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Development of a simple single reagent assay for rapid detection of bovine ketosis

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Abstract
Ketosis is a metabolic disorder of high-yielding dairy cows especially during early lactation due to negative energy balance. Bovine ketosis is an economically important disease due to heavy loss in production, diagnosis, treatment and risk of secondary diseases like mastitis, metritis etc. Numerous diagnostic kits and cow side tests are commercially available for the detection of bovine ketosis but the cost is high. Cheaper traditional method like conventional Rothera's test and Ross test is time consuming and demand freshly prepared solutions. The aim of this study was to develop a simple single reagent test for rapid detection of bovine ketosis. A total of 60 Gir cattle in early lactation were used in the study and based on serum BHBA concentration they were categorized into healthy (n=40), sub-clinical (n=12) and clinical (n=8) ketotic animals. A single reagent (30% Glycine, 15% Lactose, 50% Disodium hydrogen phosphate, and 5% Sodium nitroprusside into a fine homogeneous powder) test was developed and compared with Rothera's test and Ross test for sensitivity. The developed single reagent test was found more sensitive than Rothera's and Ross test in sub-clinical ketotic animals. This reagent can detect ketone bodies in urine at a concentration as low as 5 mg/dL. Hence, the developed single reagent test is a simple and sensitive method capable of rapid detection of bovine ketosis using urine samples.

Keywords: Gir, ketosis, BHBA, cattle, milk, urine

1. Introduction
Dairy cows often suffer from ketosis, a common metabolic disorder, especially in the first few weeks of lactation. Immediately after parturition, there is a rapid demand of energy for lactogenesis and galactopoiesis. The third to fifth week of lactation is the period of peak yield in dairy cattle and it is the period when high yielding animals generally suffer from negative energy balance (NEB). As a result of NEB, fat starts mobilizing to liver for catabolism to meet the energy demand (Leek and Reece, 2014; Seymour *et al*., 2019) [1, 8]. Ketone bodies, namely beta-hydroxybutyrate (BHBA), acetoacetate and acetone are formed in the process and may result into ketosis if the serum ketone body levels are significantly elevated. Ketosis may have a clinical or subclinical presentation in dairy cows. A clinical ketosis (CK) is characterized by an increased serum BHBA concentration (>2.9 mM) with clinical signs like anorexia, depression, pica, abnormal licking, in-coordination with abnormal gait, bellowing and aggression. However, subclinical ketosis (SCK) is characterized by an elevated level of serum BHBA (1.2-2.9 mM) without any marked clinical signs (Leblanc, 2010; Duffield *et al*., 2009) [5, 2]. Though, the mortality is low in bovine ketosis, yet it is considered as an economically important disease because it is associated with heavy production and reproductive losses. Additionally, ketosis increases the risk of other secondary diseases like mastitis, metritis etc. Sub-clinical ketosis is considered more important than clinical ketosis as it goes undetected in herd most of time and incur huge economic losses. Furthermore, there is huge losses for diagnosis and treatment of clinical as well as sub clinical ketosis. Hence, strategic programmes for hyperketonemia surveillance in dairy herds have been suggested (LeBlanc *et al*., 2005; Ospina *et al*., 2013) [4, 7] to cope up with production losses.
The ketone bodies, namely, BHBA, acetoacetate and acetone, account for 70%, 28%, and 2% of serum ketone bodies, respectively. Ketone bodies can easily diffuse across the cell membrane to provide energy (Hillreiner *et al*., 2016) [3]. Hence, it is detected in serum, urine and even milk of ketotic animals. Albeit, the gold-standard test to diagnose hyperketonemia is measuring blood BHBA concentration (van der Drift *et al*., 2012, Oetzel, 2004) [6] many biomarkers has been reported for early detection of ketosis. Now a days, electronic handheld blood ketometers have been developed that can measure blood ketone body levels and offers practical and time-related advantages (Oetzel, 2004) [6].

However, higher cost of such kits and instruments makes it less approachable for dairy farmers. Strategic programs for hyperketonemia surveillance in dairy herds have been suggested (LeBlanc *et al*., 2005; Ospina *et al*., 2013) [4, 7] to cope up with production losses due to clinical and subclinical ketosis. Hence, this study was designed to develop a simple single reagent test for rapid detection of bovine ketosis.

2. Materials and Methods

2.1 Chemicals and reagents

All the kits, chemicals, and reagents used in the present study were of high purity and analytical grade. All chemicals were obtained from Himedia (Mumbai, India), Sigma-Aldrich (St. Louis, MO, USA) and SRL Pharma (Telangana, India). The serum BHBA estimation was done with Cayman kit (USA).

