

## **Biomarkers of ketosis in dairy cows at postparturient period: acute phase proteins and pro-inflammatory cytokines**

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### **ABSTRACT**

Searching for new or additional biomarkers is an essential step toward the control of metabolic diseases such as ketosis. Acute phase proteins (APP) and pro-inflammatory cytokines were used successfully as prognostic and diagnostic biomarkers for many animal diseases. However, their use in the diagnosis of ketosis in dairy cows in the postparturient period has not been completely elucidated. Therefore, 25 cows suffering from ketosis in the postparturient period were used in the current study, together with 20 healthy cows who served as a control. Blood samples were collected from both diseased and healthy animals, and the harvested serum was used for determination of APP and proinflammatory cytokines. The obtained results indicated that there was a significant ( $P \leq 0.05$ ) increase in the levels of  $\beta$ -Hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), APP, namely: haptoglobin (HP), Serum Amyloid A (SAA), Fibrinogen (Fb), ceruloplasmin (Cp),  $\alpha$ 1-acid glycoprotein ( $\alpha$ 1-AG), and proinflammatory cytokines, namely: interleukins IL-1 $\beta$ , IL-6, IL-8, IL-12), Tumor necrosis factor-alpha (TNF- $\alpha$ ), interferon gamma (IFN- $\gamma$ ) in dairy cows affected with ketosis compared to the control. A positive correlation was observed among traditional biomarkers (BHBA, NEFA) and suggested biomarkers (APP and cytokines) in cows affected with ketosis. Conclusively, APP and pro-inflammatory cytokines could be used as promising biomarkers for ketosis in dairy cows in the postparturient period.

**Key words:** cows, ketosis, haptoglobin, fibrinogen, serum amyloid A, cytokines

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## Introduction

The transition (periparturient) period in dairy cows is an unfavorable time for dairy farmers due to the high animal culling rate and reduction of milk yield (GOFF and HORST, 1997; JORRITSMA et al., 2001). In general, it represents 3 weeks before and after parturition (CONTRERAS and SORDILLO, 2011). Ketosis and mastitis are the most metabolic and infectious diseases of this period, respectively (JONSSON et al., 2013). The occurrence of ketosis in cows in the postparturient phase is attributed to the maximum stimulus for milk production and the low available glucose to the mammary glands for this task, whereby a negative energy balance is created. As a result of the negative energy balance, lipolysis occurs, the non-esterified fatty acids (NEFA) concentration increases and  $\beta$ -Hydroxybutyrate (BHBA) produced (MELENDEZ et al., 2009; DRACKLEY et al., 2005). Therefore  $\beta$ -Hydroxybutyrate (BHBA) is the most common biomarker for evaluation of ketosis and lipid mobilization (GONZALEZ et al., 2011). Higher concentrations of non-esterified fatty acids (NEFA) induces inflammation in humans (ZHANG et al., 2006; YAQOUB and CALDER, 2007) and animals (DYK et al., 1995; SORDILLO et al., 2009) with the subsequent release of proinflammatory cytokines (interleukins, TNF- $\alpha$ , IFN- $\gamma$ ; KUSHIBIKI, 2011) from macrophages (KOJ, 1998) and other tissues, particularly the liver (BERTONI and TREVISI, 2013). These cytokines act as biomarkers of postpartum reproductive disorders (ISHIKAWA et al., 2004; ISLAM et al., 2013a; ISLAM et al., 2013b). Plasma interleukins (IL-1 $\beta$  and IL-6) were positively correlated with the severity of the inflammation, worse health status and low milk production in the early lactation period (TREVISI et al., 2012; TREVISI et al., 2015). Proinflammatory cytokines mediate the effect of APP (Hp, Fb, SAA; GRUYS et al., 2005). Acute phase proteins (APP) are a positive (up-regulated; Hp, Fb, SAA, ceruloplasmin and  $\alpha$ 1-acid glycoprotein) or negative (down-regulated; albumin, transferrin and  $\alpha$ -fetoproteins) response to stressful situations (LOUGHMILLER et al., 2007; GABAY and KUSHNER, 1999). Acute phase proteins (APP) and proinflammatory cytokines have been presented as prognostic and diagnostic biomarkers for many animal diseases by our team (EL-DEEB and EL-BAHR, 2010; EL-BAHR and EL-DEEB, 2013; EL-DEEB et al., 2014; EL-DEEB and EL-BAHR 2014a; EL-DEEB and EL-BAHR, 2014b). However, the investigation of these vital biomarkers in dairy cows in the periparturient period has not been completely elucidated yet. Therefore, the current study aimed to evaluate the diagnostic potentials of acute phase proteins and proinflammatory cytokines, as biomarkers of ketosis in dairy cows during the periparturient period.

