

<https://doi.org/10.17221/69/2019-VETMED>

## Comparative study on different field tests of ketosis using blood, milk, and urine in dairy cattle

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**Citation:** Faruk MSA, Park B, Ha S, Lee S, Mamuad LL, Cho Y (2020): Comparative study on different field tests of ketosis using blood, milk, and urine in dairy cattle. *Vet Med-Czech* 65, 199–206.

**Abstract:** This study assessed the diagnostic performance of five field tests for ketosis in the blood, milk and urine in dairy cows. Samples were collected simultaneously from 102 dairy cows to determine the accuracy of the test results. The concentration of  $\beta$ -hydroxybutyrate (BHBA) in the blood was measured quantitatively by using a portable ketone test kit. The milk and urine samples were analysed semi-quantitatively using different commercial test kits and urine sticks. The animals were categorised into a subclinical ketosis (SCK) and clinical ketosis (CK) group based on the BHBA concentration in the blood. The diagnostic performance of the milk and urine test kits was compared with the blood test kit. In the blood test, out of 102 cows, 27 and 19 cows were diagnosed with SCK and CK, respectively. The percentage agreement (kappa value) for the three different milk tests and the urine test kit was 43% ( $\kappa = -0.181$ ), 72% ( $\kappa = 0.243$ ), 63% ( $\kappa = 0.065$ ) and 64% ( $\kappa = 0.163$ ), respectively, for the SCK detection, which indicates lower sensitivity. However, a strong agreement with 88% ( $\kappa = 0.612$ ), 85% ( $\kappa = 0.505$ ), 86% ( $\kappa = 0.528$ ) and 91% ( $\kappa = 0.753$ ) was observed in the three milk tests and the urine test, respectively, for the CK diagnosis, showing a higher sensitivity. For the diagnosis of the SCK and CK, all the tested kits are generally applicable for the detection of CK in dairy cattle, but caution is needed during the interpretation of the SCK data due to the low sensitivity of the milk and urine tests for the SCK detection. Furthermore, this study suggests that milk and urine samples can be used as alternatives for the diagnosis of CK in replacement of blood tests.

**Keywords:**  $\beta$ -hydroxybutyrate; clinical ketosis; subclinical ketosis; diagnosis; semi-quantitative determination

The reduction of the occurrence, severity and consequences of a negative energy balance in the early postpartum period has become an important issue for the dairy industry. One of the consequences of a negative energy balance is ketosis (Andersson 1988). This condition occurs in the transition pe-

riod of cattle due to disturbances of the carbohydrate and fat metabolism characterised by increased concentrations of ketone bodies in the blood (ketonemia), milk (ketolactia), urine (ketonuria), and other body fluids. The major ketone bodies are acetone, acetoacetate, and  $\beta$ -hydroxybutyrate (BHBA).

Supported by a grant from the National Research Foundation of Korea (NRF-2018R1C1B6004589), and by the Cooperative Research Program for Agriculture Science and Technology Development (PJ01269704) funded by the Rural Development Administration, Republic of Korea.

<https://doi.org/10.17221/69/2019-VETMED>

Ketosis can be classified as subclinical ketosis (SCK) or clinical ketosis (CK) based on the concentration of the ketone bodies in the tissues, bodily fluids, and its clinical signs. The prevalence of SCK is comparatively higher than CK (Duffield 2000). During the SCK period, the milk production from each cow decreases by 1 l to 4 l daily (Gustafsson and Emanuelson 1996). In addition, cows with SCK have an increased risk of postpartum diseases, such as displaced abomasum, cystic ovarian disease (Gustafsson and Emanuelson 1996), clinical ketosis (Duffield et al. 1999), and metritis (Francos et al. 1997). The losses caused by undiagnosed subclinical ketosis exceed the losses caused by clinical ketosis. On the other hand, losses due to subclinical ketosis can be minimised by the early detection and treatment of the affected cows (Geishauser et al. 2000).

