Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

REVIEW ARTICLE

Detection of Subclinical Ketosis in Dairy Cows

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ARTICLE HISTORY

Received: July 16, 2011
Revised: October 02, 2011
Accepted: October 24, 2011

Key words:
Cowside tests
Dairy Cows
Ketone
Laboratory tests
Subclinical ketosis

ABSTRACT

Ketosis is a common metabolic disorder frequently observed in dairy cows during the early lactation period. It is characterized by increased levels of ketone bodies in the blood, urine, and milk. Subclinical ketosis (SCK) in dairy cattle is an excess level of circulating ketone bodies in the absence of clinical signs of ketosis. Usually, detection of SCK is carried out by testing the ketone concentrations in blood, urine, and milk. Here, This review overview the detection methods for SCK in dairy cows, including cowside and laboratory tests.

To Cite This Article: Zhang Z, G Liu, H Wang, X Li and Z Wang, 2012. Detection of subclinical ketosis in dairy cows. Pak Vet J, 32(2): 156-160.

INTRODUCTION

Ketosis is a common metabolic disorder frequently observed in dairy cows during the early lactation period. It is characterized by increased levels of ketone bodies in the blood, urine, and milk (Tehrani-Sharif *et al*., 2011). There are many theories on the cause, the biochemical (Kinoshita *et al*., 2010) and hormonal pathogenesis of ketosis, and the importance of predisposing factors (El-Deeb and Younis, 2009; Ghanem and El-Deeb, 2010; Liu *et al*., 2010). It is generally accepted that clinical ketosis occurs in ruminants when they are subjected to demands on their resources of glucose and glycogen that cannot be met by their digestive and metabolic activity (Ospina *et al*., 2010b; Wagner and Schimek, 2010).

Ketone bodies comprise beta-hydroxybutyrate (BHBA), acetoacetate (AcAc), and acetone (Ac), at 70, 28 and 2%, respectively. BHBA is the predominant circulating ketone body in ruminants, and there is a strong correlation between the whole blood concentrations of BHBA and AcAc (Kauppinen, 1983). Ketone bodies can freely diffuse across the cell membrane and provide energy during prolonged fasting (Laffel, 1999). Increased BHBA concentrations in blood indicate stimulation of lipolysis or excess absorption of butyrate from feeding on spoiled silage.

Clinical ketosis has visible clinical symptoms and typically occurs within the first six to eight weeks post-calving, resulting in anorexia, licking and blindness, hard dry feces, rapid loss of condition, and decreased milk production (Youssef *et al*., 2010). In addition, the milk fat yield of ketotic cows is increased due to the availability of BHBA and fatty acids. Clinical ketosis is easy to diagnose by its clinical symptoms.

The case definition of subclinical ketosis (SCK) in dairy cattle is an excess level of circulating ketone bodies in the absence of the clinical signs of ketosis (Andersson, 1988). The risk of abomasal displacement was identified at serum BHBA concentrations of 1.2 mM (Geishauser *et al*., 1997; Leblanc *et al*., 2005; Duffield *et al*., 2009). Furthermore, associations between elevated concentration of circulating ketones and periparturient uterine disease were described by Reist *et al*. (2003) Cheong *et al*. (2011). When lower milk production, the increased risk of disease (Grinberg *et al*., 2008), and reduced reproductive performance (Walsh *et al*., 2007; Elisabeth *et al*., 2010; Leblanc, 2010; Ospina *et al*., 2010a) are considered, the cost of one case of SCK has been estimated to be $78 (Geishauser *et al*., 2001).

SCK has a prevalence of around 7 to 41% (Geishauser *et al*., 1998; Enjalbert *et al*., 2001). Because of the economic consequences, it is very important to diagnosis SCK in dairy cows, especially during early lactation. Here, we review the detection methods for SCK in dairy cows, including cowside and laboratory tests.

Laboratory test

Enzyme catalysis: Williamson *et al*. (1962) developed an enzyme catalysis method to test serum BHBA. Based on this method, a test kit was manufactured. The test kit required the use of an ultraviolet spectrophotometer or biochemistry analyzer and could be used to test the serum BHBA levels of humans and animals (Chung *et al*., 2009; Al-Qudah, 2011; Křížová *et al*., 2011 Zhang *et al*., 2011).

The blood BHBA concentration has also often been used for this type of detection, and several authors have used a cut-off point of 1.2 mM (Geishauser *et al*., 1998; Xu and Wang, 2008; Van Knegsel *et al*., 2010; Xu *et al*., 2010), 1 mM (Goldhawk *et al.,* 2009; Kinoshita *et al*., 2010) or 1.4 mM (Geishauser *et al*., 2000; Carrier *et al*., 2004; Iwersen *et al*., 2009) to discriminate between healthy cows and cows affected by SCK.