## Material and methods

*Animals.* This study was carried out on a total number of 45 cows (3-9 weeks post parturient), aging from 4 to 7 years old, with average body weight of  $650 \pm 15$  kg from a private farm. The selected cows were assigned to two groups: the first group represented control cows ( $n = 20$ ), whereas the second group ( $n = 25$ ) consisted of ketotic cows. All

the cows were clinically examined every day until 4 weeks after parturition (RADOSTITS et al., 2007).

*Detection of ketonuria.* The presence of ketone bodies in the urine was detected by commercial kits (Fujisawa pharmaceutical Co., Osaka, Japan).

*Samples collection.* Blood samples were collected from the jugular vein of both groups into plain and sodium citrate vacutainers (sodium citrate 3.8%). Plasma was used for measurement of fibrinogen. Sera were harvested and stored frozen at -20 °C until assayed for non-esterified fatty acids (NEFA),  $\beta$ -Hydroxybutyrate (BHBA), haptoglobin (HP), serum amyloid A (SAA), fibrinogen (Fb), interleukins (IL-1 $\beta$ , IL-6, IL-8, IL-12), interferon - $\gamma$  (IF- $\gamma$ ), and tumor necrosis factor - $\alpha$  (TNF- $\alpha$ ).

*Biochemical analysis.* Serum concentrations of NEFA were found using commercially available test kits supplied by Randox laboratories Ltd, Crumlin Co., Antrim, UK. Uric acid was determined by the uricase-POD enzymatic colorimetric method, using kits provided by Spinreact, Spain. Serum concentrations of BHBA were determined by the kinetic enzymatic method, using a commercially available kit (Ranbut D-3- hydroxybutyrate, Randox laboratories Ltd, Crumlin Co., Antrim, UK). The assay was based on the reversible reaction between 3-hydroxybutyrate and NAD1, catalyzed by 3-hydroxybutyrate dehydrogenase, and the change in NADH concentration was measured by changes in the absorbance at 340 nm. Serum Hp was measured spectrophotometrically with a commercially available colorimetric kit (Phase HP kit, Tridelta Ltd., Ireland), according to the manufacturer's instructions. Serum SAA was measured with a commercially available ELISA kit (Phase SAA kit, Tridelta Ltd., Ireland), according to the manufacturer's instructions. Fibrinogen concentrations in the plasma were measured with a commercial ELISA kit (USCN, Life Science, USA) according to the manufacturer's instructions. Ceruoloplasmin activity was measured using a commercial kit (MyBiosource, USA). Serum  $\alpha$ 1-acid glycoprotein was analyzed using a commercial radial immunodiffusion kit manufactured by Ecos Institute (Furukawa, Miyagi, Japan). Interleukins (IL-1 $\beta$ , IL-6, IL-8, IL-12), tumor necrosis alpha (TNF- $\alpha$ ), and interferon gamma (IFN- $\gamma$ ) levels were determined from undiluted serum samples, using a commercially available ELISA Kits (Biosource International, California, USA). The plates were read at 450 nm on a computerized automated micro plate ELISA reader (Bio TEC, ELX800G, USA). All measurements were made in duplicate.

*Statistical analysis.* All data were presented as mean  $\pm$  standard error of mean using one way analysis of variance (ANOVA). All tests were performed using the computer package of the statistical analysis system (SAS, 2002).

## Results

*Clinical examination.* The diseased cows showed Anorexia, ruminal stasis, constipation and significant reduction in milk yield.

*Biochemical analysis of NEFA, BHBA and acute phase proteins.* The concentrations of acute phase proteins are shown in Table 1. The presented data indicate a significant increase in the levels of  $\beta$ -Hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), haptoglobin (Hp), serum amyloid A (SAA), fibrinogen (Fb), ceruloplasmin and  $\alpha$ 1-acid glycoprotein in cows affected with ketosis compared to the control.