The early stage detection of metabolic diseases is important for the optimal herd management to prevent outbreaks of clinical diseases. Laboratory tests are usually used for ketosis diagnosis; however, it is labour-intensive, time-consuming, and requires skilled personnel. Hence, commercialised field test kits have been developed to diagnose ketosis, which are simple, convenient, accurate, and provide an immediate result (Carrier et al. 2004). Blood samples are commonly used in the field for the diagnosis of ketosis, but sometimes clinicians and farm producers prefer non-invasive samples, such as from the milk or urine. In addition to the blood test, the results of the milk and urine tests also have an additional diagnostic value for the detection of ketosis. Evaluations of these cow-side tests have been reported (Geishauser et al. 1998; Jorritsma et al. 1998), but there are few reports in the literature that provide extensive data for comparing the concentrations of the ketone bodies in the blood, milk, and urine samples from the same animal. Therefore, the goal of this study was to evaluate the comparative diagnostic performances among different ketosis field test, based on the BHBA concentration in the blood, milk, and urine samples.

## MATERIAL AND METHODS

### Cows

To evaluate the diagnostic performance of the ketosis tests, 102 Holstein cows at the early lactation

period (0 day to 60 days in milk, DIM) were selected from fifteen dairy herds. The animals were kept in a free-stall housing system and fed the TMR diet. Drinking water was available *ad libitum*. The samples were obtained simultaneously from each cow, we analysed a total of 102 blood and milk samples and 78 urine samples. All the tests were performed by veterinary clinicians throughout the study.

### Blood sampling for the measurement of BHBA

Approximately 5 ml of blood was collected from the coccygeal vein of each animal using a 19-gauge, 2.54 cm needle and a 10 ml syringe. The BHBA testing was conducted according to a portable ketone test meter (FreeStyle Optium Neo H-ketone meter; Abbot Diabetes Care Ltd., Witney, Oxon, UK) and the respective strip (Precision extra ketone test strips) instructions and was performed immediately after the blood collection. For each sample test, a drop of blood was placed in the chamber of the ketone strip and one must then wait for 10 seconds. During this time, a chemical reaction occurs within the test strips as follows: the BHBA in the blood sample is oxidised to acetoacetate in the presence of the BHBA enzyme dehydrogenase, with a concomitant reduction of  $\text{NAD}^+$  to NADH. The NADH is then re-oxidised to  $\text{NAD}^+$  by a redox mediator. This chemical reaction produces electrons and generates an electric current, which is directly proportional to the concentration of the BHBA (Weng et al. 2015; Ghanem et al. 2016). Then, the meter showed the concentration of the BHBA and the value was recorded. A blood BHBA of 1.2 mmol/l to 2.9 mmol/l was diagnosed as SCK, and the clinical ketosis was diagnosed as  $\text{BHBA} \geq 3.0$  mmol/l (McArt et al. 2012; Al Faruk et al. 2018).

### Milk sampling for the measurement of BHBA

The composite milk samples, including milk from all quarters, were tested for BHBA using three different commercial test kits: A Porta BHB strip (USA), a Healthmate BHB strip (Republic of Korea) and a Wizcheck BHB strip (Republic of Korea). The test procedures of these three commercial milk

<https://doi.org/10.17221/69/2019-VETMED>

test kits were very similar. The milk sample was collected and a test strip was then dipped into the milk and waiting for 60 seconds. Subsequently, the test strip colour was compared with the standard colour chart and the value of the milk BHBA was recorded. Based on the colour of the milk test strip, the BHBA levels were denoted as 0, 50, 100, 200, 500, and 1 000  $\mu\text{mol/l}$  in the milk according to the manufacturer's description. Dubious colour changes were categorised as negative in the case of all three milk tests. The BHBA concentrations of < 100, 100 to 200, and > 200  $\mu\text{mol/l}$  in the milk were diagnosed as normal, SCK, and CK, respectively (Geishauser et al. 1998).