Fourier transform infrared (FTIR) spectrometry: Hansen (1999) and Heuer *et al*. (2001) reported a method for screening cows for SCK by determining the milk Ac concentration using FTIR spectrometry. FTIR spectrometry is fast, inexpensive, and easy to implement on a large scale.

FTIR spectrometry was used to measure the concentration of BHBA and Ac in milk to detect SCK. De Roos *et al*. (2007) reported that using thresholds of 0.15 mM for Ac and 0.10 mM for BHBA, high values for Ac or BHBA were detected with a sensitivity of 69 to 70%, a specificity of 95%, with 25 to 27% false positives and 6 to 7% false negatives. In the research reported by Van Knegsel *et al*. (2010), the reference test for hyperketonemia was defined as a plasma concentration of BHBA of ≥1.2 mM, whereas when testing the BHBA and Ac concentrations in milk by FTIR, higher sensitivity (80%) and specificity (71 and 70%, respectively) were obtained in the detection of SCK. According to previous research, it is therefore proposed that FTIR predictions based on Ac and BHBA levels are valuable in the screening of cows for SCK.

***Fluorometric determination of BHBA levels*:** Larsen and Nielsen (2005) described the fluorometric determination of BHBA levels in milk and blood plasma. The analyses of milk BHBA concentrations were based on an enzymatic method introduced by Williamson and Mellanby (1974); followed by a second process that was coupled to the oxidation of BHBA (Guilbault *et al*., 1969).

Data obtained using this fluorometric method correlated closely to those obtained by the traditional spectrophotometric method (r=0.987). The advantages of the fluorometric determination of BHBA are that the detection results are not affected by the hemolysis of blood samples, and whole milk samples do not need to be pretreated. This assay is also easily automated to permit the handling of large numbers of samples, especially the large-scale in-line sample detection of milk (Larsen and Nielsen, 2005).

Gas liquid chromatography to test Ac levels: Ac levels in milk and serum have been analyzed by gas liquid chromatography, using N-propanol as an internal standard (Enjalbert *et al*., 2001), best threshold, sensitivity, specificity of blood Ac were 175 μM, 91.7%, 68.3%, and milk Ac were 160 μM, 91.7%, 57.4%, respectively (assuming that true positive were animals with blood BHBA concentration >1.2mM). The threshold level for Ac in milk was 0.4mM or higher for ketotic cows (Cook *et al*., 2001), and the same threshold level was used in a detection of acetone in milk by qualitative and quantitative salicylaldehyde test for diagnosis of SCK in dairy cows (Venkateswarlu and Choudhuri, 2001).

Nuclear magnetic resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS) to test Ac, BHBA levels: High-resolution NMR spectroscopy and GC-MS were used to test Ac and BHBA levels (Klein *et al*., 2010). However, in their research, Ac and BHBA were only used as biomarkers of energy metabolism of dairy cows during early lactation, and detection effect of SCK was not investigated.

Cowside test

Biosensor: In human medicine, electronic hand-held blood glucose and ketone measuring biosensors (Precision Xtra™ meter) are widely used for diabetes monitoring (Ham *et al*., 2004; Guerci *et al*., 2005), and are approved for home use for the early detection of diabetic ketoacidosis. The Precision Xtra test system includes an Abbott Precision Xtra meter, an Abbott Precision Xtra blood glucose test strip and Abbott Precision Xtra blood ketone test strip. A small amount of blood (1.5 μL) is applied to the end of the ketone test strip and the strip draw the blood into a small sample well. The ketone test strip contains BHBA dehydrogenase, which oxidizes BHBA to AcAc. This reduces NAD^+ to NADH, and NADH is then reoxidized to NAD^+ by an electron transfer mediator molecule. The electrical current generated by this conversion is measured by the meter and is directly proportional to the BHBA concentration in the sample. The meter displays the results after 10 s and BHBA results are displayed as mmol/L.

The Precision Xtra™ meter is a useful cowside ketone test for the diagnosis of SCK in postpartum dairy cows (Heuwieser *et al*., 2007; Konkol *et al*., 2009). Cows with blood BHBA levels above 1.4 mM (14.4 mg/dl) are considered positive for ketosis according to the reference provided by the manufacturer. The first report to use an electronic human BHBA meter for dairy cows described a high correlation (r^2=0.99) with BHBA concentrations determined spectrophotometrically and considered the test suitable for detecting SCK in dairy cows (Jeppesen *et al*., 2006). Iwersen *et al*. (2009) reported that the Precision Xtra test had sensitivity levels of 88 and 96% at 1.2 mM and 1.4 mM BHBA of whole blood, respectively, and specificity levels of 96 and 97%, respectively. In the study of Voyvoda and Erdogan (2010), when SCK was defined as plasma BHBA levels above 1.2mM, the sensitivity and specificity of the hand-held meter ketone testing in determining SCK were 85 and 94%, respectively; raising the threshold of the laboratory method to above 1.4 mM, the sensitivity and specificity incremented to 0.90 and 0.98, respectively.

