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A RAPID POINT-OF-CARE TESTING FOR DIAGNOSING SUBCLINICAL KETOSIS IN DAIRY COWS

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Abstract: Ketosis is a common metabolic disorder of dairy cows that occurs mainly due to negative energy balance (NEB) and its prevalence results in reduced milk yield and poor reproductive performance. The diagnosis of the ketosis in the subclinical stage will prevent metabolic deterioration and stops production losses. In the present study, an in-house bovine ketosis diagnostic kit (KetoQuant) that detects β-hydroxybutyrate (BHB) in milk samples was developed and its diagnostic sensitivity and specificity were estimated in comparison with a commercially available milk strip test. Of 702 milk samples screened, using 0.2 mmol of BHB as a cut-off for diagnosing sub-clinical ketosis, the diagnostic sensitivity and specificity were estimated to be 99.5% and 99.2% respectively with the positive predictive value of 99.5% and a Kappa value of 0.988. Thus, the developed KetoQuant kit is useful as an affordable cow-side test for BHB detection in milk samples for routine monitoring of ketosis in cows.

Index Terms **- Subclinical ketosis, milk, β-hydroxybutyrate, diagnosis, bovine, colorimetry**

I. Introduction

One of the most important problems associated with postpartum cows is the negative energy balance (NEB) resulting in a metabolic condition termed as ketosis (Gross and Bruckmaier, 2019; Overton et al., 2017). The sudden change in the metabolic status during the pregnancy to lactation transition and deficient feed intake leads to the NEB in cows that are in early lactation or in postpartum period which has become a serious issue in milking industry (Gohary et al., 2016; Mostert et al., 2018; Mozduri et al., 2018). Moreover, the asymptomatic nature of the condition, reduced milk yields with sudden energy loss and associated sickness make the cows more susceptible to infections and poor reproductive performance in the later stages (Pascottini and LeBlanc, 2020; Raboisson et al., 2014; Xu et al., 2018). Unnoticed or poor management during the early days of lactation reduces the milking efficiency that directly leads to production loss that impacts economic growth (Mostert et al., 2018; Raboisson et al., 2015). Moreover, NEB impact the reproductive ability of the cows that in the recovery stage after parturition that predispose animals to acute and recurrent inflammatory attacks.

Major indications of NEB are drop in milk yield, reduction in body weight, inappetence with occasional neurological signs and altered blood levels of β-hydroxybutyrate (BHB), a short chain fattry acid that belong to the group, non-esterified fatty acid (NEFA), gluconeogenic precursors, rumen-choline level and somatotropins (Ceciliani et al., 2018; Shahzad et al., 2019). In general, the increase in blood BHB leading to acetonemia in severe ketosis condition is classified as clinical ketosis. While the increase in blood BHB without clinical signs of illness is termed as subclinical ketosis. Nevertheless, financial damages due to subclinical ketosis or NEB can be minimized by constant or early detection and treatment of cows in the lactation period. Among the various cellular indicators that are altered, the thresholds of ketone bodies such as BHB, acetoacetate (AA), and acetone (Ac) has been used for the identification of clinical condition and its severity. Clinical ketosis is well defined as having BHB blood level of ≥2.5 mmol/l (26.2 mg/dl) and generally affects up to 15% of cows, whereas sub-clinical ketosis begins at ≥1.2 mmol/L (12.4 mg/dl), and shows a prevalence of over 40% of cows in contemporary commercial herds. Apparently normal cows had about 150 µmol/L average concentrations of BHB in the milk which was about ten times lower than blood concentrations. These levels increased up to 1mmol/L in clinical ketosis (Benedet et al., 2019; Jezek et al., 2017). The statistically profound positive equavalence between the concentration of BHB in blood and milk was found during NEB or ketosis. Hence, identification of milk ketosis was found to be relevant and encouraging because of its ease of sampling and the possibility for routine monitoring without any invasive method.

Existing methods for bovine ketosis diagnosis depend on the availability of testing kits and the affordability of the animal owners. For instance, test kits that are in use are Rothera's powder, ketone meters, acetone or acetoacetate test strips, colorimetric and spectrophotometric methods (Oetzel, 2004; Overton et al., 2017). Moreover, researchers have also developed testing methods with super-specific identification of molecules using quantum dots phosphor particles (Weng et al., 2015a), Fourier-transform infrared spectroscopy (FTIR) based methods (Pralle et al., 2018), Nuclear magnetic resonance (NMR) (Basoglu et al., 2020) and microfluidics technologies (Weng et al., 2015b). Despite demonstrable diagnostic accuracy, drawbacks such as high costs, high turnaround times, and requirement of expertise make them less attractive for field applications. Hence, in order to develop a portable a milk-based ketosis diagnosis method has been developed based on colorimetric detection that demonstrated the limit of detection (LOD) in the milk samples equivalent to commercial kits. The developed kit substantiated with good sensitivity in diagnosing complex milk samples, and this platform can be used in testing other body fluid that are of clinical interest. The developed method provides a instant, sensuous and assured tool for the continual observation of cows for subclinical ketosis as a point-of-care (POC) assay.