### Urine sampling for the measurement of BHBA

Urine samples were collected aseptically from each animal by using a urinary catheter and then analysed it using urine stick (URiSCAN10 SGL strip; YD Diagnostics, Yongin, Republic of Korea). The urine samples were tested on the farm by dipping the test strip into the fresh urine, waiting for 60 s, and then the strip colour was compared with the recommended standard colour chart. The scores were –,  $\pm$ , 1+, 2+ and 3+ for the normal, trace, positive, high positive, and very high positive BHBA levels, respectively. The positive test results were defined as  $\pm$  or 1+ for the SCK, and 2+ or 3+ for the CK.

### Data management and statistical analysis

The diagnostic performance of all the milk and urine tests were evaluated by comparing it with the blood BHBA concentration, and the sensitivity (proportion of the true positive cows testing positive) and specificity (proportion of the true negative cows testing negative) were determined for all the ranges of the milk and urine tests. The *P*-value was calculated using online Open Epi software (Sullivan et al. 2009) and the differences were considered to be significant if *P* < 0.05. The performance of the milk and urine test kits was compared with that of the blood test kit by calculating the percentage agreement and the  $\kappa$  value as follows:

$$\% \text{Agreement} = \frac{[\text{agreed pos} + \text{neg}/\text{total tests}]}{(n = 37)} \times 100 \quad (1)$$

$$\kappa = \frac{[Pr(a) - Pr(e)]}{[1 - Pr(e)]} \quad (2)$$

where:

*Pr(a)* – relative observed agreement between the tests;

*Pr(e)* – probability that the agreement is due to chance.

If the tests are in complete agreement, then  $\kappa = 1$  and  $\kappa \leq 0$  if there is no agreement. The interpretation of the  $\kappa$  value was based on the guide provided previously (Landis and Koch 1977): poor ( $\kappa = 0.00$ ); slight ( $0.01 < \kappa < 0.20$ ); fair ( $0.21 < \kappa < 0.40$ ); moderate ( $0.41 < \kappa < 0.60$ ); substantial ( $0.61 < \kappa < 0.80$ ); almost perfect agreement ( $0.81 < \kappa < 1.00$ ).

## RESULTS

The blood ketone test kit was compared with the milk and urine test kits for the detection of SCK (Table 1). The blood test was determined to be the gold standard for diagnosis of ketosis. Among all of the experimental samples, the blood test kit detected 27 samples as positive for SCK in which the Porta BHB milk test kit detected 19 samples as negative. The blood test kit also detected 75 samples as negative for SCK, whereas 36 samples were negative by both the blood and Porta BHB milk kit for the SCK detection. Accordingly, the test agreement between the blood test kit and the Porta BHB milk test kit was 43% (44/102), and the  $\kappa$  value was –0.181.

In comparison between the blood and Healthmate BHB milk test kit, the blood test kit identified 16 more samples as positive for SCK compared to the Healthmate BHB milk test kit, whereas 13 positive samples by the Healthmate BHB milk test kit were negative by the blood test. The test agreement between the two tests was 72% (73/102), and the  $\kappa$  value was 0.243. In comparison between the blood and the Wizcheck BHB milk test kit, the blood test identified 18 more positive samples for SCK, whereas twenty positive samples by the Wizcheck BHB milk test kit were negative by the blood test. The agreement between the Wizcheck BHB milk kit and the blood test was 63% (64/102), and the  $\kappa$  value was 0.065. In the case of the urine test, the URiSCAN strip detected 27 samples as a positive, in which 17 samples were negative by the blood test. The agreement between the blood and urine test kit was 64% (50/78), and the  $\kappa$  value was 0.163.

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Table 1. The comparative performance of the blood ketone test kit with the three milk and urine test kit assays in detecting the subclinical ketosis

		Blood test		Total	%Agreement ( $\kappa$ value)	
		positive	negative			
Milk test	<b>Porta</b>					
	positive	8	39	47	43% ( $\kappa = -0.181$ )	
	negative	19	36	55		
	total	27	75	102		
		<b>Healthmate</b>				
	positive	11	13	24	72% ( $\kappa = 0.243$ )	
	negative	16	62	78		
	total	27	75	102		
		<b>Wizcheck</b>				
positive	9	20	29	63% ( $\kappa = 0.065$ )		
negative	18	55	73			
total	27	75	102			
Urine test	<b>URiSCAN Strip</b>					
	positive	10	17	27	64% ( $\kappa = 0.163$ )	
	negative	11	40	51		
	total	21	57	78		