Strip test, Pink test liquid, ketone powder test, urine ketone paper strip test: The Ketolac strip (also known as the KetoTest, and Sanketopaper in different countries) test has also been used to test the milk BHBA concentration (Larsen and Kristensen, 2010; Samiei *et al*., 2010). The diagnosis value stated by the manufacturer is 200 μM. This strip includes a support pad and reaction module. Ketolac results that indicated ≥200 μM of BHBA of milk were taken as a positive result, the sensitivity of Ketolac

for SCK was 75-88%, and the specificity was 63-93% (Jorritsma *et al*., 1998; Geishauser *et al*., 2000; Duffield *et al*., 2003). The simplicity of use of the Ketolac strip test makes it a valuable way to investigate SCK (Dirksen and Breitner, 1993).

In a previous report, milk samples were tested semi-quantitatively for AcAc using Pink test liquid (Geishauser *et al*., 2000). In this study, a BHBA concentrations of >1.4 mM in blood, and a AcAc concentration of ≥100 μM in milk were considered to indicate a positive SCK result. The sensitivity of the Pink test for SCK was 76%, and the specificity was 93%.

Cowside milk ketone powder and urine ketone paper strips (Ketostix urine strip) can also be used to diagnosis SCK (Larsen and Kristensen, 2010; Krogh *et al*., 2011). Their major components comprise sodium carbonate, ammonium persulfate, and sodium nitroprusside. The test principles are based on a simple chemical reaction, AcAc reacts with sodium nitroprusside under alkaline conditions, resulting in a color change from white to purple (Nielen *et al*., 1994). Greater the amount of ketones, the darker/purple color and the higher the degree of ketosis will be. Although they are inexpensive, rapid, and easy to use, cowside milk ketone powder and urine ketone paper strip tests have been reported to have lower sensitivity and specificity than other available tests for SCK (Geishauser *et al*., 1998; Geishauser, *et al*., 2000; Carrier *et al*., 2004), and they rely on visual interpretation of a color change.

Other indices for the detection of SCK in dairy cows

Fat and protein in milk: Cows during early lactation exist in a state of negative energy balance, which results in the cows mobilization of body fat to meet their energy requirements (Elitok *et al*., 2010). A portion of the fatty acids that are mobilized are directly incorporated into milk fat, resulting in an increase in the percentage of fat in the milk. By contrast, the percentage of protein in the milk will fall slightly in these cows because of a reduction in energy supply. Therefore, the ratio between the percentage of milk fat and milk protein is used to monitor the prevalence of SCK in herd (Eicher, 2004; Richardt, 2004; Gantner *et al*., 2009). Čejna and Chládek (2005) reported that a fat to protein ratio higher than 1.5 indicates SCK whereas a fat to protein ratio lower than 1.1 indicates mean suspected rumen acidosis. Using a serum BHBA level of 1.2 mM or higher as a threshold concentration, both the test-day fat percentage and the test-day protein percentage were significantly associated with SCK (Duffield *et al*., 1997). Krogh *et al*. (2011) reported that the specificity (analyzed with a modified Hui-Walter model) of fat to protein ratio for detection of SCK was lower [0.79 (0.77–0.81)] than the KetoLac BHB test and the KetoStix test [0.99 (0.97–0.99)].

Oleic acid and long-chain fatty acids in milk fat: In research performed by Van Haelst *et al*. (2008), the milk fatty acid profiles of cows classified as healthy or as subclinically ketotic based on a blood plasma BHBA threshold concentration of 1.2mM were compared. Subclinically ketotic cows showed an elevated level of C18:1 *cis*-9 in milk fat during the first nine weeks of lactation.

Nonesterified fatty acid (NEFA): In the research of Asl *et al*. (2011), cows with BHBA concentrations higher than 1.2 mM were classified as having SCK. The optimal cutoff point was set, by the receiver operating characteristic analysis method, to >0.26 mmol/L for NEFA in serum, with corresponding 82.54% sensitivity and 91.89% specificity for NEFA.