II. Materials and methods

II.1. Materials

BHB dehydrogenase (BHBDH), BHB, Tetrazolium salt, Nicotinamide adenine dinucleotide, All other reagents used were of analytical grade.

II.2. Assay principle and standardization

The assay is based on the chemical reaction between BHBDH enzyme with the substrate, BHB resulting in color development which is proportionate to the BHB level in the reaction mixture (Figure 1A). For the detection of ketosis, 50µl of reaction mixture containing NAD+ (5 mg in 50 µL of 0.1M phosphate buffer, pH 7.5), BHBDH (10 mg in 1000 µL of 0.1 M phosphate buffer, pH 7.5), tetrazolium salt (1mg in 50ml of 0.1 M Tris buffer, pH 7.5) was mixed with equivalent part of milk sample. The color developed in 10 seconds was read at 450nm in the spectrophotometer. For the initial standardization, varying known concentrations of BHB were spiked in milk samples which were tested (commercial assay) negative for BHB and the standard graph was drawn and the amount of BHB and color developed was noted for reference color chart preparation.

For the kit preparation as a POC, 50µl of the reaction mixture was aliquoted in 0.2ml vials. For the field milk sample testing, one drop of milk (~50µl) was added in the tube, mixed well and the purple color developed in 10 seconds was read visually compared with the color chart made using known BHB during standardization as above. For the kit validation, the results were compared with Porta milk BHB strips as per the manufacturer's instructions. Briefly, the milk sample was directly placed onto a test strip or the strips were dipped into the milk collected in a clean labeled container and the excess milk was shaken off; the color developed was compared to color chart after 60 seconds.

II.3. Field samples testing and data analysis

A total of 702 cows from various herds in the vicinity of Chennai were used for this study. Milk collected from cows that are in early lactation (up to 6 weeks) after calving. For this purpose, the herds were visited weekly. The milk samples were collected and tested using in-house developed kit and Porta milk BHB strips. Both the in-house developed BHB test and the commercial test displayed the results to classify cows as normal, or sub-clinical or clinical ketosis based on the color intensity. Data analyses compared whether or not the BHB tests categorized the same cows as ketotic or non-ketotic or discordant on their ketosis status. Further, the developed kit compared with the Porta milk BHB test strips and various assay parameters were analyzed using the below formula using MedCalc Software (Version 15.0).

- Prevalence = Number of cases/ Population size * 100
- Sensitivity = True positives / (True positives + False negatives) * 100
- Specificity = True negatives / (True negatives + False positive) * 100
- PPV = True positives/ (True positives + False positive) * 100
- NPV = True negatives/ (True negatives + False negatives) * 100

The agreement between test methods was calculated by kappa value (Graph Pad Prism, version 8.0). Further, from samples classified as having clinical or subclinical ketosis in the above mentioned tests done with milk (n=185), additionally blood BHB levels were also monitored with Abbott Laboratories blood ketone meter (Abbott Park, USA). The results were expressed as average ± SD for denoting blood BHB level.

III. Results and Discussion

For the ketosis measurement using the in-house developed kit, the BHB level was analysed by the chemical reaction of BHBDH catalyzed oxidation of BHB to AA (Harano et al., 1984) using NAD under alkaline conditions releases a purple-colored product which can be compared with the color chart (Figure 1A and 1B). A linear rise in the color in the reaction mixture was observed with good correlation (R^2=0.98) from the BHB concentration ranges from 0.05 mM to 2mM (Figure 1C).

Of the 702 cows studied, 35% were primiparous and 65% were pluriparous. The results of in-house developed milk ketosis assay demonstrated that 13.3% of animals had clinical ketosis (>0.5mM) and 63.2% of animals (>0.2mM) had subclinical ketosis. The in-house diagnostic kit demonstrated the sensitivity and specificity of 99.5% and 99.2% respectively for detecting sub-clinical ketosis with the PPV of 99.55% and the NPV of 99.22% and accuracy of 99.43% for subclinical ketosis with 0.2mM cutoff (Table 1A and B). With the cut off set at 0.5mM for detecting clinical ketosis, the diagnostic sensitivity was 97.9%; specificity of 99.8% the PPV and NPV were found to be 98.95% and 99.67% respectively with an accuracy of 99.57% (Table 2A

and B). The Kappa statistics value for observer agreement was 0.98 (SE of kappa = 0.006, 95% confidence interval: From 0.976 to 1.000).

For further confirmation, those animals which showed the presence of BHB in milk also had higher levels in the blood. The analysis in animals with clinical ketosis (n = 94) displayed the blood BHB level of 3.0 ± 0.5 mM, but the equivalent milk BHB level was found to be >0.5 mM (maximum color in milk assays). Whereas, the cows tested as subclinical ketosis (n=91) displayed the blood BHB level of 1.8 ± 0.5 mM with a milk BHB level of 0.31 ± 0.1 mM.