The blood test was undertaken using a FreeStyle Optium Neo H-ketone meter and the respective strip (Precision extra ketone test strips)

Table 2. The comparative performance of the blood ketone test kit with the three milk and urine test kit assays in detecting the clinical ketosis

		Blood test		Total	%Agreement ( $\kappa$ value)	
		positive	negative			
Milk test	<b>Porta</b>					
	positive	13	6	19	88% ( $\kappa = 0.612$ )	
	negative	6	77	83		
	total	19	83	102		
		<b>Healthmate</b>				
	positive	11	7	18	85% ( $\kappa = 0.505$ )	
	negative	8	76	84		
	total	19	83	102		
		<b>Wizcheck</b>				
positive	11	6	17	86% ( $\kappa = 0.528$ )		
negative	8	77	85			
total	19	83	102			
Urine test	<b>URiSCAN Strip</b>					
	positive	15	6	21	91% ( $\kappa = 0.753$ )	
	negative	1	56	57		
total	16	62	78			

The blood test was undertaken using a FreeStyle Optium Neo H-ketone meter and the respective strip (Precision extra ketone test strips)

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Table 2 compares the blood test kit with the three milk and the urine test kits for the detection of the CK. The diagnostic performance of the Porta BHB strip, the Healthmate BHB strip and the Wizcheck BHB strip were almost identical for the CK diagnosis. The blood test kit detected 19 samples as positive for CK, whereas 13 samples were positive in both the blood and Porta BHB milk test kit. The test agreement between the blood and the Porta BHB milk test kit was 88% (90/102), and the  $\kappa$  value was 0.612. The Healthmate BHB milk test kit identified 18 samples as positive for CK, in which 7 samples were negative by the blood test. Consequently, the test agreement between the two tests was 85% (87/102) and the  $\kappa$  value was 0.505. In comparison between the blood and Wizcheck BHB milk test kit, the blood test identified eight more positive samples for CK, whereas six positive samples by the Wizcheck BHB milk test kit were negative by the blood test. The agreement between the Wizcheck BHB milk kit and the blood test was 86% (88/102), and the  $\kappa$  value was 0.528. In comparison between the blood and urine test kit, the urine test kit detected CK in 21 samples in which six samples were negative in the blood test, whereas a total of 56 samples were negative for CK in both the blood and urine test. The test agreement between the blood and urine test kit was 91% (71/78), and the  $\kappa$  value was 0.753.

The sensitivity and specificity of the milk and urine test kit were also evaluated in the SCK (Figure 1A) and CK (Figure 1B) animals. For the detection of the SCK, the sensitivity of the Porta and Wizcheck BHB milk test kits were 29.6% and 33.3%, respectively, which was slightly lower than the Healthmate BHB milk kit. The specificity of the Porta, Healthmate and Wizcheck BHB milk test kits for the SCK diagnosis were 48.0%, 82.7%, and 73.3%, respectively. The *P*-value of the sensitivity and specificity of the three milk tests were 0.242 and < 0.001 for the diagnosis of SCK. The *P*-value of sensitivity (*P* = 0.242) for these three milk test kits indicated no significant difference present among the sensitivity of the three milk test kits for the SCK diagnosis. The sensitivity and specificity of the urine stick for the SCK were 47.6% and 70.2%, respectively. In the case of the CK, the sensitivity of the Porta, Healthmate and Wizcheck BHB milk kits were 68.4%, 57.9% and 57.9%, respectively, and the specificity were 92.8%, 91.6% and 92.8%, respectively. The *P*-value of the sensitivity and specificity were 0.210 and 0.934, respectively,