Correlation between the blood & milk concentrations of ketone bodies: The ketone concentrations in milk are reportedly much lower than in blood. Andersson (1984) reported that the concentration of milk Ac (MAc) was similar to that of blood Ac (BAc) relationship (r=0.96), and with a mean MAc/BAc ratio of 0.95. Enjalbert *et al*. (2001) reported that the concentration of MAc was similar to that of BAc, with a mean MAc/BAc ratio of 1.04. Andersson (1984) also found a low correlation coefficient between blood AcAc and milk AcAc (r=0.45). Geishauser *et al*. (1998) reported that the correlation between blood BHBA and milk BHBA ranged from 0.00 to 0.87. In a study reported by Enjalber *et al*. (2001), the mean milk AcAc to BAcAc ratio and the mean milk BHBA to blood BHBA ratio reported (0.40 and 0.12, respectively) were both similar to the values (0.45 and 0.13, respectively) reported by Andersson (1984). The use of milk BHBA to discriminate ketotic cows proved to be almost as reliable as BAc (Enjalbert *et al*., 2001).

DISCUSSION

Transition dairy cows experience the highest risk for production disease (Mulligan and Doherty, 2008). SCK is an important production disease of dairy cows and continue to cause significant economic losses to the dairy industry (Ileri-Büyükoğlu *et al*., 2009). The highest prevalence of SCK of dairy cows occurs in the first two months after calving (Akamatsu *et al*., 2007). The detection of cows with SCK is important with regard to treating individual cows and improving their diet, thereby, decreasing the economic losses caused by SCK in dairy cows (Oetzel, 2004). Ketone bodies levels in blood, urine, and milk can be monitored to detect SCK in cows, and to increase their chances of successful lactation.

Among the ketone bodies, Ac is volatile and present in lower concentrations, and AcAc is unstable. Therefore, BHBA is the most suitable ketone for detection. A number of cowside tests have been developed for the detection of ketone levels in serum, milk, and urine. Most of these tests lack sensitivity and specificity compared with the analysis of serum BHBA, which remains the gold standard for studying ketosis. Many thresholds (e.g. 1, 1.2, or 1.4mM) that have been proposed to distinguish healthy cows from cows with SCK (Van Haelst, *et al*., 2008; Rollin *et al*., 2010; Seifi *et al*., 2011). A definitive cutoff point for the detection of SCK is still needed.

Milk and urine ketone tests are quick and easy cowside tests; however, accurately quantifying ketone levels can prove difficult. Discrepancies between reports show that cowside tests based on a color change may be prone to subjective interpretation and are difficult to standardize (Carrier *et al*., 2004). The simplicity of the Ketolac strip test makes it a valuable method of investigating SCK. Using whole blood and a cutoff value

of ≥1.4 mM of BHBA/L of blood, the Precision Xtra test achieved a higher diagnostic performance than other cowside tests. The cowside BHBA test, which uses a hand-held meter, can replace the need to submit serum or plasma samples to a laboratory for BHBA testing, and can be used to detect individual cows with SCK or monitor the herd prevalence of SCK. The advantages of using milk instead of blood for the analysis of ketosis are the noninvasive nature of milk sampling and the convenience. The sensitivity and specificity of the Precision Xtra test was considerably lower in milk or urine. If the diagnostic performance of the Precision Xtra test using milk as a substrate could be improved, it will become a valuable test for the detection of SCK.

High milk somatic cell count levels and feeding cows mal fermented silage may inflate the results of milk ketone tests (Iwersen *et al*., 2009). Blood and milk ketone concentrations may vary during the day, and these fluctuations were found to be smaller in milk than in blood (Andersson, 1988). The effect of diurnal variations on milk ketone test results may be minimized by routinely testing milk at the same time each day (Geishauser *et al*., 2000).

Because of the high proportion of false-positive test results, there are concerns about the practical applicability of FTIR predictions of Ac and BHBA in milk for detecting SCK in cows. The fat to protein ratio is a good measure of SCK at the whole herd level, but is not sensitive enough for the individual diagnosis of cows with SCK. The test-day fat percent and the test-day protein percent, used alone or in combination, were proven to be ineffective screening tests for identifying cows with SCK (Duffield *et al*., 1997). Variations in the level of C18:1 *cis*-9 milk fat was found to be affected by lactation stage and feed composition, and the validity of using the milk C18:1 *cis*-9 concentration as a diagnostic tool for predicting SCK requires further study.

Conclusions: The gold standard diagnostic test for SCK is the measurement of blood BHBA levels in serum or plasma. The cowside BHBA test, using a hand-held meter, confers higher levels of sensitivity and specificity than other cowside tests, and can replace the need for submitting blood samples to laboratories for BHBA testing. The Ketolac BHBA test strip (with a cut-off threshold of 200 mM of BHBA in milk) is potentially useful tools for the routine monitoring of SCK in early postpartum dairy cows.

Acknowledgments: This study was supported by the Science and Technology Research Program of Department of Education of Heilongjiang Province (12511030) and National Science Foundation Committee of China (31101868).

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