Hence, the present study demonstrated a proof-of-concept for a portable bovine ketosis diagnosis kit for the continuous monitoring of herds. Thus, the result provides information that the in-house developed kit is a useful cow-side milk ketone test for the detection of subclinical ketosis in postpartum dairy cattle. Hence, the developed kit may be used for monitoring cows for subclinical ketosis regularly. The study results demonstrated the likelihood of minimal concentrations to detect subclinical ketosis is 0.05 mM of BHB in milk samples with the specificity equal to that of commercial assay kit.

It has been reported that the concentrations of blood and milk ketone bodies may vary during the day and the fluctuations in milk were smaller than in blood (Geishauser et al., 2000). Hence, a variation on milk ketones and tremendous changes during the postpartum can be monitored by continuous testing. The existing methods for ketone detection include sodium nitroprusside strips that detect AA (Chong and Reineke, 2016), spectrophotometric (Zhang et al., 2009), fluorometric (Larsen and Nielsen, 2005) and electrochemical devices (Bach et al., 2016; Helmersson-Karlqvist et al., 2019) that require instrumentation, and microfluidic biosensor (Weng et al., 2015b) and quantum dots (Weng et al., 2015a) test that requires preparation and characterization of particles. Even though the LOD demonstrated by these methods is lower, the need for the sophisticated facility and maintenance of systems and devices was quite expensive making these methods less field applicable.

The blood levels of BHB were much higher than those in milk samples in animals with clinical or subclinical ketosis. This is due to the utilization of BHB in mammary epithelial cells for fatty acid synthesis in high milk yielders (Zhang et al., 2015). Conversely, the present study results demonstrating high blood BHB levels with many-fold reduction in milk BHB corroborate with other studies that reported high correlation coefficients comparing the blood BHB with blood NEFA and between blood and milk BHB (Jezek et al., 2017; Samiei et al., 2010).

However, knowing the fact of susceptibility to pathogens because of persistent ketosis (Grinberg et al., 2008; Janosi et al., 2003), the simple metabolic disorder may be misinterpreted as mastitis due to reduced milk yield. Thus, the major problem of misdiagnosis leads to the unwanted administration of antibiotics to the condition of ketosis where simple feed management is the key treatment. Moreover, the persistent or resumption of postpartum NEB are majorly associated with reproductive disorders, and decreased reproductive performance in dairy cows (Shin et al., 2015). Due to these facts, to aid continuous ketosis monitoring, a simple and POC colorimetric diagnostic was developed which exhibits good sensitivity and specificity in diagnosing field milk samples.

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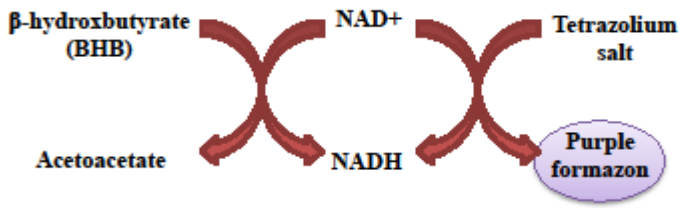
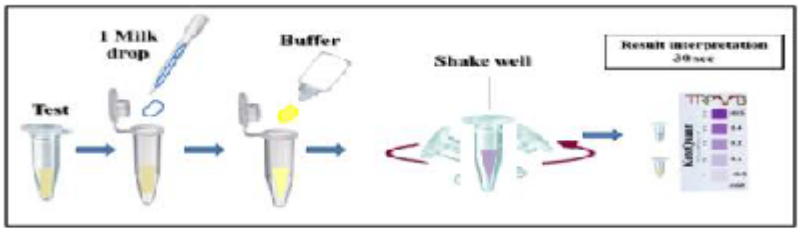
Figure 1

A



B



C

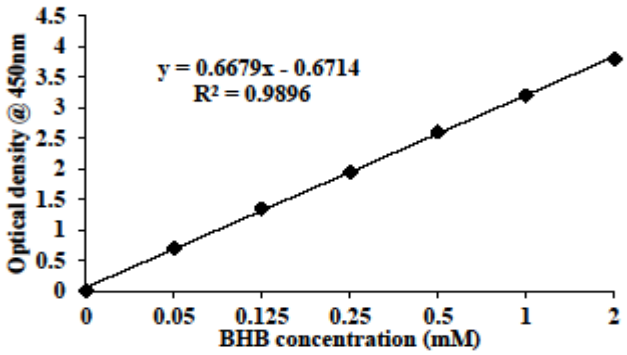


Figure 1: A). The enzymatic reaction BHBDH oxidation of BHB in the presence of co-factors. B). The procedure of assay performed in the field samples. C) The correlation graph of BHB spiked in the milk samples and optical density values.