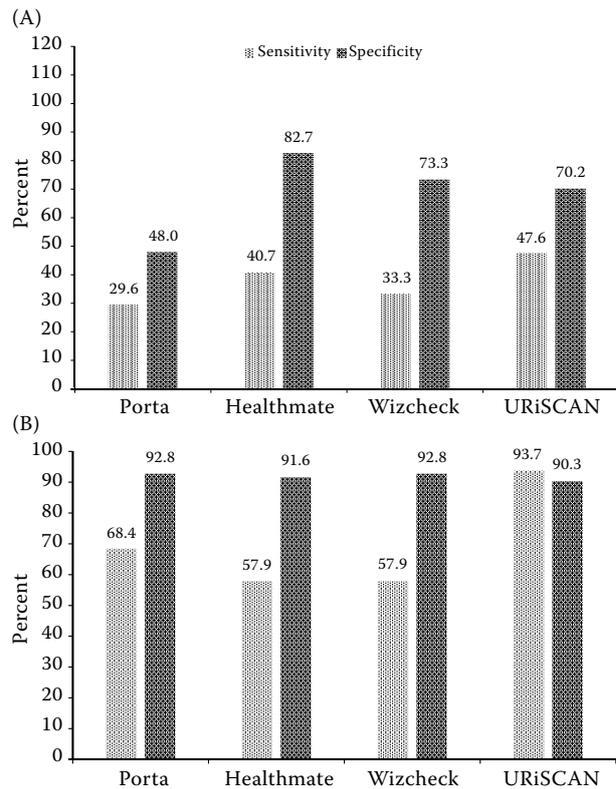


Figure 1. The evaluation of the sensitivity and specificity of the milk and urine test kit for the detection of the SCK (A) and the CK (B). The Porta, Healthmate and Wizcheck kits were used for the milk test and a URiSCAN strip was used for the urine analysis. The *P*-values of the sensitivity and specificity of the three milk tests were 0.242 and < 0.001 for the diagnosis of the SCK and the *P*-values were 0.210 and 0.934 for the CK, respectively

ly, in the three milk tests for the diagnosis of the CK. These *P*-values (*P* > 0.05) indicated having no significant difference present between the sensitivity and specificity of these three milk test kits. The higher percentage of the sensitivity and specificity was observed in the urine test for the detection of the CK.

## DISCUSSION

The blood, milk and urine samples were collected simultaneously in this study to avoid a test bias and to eliminate the possibility of time dependent changes in the physiological parameters of the blood, milk and urine. The level of the ketone body also changes with time and there are many factors that are involved with the ketone production in an animal's body. During starvation, the ketone

level will increase because, at that time, the fat store of the body will break down and form three types of ketone bodies (Grabacka et al. 2016). Although the ketone bodies formed at the time of fasting, different factors including the tissue type (skeletal muscle vs brain) during exercise and the circulating ketone bodies concentrations also effect the ketone body metabolism (Evans et al. 2017). Therefore, the simultaneous collection of the blood, milk and urine is important to evaluate the actual BHBA level in these three different samples and the diagnostic performance of the ketosis test (Carrier et al. 2004).

The major advantages of a field ketosis test are that it can provide quick results on the farm and clinicians can apply treatment immediately after the diagnosis. A rapid and accurate diagnosis is essential on a field level to prevent ketosis from occurring at the farm and reduce the economic losses caused by it. In this regard, an animal-side or pen-side rapid test is highly desirable if the kit has an appropriate and predictable sensitivity and specificity.

This study examined the diagnostic performance of three commercially available milk test kits and one urine test kit for the detection of ketosis. A blood BHBA test is the standard method for ketosis detection because BHBA is more stable in the blood (Oetzel 2007). In the case of the SCK detection, the low agreement between the milk and blood test kits (Table 1) was attributed to the lower sensitivity of the milk test. The Porta BHB milk test kit showed 43% agreement with the blood test and its negative  $\kappa$  value ( $\kappa = -0.181$ ) indicated no effective agreement between these two tests for the SCK detection. The Healthmate and Wizcheck milk test kits also showed low agreement with the blood test kit (Table 1) because of their lower sensitivity. The sensitivity of the Porta, Healthmate and Wizcheck BHB milk test kits (Figure 1A) was 29.6%, 40.7%, and 33.3%, respectively. The lower sensitivity of the milk test also indicates that the milk test has less capacity to detect SCK animals, which are truly positive for SCK. Sometimes a true SCK is observed as being a normal in milk test because lots of noisy compounds are present in the milk, like proteins, fats, minerals, etc. (Cheruiyot et al. 2018). In the case of the urine test, a lower sensitivity and specificity was observed for the SCK detection which was 47.6% and 70.2%, respectively, which represents the lower agreement of this test (64% agreement; a  $\kappa$  value of 0.163). This low agree-