2.2 Animals

A total of 60 Gir cows in the early stage (3-5 weeks) of lactation were used in this study. The animals used in the study were reared under standard feeding and management protocols in an organized cattle breeding farm at Junagadh district of Gujarat state in India.

2.3 Sample collection

Blood samples were collected into blood collection vials with clot activator from all the animals and serum was harvested for estimation of serum BHBA concentration using BHBA assay kit to classify animals into healthy, subclinical ketotic and clinical ketotic groups.

Urine samples were collected in a clean and sterile plastic urine collection container and immediately transferred to the laboratory for the assay. The obtained samples were tested qualitatively for ketone bodies using the single reagent test and Rothera's test whereas it was tested quantitatively using modified Nitroprusside test.

For collection of milk samples, udder was washed and wiped by clean towel before milking. First few streaks of milk were discarded and then milk samples were collected in clean containers. The collected samples were subjected to Ross test for detection of ketone bodies.

2.4 Development of a qualitative single reagent test

To develop a simple and rapid test for the detection of ketone bodies in urine, a single reagent was prepared by combining and crushing Glycine, Lactose, Disodium hydrogen phosphate, and Sodium nitroprusside into a fine homogeneous powder. On a clean glass slide, 5 drop of standard/ urine sample was added to a pinch of prepared powder and allowed to develop color for 2 min. Development of purple color indicated the presence of ketone bodies. A gradient (0-100 mg/dL) of Acetone in distilled water was prepared to serve as standard. Experiments were carried out to find a suitable combination of reagents that gave best result using acetone standard solution.

2.5 Detection of ketone bodies in urine and milk

Ketone bodies were qualitatively detected in urine and milk samples using Rothera's test and Ross test procedure, respectively and compared with the detection of ketone body in urine by developed single reagent test. Furthermore, the urine ketone body concentration was estimated quantitatively using modified nitroprusside test using acetoacetate as standard.

2.6 Quantitative modified nitroprusside test

The modified Nitroprusside test is a sensitive colorimetric assay for the quantitative evaluation of ketone bodies in urine. The test was created by mixing 20 µL of glycine (10-50 mM), 100 µL of nitroprusside (5-10% in 0.1 M Na_2HPO_4), and 10 µL of lactose (1-5% w/v) to create a purple complex. The curve generated using 70 µL of standard acetoacetate (5-100 mg/dL) was used to compute the ketone body concentration of the urine samples.

3. Results and Discussion

3.1 Serum BHBA levels

The serum BHBA concentration is considered as a gold standard for detection of ketosis in dairy cows. Out of the 60 animals screened by BHBA assay kit, forty (67%) animals were categorized as healthy, twelve (20%) as sub-clinical ketotic and eight (13%) as clinical ketotic animals, based on serum BHBA levels (Figure. 1).

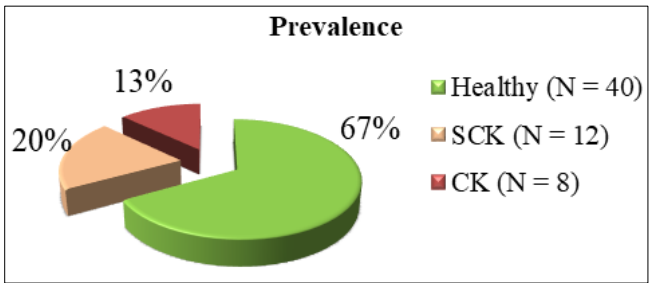

Fig 1: Prevalence of healthy, sub-clinical ketotic and clinical ketotic animals based on serum BHBA concentration between three to five weeks of lactation.

3.2 Development of single reagent test

A single reagent containing combination of Glycine, Lactose, Disodium hydrogen phosphate, and Sodium nitroprusside in a proportion of 30%, 15%, 50% and 5% w/w, respectively gave the maximum color. The acetone solution, rapidly exhibited a shift in color to lavender-purple when added to homogenous powder of single reagent. The acetone solution, with increasing concentrations from 5-100 mg/dL showed a gradual increment in the color intensity. Once the ideal combination was achieved the powdered single reagent was tested with urine samples. Based on the intensity of purple color development the test was scored between 0-4 (Figure. 2). If no purple color developed, the test was scored as zero and considered as negative. Similarly, for faint purple, moderate purple, purple and dark purple the scores were 1, 2, 3 and 4, respectively. The principle of this test is based on Rothera's test with modifications. When Acetone reacts with sodium nitroprusside at moderate alkaline pH (~8.2) in presence of glycine and lactose rapidly forms a purple-colored complex. Rather than making fresh solutions of sodium nitroprusside and disodium hydrogen phosphate we used chemicals (Glycine, Lactose, Disodium hydrogen phosphate and Sodium nitroprusside) as such and crushed it into fine powder. The glycine and lactose were added to enhance color of the reaction. The single reagent so formed in a homogenous fine powder is stable at room temperature for months and it can be used instantly for rapid detection of ketone bodies in urine.