Table 1. Acute phase proteins in serum of control and cows affected with ketosis

Variables	Ketosis	Control
NEFA (mg/ dL)	623.32 $\pm$ 9.32*	365.32 $\pm$ 11.2
BHBA (mmol/L)	1.9 $\pm$ 0.023*	0.6 $\pm$ 0.01
HP (g/L)	1.7 $\pm$ 0.12*	0.1 $\pm$ 0.01
SAA (mg/L)	46.32 $\pm$ 1.23*	23.9 $\pm$ 0.56
$\alpha$ 1 Acid glycoprotein (g/L)	2.32 $\pm$ 0.23*	1.1 $\pm$ 0.44
Ceruloplasmin (g/L)	0.72 $\pm$ 0.12*	0.24 $\pm$ 0.36
Fibrinogen (g/L)	1.76 $\pm$ 0.53	1.58 $\pm$ 2.94

Values are expressed as mean  $\pm$  SEM; \*Means are significantly different at the level ( $P \leq 0.05$ )

*Biochemical analysis of proinflammatory cytokines.* The values of proinflammatory cytokines are presented in Table 2. The data summarized in this table revealed significant elevations in the levels of interleukins (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-12), tumor necrosis factor alpha (TNF- $\alpha$ ) and interferon gamma (IFN- $\gamma$ ) in dairy cows affected with ketosis compared to the control.

Table 2. Proinflammatory cytokines in serum of control and cows affected with ketosis

Variables	Ketosis	Control
IF- $\gamma$ (pg/mL)	44.76 $\pm$ 2.56*	22.32 $\pm$ 2.36
IL-1 $\beta$ (pg/mL)	168.3 $\pm$ 2.36*	102.43 $\pm$ 2.45
IL-6 (ng/mL)	12.23 $\pm$ 1.33*	5.92 $\pm$ 0.65
IL-12 (ng/mL)	14.36 $\pm$ 0.89*	7.88 $\pm$ 0.67
IL-8 (pg/mL)	3.21 $\pm$ 0.32*	1.82 $\pm$ 0.23
TNF- $\alpha$ (ng/mL)	2.55 $\pm$ 0.12*	0.42 $\pm$ 0.04

Values are expressed as mean  $\pm$  SEM; \*Means are significantly different at the level ( $P \leq 0.05$ )

Table 3. Spearman's correlation coefficient among traditional (BHBA, NEFA) and suggested (TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-6, IL-12, IL-8) biomarkers of ketosis in cows affected with ketosis

Variables	BHBA (mmol/L)	NEFA (mg/dL)	TNF- $\alpha$ ( $\mu$ g/L)	IFN- $\gamma$ (pg/ml)	IL-1 $\beta$ (pg/ml)	IL-6 (ng/ml)	IL-12 (ng/ml)
BHBA (mmol/L)							
NEFA (mg/dL)	0.974***						
TNF- $\alpha$ ( $\mu$ g/L)	0.935***	0.960***					
IFN- $\gamma$ (pg/mL)	0.963***	0.966***	0.948***				
IL-1 $\beta$ (pg/mL)	0.964***	0.980***	0.948***	0.975***			
IL-6 (ng/mL)	0.294*	0.296*	0.370*	0.307*	0.335*		
IL-12 (ng/mL)	0.904***	0.927***	0.896***	0.935***	0.944***	0.283*	
IL-8 (pg/mL)	0.860***	0.835***	0.822***	0.869***	0.862***	0.429**	0.836***

BHBA: beta hydroxyl butyric acids; NEFA: Non esterified free fatty acids; TNF- $\alpha$ : Tumor necrosis factor  $\alpha$ ; IFN- $\gamma$ : interferon gamma; IL-1 $\beta$ : interleukins 1 $\beta$ ; IL-6: interleukins 6; IL-12: interleukins 12; IL-8: interleukins 8. \*Significant correlations at P<0.05; \*\*Significant correlations at P<0.01 and \*\*\* Significant correlations at P<0.001.

Table 4. Spearman's correlation coefficient among traditional (BHBA, NEFA) and suggested (Hp, SAA,  $\alpha$ 1-AG, Cp, Fb) biomarkers of ketosis in cows affected with ketosis

Variables	BHBA (mmol/L)	NEFA (mg/dL)	HP (g/L)	SAA (mg/L)	$\alpha$ 1-AG (g/L)	Cp (g/L)
BHBA (mmol/L)						
NEFA (mg/dL)	0.974***					
HP (g/L)	0.927***	0.969***				
SAA (mg/L)	0.949***	0.959***	0.942***			
$\alpha$ 1-AG (g/L)	0.714**	0.717**	0.690**	0.739**		
Cp (g/L)	0.647**	0.694***	0.668**	0.705**	0.550**	
Fb (g/L)	0.198*	0.214*	0.171*	0.197*	0.037*	0.060*

BHBA: beta hydroxyl butyric acids; NEFA: Non esterified free fatty acids; Hp: Haptoglobin; SAA: serum amyloid A;  $\alpha$ 1-AG:  $\alpha$ 1 Acid glycoprotein; Cp: Ceruloplasmin; Fb: Fibrinogen; \*Significant correlations at P<0.05; \*\*Significant correlations at P<0.01 and \*\*\* Significant correlations at P<0.001.

