for protein synthesis, selective catabolism, or synthesis of other specific molecules [18;.22].

In wavelengths analysis using FTIR, 1300-900 $cm^{-1}$, 1630-1570 $cm^{-1}$, 1720-1600 $cm^{-1}$ and 2880-2850 $cm^{-1}$ wavelengths are typically used to identify functional groups and each of its characteristics, from CO (lactate, glycerol, saccharides), $NH_2$ (amine), CH (amide) and $CH_3$ (methyl) bending vibration [24]. Tsunoda [37] who measured catecholamine levels in mice using HPLC (High Performance Liquid Chromatography) method stated that it can be used to determine the mechanism of catecholamines in regulating blood pressure.

According to Prabahari [26], FTIR method is able to recognize an increase in the absorption of functional group in cortisol and serum, such as the methyl group (= CH3), methylene group (= CH2), hydroxyl group (-OH), carbonyl group (ketone) (C=O), primary amines (NH), secondary amines (NHR), carboxyl group ($COO^-$), glucose and urea, which increased due to stress. In this study, not all of the functional groups increase. These findings were in accordance with the result of ELISA test which showed that the catecholamine levels were slightly below normal, and also with the result of cortisol test by Titisari [36] who stated that the cortisol levels in all three milking treatments were normal.

The physiological response to stress is more than just behavioral changes. However, there are no physiological parameter to measure the stress response that have been identified yet [7]. In humans and animals, some parameters that have been used to study the stress response were measuring the hormone levels which were released from brain and other organs, white blood cells fluctuations, changes in heart rate and blood vessel elasticity. A stress stimulus that lasts a few seconds up to one minute is able to increase heart rate, respiratory rate and causing indigestion or decreasing food intake [25].

Animal response toward stress depends on genetic inheritances, lifetime experiences and physiological responses. This is related to the function of HPA (hypothalamic-pituitary-adrenal) axis, sympathetic nervous system, and the immune system [2]. Animals acquire stress stimulus from the environment through vision, hearing, smelling and physical contact. In some situation like the first lactating experience and being in unknown environment, animals take these experiences as negative stimuli which is able to create stress [10]. During these stressful conditions, there are some nervous and hormonal changes in the animals that might be causing some changes in their behavior, reproduction, productivity and immunity.

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