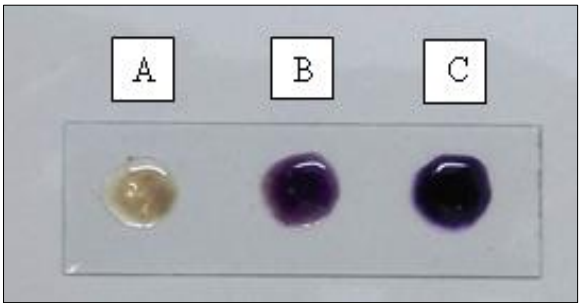

Fig 2: Solid phase single reagent test, A: Negative (score 0), B: Moderate purple (score 2), C: dark purple (score 4).

3.3 Qualitative detection of ketone bodies in urine and milk

Qualitative detection of ketone bodies in urine using the developed single reagent test showed that the sensitivity of this test was 100% in all clinical ketotic and sub-clinical ketotic urine samples. Similarly, the sensitivity of Rothera's test and Ross test was 100% for clinical ketotic animals. However, the sensitivity of Rothera's test in urine and Ross test in milk were 66.66 and 41.66%, respectively for sub-clinical ketotic animals (Table.1). Few of the urine and milk sample from sub-clinical ketotic animals did not give positive reaction with Rothera's and Ross test, respectively. However, the same sample tested positive with single reagent test. The single reagent is able to detect the presence of ketone bodies in urine. Albeit, it is difficult to distinguish clinical and sub-clinical ketosis merely based on color development using this test. One must look for clinical signs and symptoms to distinguish between the two. Again it is just a rapid test to detect bovine ketosis using urine samples, qualitatively.

Table 1: Comparative sensitivity of Rothera's test, Ross test and single reagent test to detect ketone bodies in urine or milk of sub-clinical ketotic animals.

Sr. no. of Animal	Rothera's test (Urine)	Ross test (Milk)	Solid phase single reagent test (Urine)
1	+	-	++
2	-	-	+
3	-	-	+
4	+	+	++
5	+	+	+
6	+	-	++
7	+	-	++
8	++	+	+++
9	-	+	++
10	+	+	++
11	-	-	+
12	+	-	+

3.4 Quantitative estimation of ketone bodies in urine

The mean ketone body concentrations of healthy, sub-clinical ketotic and clinical ketotic animals were 5.04±0.46, 42.51±4.89 and 74.29±7.59 mg/dL, respectively. We observed a significant (p<0.05) difference in urine ketone body concentration among the groups (Figure.3). A highly significant positive correlation (p<0.001; r = 0.88) was recorded between serum BHBA and urine ketone body concentration. The lowest detection level of single reagent test was found to be 5mg/dL in urine. Though the reagent was able to detect the ketone body at a concentration of 5mg/dL the color developed was very faint. A distinct color develops when the score is between 2 to 4.

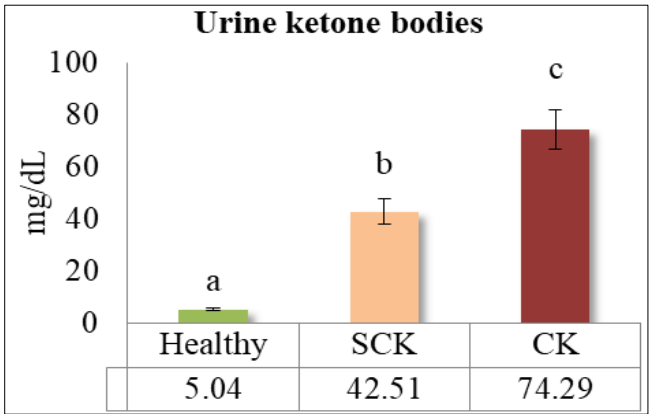

Fig 3: Urine ketone bodies (Mean ± SE) of healthy, sub-clinical ketotic (SCK) and clinical ketotic (CK) animals. Small superscripts (alphabet) indicate level of significance (P < 0.05) among the groups.

4. Conclusion

The single reagent (30% Glycine, 15% Lactose, 50% Disodium hydrogen phosphate, and 5% Sodium nitroprusside) test can detect ketone bodies in urine with high sensitivity. Hence, the developed single reagent test is a simple and sensitive method capable of rapid detection of bovine ketosis using urine samples.

5. Acknowledge

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6. Conflict of Interest: None.

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