*The correlation between traditional (BHBA, NEFA) and suggested (acute phase proteins and proinflammatory cytokines) biomarkers of ketosis in cows affected with ketosis.* The data summarized in Table 3 indicated that,  $\beta$ -Hydroxybutyrate (BHBA) was positively correlated with non-esterified fatty acids (NEFA) ( $r = 0.974$ ;  $p = 0.000$ ). Further, both  $\beta$ -Hydroxybutyrate BHBA and non-esterified fatty acids (NEFA) were positively correlated with tumor necrosis alpha (TNF- $\alpha$ ) ( $r = 0.935$ ;  $0.960$ ), interferon gamma (IFN- $\gamma$ ) ( $r = 0.963$ ;  $0.966$ ), and all interleukins: IL-1 $\beta$  ( $r = 0.964$ ;  $0.980$ ), IL6 ( $r = 0.294$ ;  $0.296$ ), IL12 ( $r = 0.904$ ;  $0.927$ ) and IL8 ( $r = 0.860$ ;  $0.835$ ). The data summarized in Table 4 indicated that both  $\beta$ -Hydroxybutyrate (BHBA) and non-esterified fatty acids

(NEFA) were positively correlated with haptoglobin (Hp) ( $r = 0.927; 0.969$ ), serum amyloid A (SAA) ( $r = 0.949; 0.959$ ), alpha-1 acid glycoprotein ( $\alpha$ 1-AG) ( $r = 0.714; 0.717$ ), fibrinogen (Fb) ( $r = 0.198; 0.214$ ) and ceruloplasmin (Cp) ( $r = 0.647; 0.694$ ).

## Discussion

The clinical signs observed in cows affected with ketosis in the current study are in accordance with those obtained earlier in cows (TÓTHOVÁ et al., 2014) and buffaloes (GHANEM and EL-DEEB, 2010; YOUSSEF et al., 2010). Beside the clinical signs observed in ketotic cows in the current study, ketosis was proven by a positive test for ketone bodies in the urine, ketonuria. In addition, the significant increase of non-esterified fatty acids (NEFA) and  $\beta$ -Hydroxybutyrate BHBA in the serum of ketotic cows in the postparturient period confirmed the observed clinical findings. Although many studies (KEHRLI et al., 1989; GILBERT et al., 1993; MALLARD et al., 1998; KIM et al., 2005) have reported the role of the reduced functioning of neutrophils and lymphocytes in the postparturient period and the subsequent high susceptibility to infectious disease, data regarding their association with ketosis have not been completely elucidated. Therefore, the current study suggests proinflammatory cytokines and acute phase proteins as prognostic and diagnostic biomarkers of ketosis in this period. The use of acute phase proteins to monitor reproductive disorders (KRAKOWSKI and ZDZISINSKA, 2007) and ketosis (FATHI et al., 2013) in cows has already been documented. Disturbances in homeostasis in the transition period are the animal's reaction, known as the acute phase response (PINEIRO et al., 2003). Changing concentrations in acute phase proteins (APP) is the most striking phenomenon (PETERSEN et al., 2004). Haptoglobin (Hp) and serum amyloid A (SAA) are the most important acute phase proteins (APP) in cattle (KRAKOWSKI and ZDZISINSKA, 2007; PAULINA and TADEUSZ, 2011). They play an important role in the reconstitution of tissues damaged during inflammation (REGASSA and NOAKES, 1999). The significant elevation of Haptoglobin (Hp) and serum amyloid A (SAA) concentrations in the serum of ketotic cows in the current study compared to the control has been reported earlier in cows around (GYMNICH et al., 2003; TÓTHOVÁ et al., 2014) and after (AMETAJ, 2005; CHAN et al., 2010) delivery. The significant positive correlation between both  $\beta$ -Hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFA) with acute phase proteins (APP) is in accordance with the findings of a previous report (HARDARDOTTIR et al., 1994). The current findings comply with these findings as a significant increase in Haptoglobin (Hp), serum amyloid A (SAA), fibrinogen (Fb), ceruloplasmin (Cp) and  $1\alpha$ -glycoprotein ( $1\alpha$ -AG) was observed in ketotic cows compared to the control in the postparturient period. The significant increase in proinflammatory cytokines levels (IL- $1\alpha$ , IL- $1\beta$ , IL-6, IL-8, IL-12, TNF- $\alpha$  and IFN- $\gamma$ ) in ketotic cows compared to the control animals, as reported in the current study, is in accordance with previous reports in cows (JONSSON et al., 2013; TREVISI et al., 2015). In addition, plasma interleukins were positively correlated with the severity of the inflammation (TREVISI et al., 2015). Higher levels of proinflammatory

cytokines (IL, TNF- $\alpha$ , IFN- $\gamma$ ), perhaps released as a result of high NEFA, were observed in the serum of ketotic cows in the current study (DYK et al., 1995; SORDILLO et al., 2009). This suggestion is confirmed by the significant correlation observed among both the traditional biomarkers of ketosis ( $\beta$ -Hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFA)) and cytokines in the serum of the cows affected with ketosis in the present study. Based on the results presented in Table 3 and Table 4, it may be said that  $\beta$ -Hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFA) were positively correlated with acute phase responses and cytokine release in cows affected with ketosis. The current study may conclude that in the postparturient period in cows, lipid mobilization and fatty acid oxidation increased with the subsequent increase in ketone bodies  $\beta$ -Hydroxybutyrate (BHBA), creating a state of inflammation. As a result of that inflammation, proinflammatory cytokines may release and stimulate the release of acute phase proteins (Haptoglobin, Serum amyloid A, fibrinogen, ceruloplasmin and  $\alpha$ 1-acid glycoprotein) from macrophages or other organs. Acute phase proteins may reconstitute the damaged inflamed tissues. Therefore, acute phase proteins (APP) and proinflammatory cytokines could be used as promising biomarkers for ketosis in dairy cows in the postparturient period. In addition, anti-inflammatory therapy may be useful in the treatment of ketosis in cows in the postparturient period.

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**EL-DEEB, W. M., S. M. EL-BAHR: Biomarkeri ketoze u mliječnih krava u postpartalnom razdoblju: proteini akutne faze i proupalni citokini. *Vet. arhiv* 87, 431-440, 2017.**

**SAŽETAK**

Pronalaženje novih ili dodatnih biomarkera bitni je korak u kontroli metaboličkih bolesti kao što je ketoza. Proteini akutne faze i proupalni citokini uspješno se koriste kao prognostički i dijagnostički biomarkeri za mnoge životinjske bolesti. Ipak, njihova primjena u dijagnostici ketoze u mliječnih krava u postpartalnom razdoblju nije u potpunosti razjašnjena. Stoga je u ovo istraživanje bilo uzeto 25 krava oboljelih od ketoze u postpartalnom razdoblju, zajedno s 20 zdravih kontrolnih krava. Uzorci krvi bili su prikupljeni od bolesnih i zdravih životinja te su uzorci seruma bili pretraženi na proteine akutne faze i proupalne citokine. Dobiveni rezultati pokazali su da se u krava s ketozom u odnosu na kontrolnu skupinu značajno ( $P \leq 0,05$ ) povećala razina  $\beta$ -hidroksibutirata (BHBA), neesterificiranih masnih kiselina (NEMK), proteina akutne faze i to haptoglobina (HP), serumskog amiloida A (SAA), fibrinogena (Fb), ceruloplazmina (Cp),  $\alpha 1$ -kiselog glikoproteina ( $\alpha 1$ -AG) i proupalnih citokina kao što su interleukini (IL-1 $\beta$ , IL-6, IL-8, IL-12), faktor tumorske nekroze alfa (TNF- $\alpha$ ) i interferon gama (IFN- $\gamma$ ). Pozitivna korelacija utvrđena je između postojećih biomarkera (BHBA, NEMK) i novih predloženih na osnovi ovog istraživanja (proteina akutne faze i citokina) u krava oboljelih od ketoze. Zaključuje se da bi proteini akutne faze i proupalni citokini mogli biti obećavajući biomarkeri ketoze u krava u postpartalnom razdoblju.

**Cljučne riječi:** krave, ketoza, haptoglobin, fibrinogen, serumski amiloid A, citokini

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