ment of the urine test indicates that the ketone bodies concentration in the urine sometime varies. If the animals are dehydrated, the urine ketone concentration will be high and give “false positive” results. This usually occurs when the ketone levels are tested in the morning. Similarly, the ketone concentration can be lower in the urine of the cows that drinks substantial amount of fluids, giving “false negative” results (Sharma et al. 2013). Additionally, the reagent strip can also influence the urine ketone test results. Most commercially available urine test kits rely on sodium nitroprusside in the reagent strip and it produces a purple colour in the presence of acetone and acetoacetate in the urine sample. None of the tests detected BHBA in the urine, resulting in the fact that the total ketone content might be underestimated. Therefore, the ketone test results in the urine sample might vary especially in the SCK in comparison to the blood BHBA (Goldstein et al. 2004).

In the case of the CK, the three milk tests were compared with the blood test and a moderate agreement were observed ( $\kappa$  value  $> 0.5$ ), suggesting that these kits have a strong capacity to detect the CK in field conditions. The sensitivity and specificity of the three milk test kits were also good for the CK. The urine test also has strong agreement when compared to the blood test result (91% agreement;  $\kappa$  value  $> 0.6$ ), suggesting that the urine test has a very good capacity for the detection of the CK. Furthermore, the diagnostic sensitivity and specificity of the urine test were also high, which was 93.7% and 90.3%, respectively. Compared to the milk and urine test, the urine sample is good for the diagnosis of ketosis because it is more purified than the milk. After the glomerular filtration, an aqueous solution of urine becomes clearer with more than 95% water with a minimum of other constituents (Skotnicka et al. 2007). In the milk, however, many noisy compounds, such as fats, casein, lactose and somatic cells are present, which can inhibit the colorimetric reaction of the milk test kit (Cheruiyot et al. 2018). Therefore, urine samples are comparatively better for the evaluation of the ketosis in dairy cattle than milk samples. However, the collection of urine is difficult by a urinary catheter which often requires a skilled veterinarian, which should also be kept in consideration.

Furthermore, the comparison between the diagnosis of the SCK and CK indicates that the sensitivity and specificity for the detection of the CK was

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good in the milk and urine tests (Figure 1) because of the larger amount of BHBA present in the CK animals, which can give a strong reaction on the immuno-chromatographic ketone test strip and easily identify the CK positive cases. Conversely, having a low amount of BHBA present in the SCK which cannot react properly on the immuno-chromatographic ketone test strip can, thus, increase the false positive and negatives results in the SCK detection (Enjalbert et al. 2001). Some improvement in the quality of these colorimetric strips is needed due to the limitations of the milk and urine test strips. Also, precaution should be taken during the data interpretation of the SCK screening in a dairy herd. In comparison between the milk and urine sample, urine is good because the urine is more purified after the glomerular filtration than the milk although its collection is difficult. The milk and urine test kits also have some advantages because of the low price, rapid and easy diagnostics procedure. Also, farm producers do not prefer blood sampling because of its invasive collection procedure. Hence, a milk and urine test can be applicable as a useful tool to identify cows suffering from clinical ketosis, but they need some attention for the SCK data interpretation.

In summary, the results of this study help to inform the clinicians, farm animal practitioners, and farm producers that all the tested kits of the milk and urine are useful for the detection of ketosis on a field level. The milk and urine test kits can detect CK easily, but caution should be used when interpreting the SCK data because of the low sensitivity of the milk and urine tests. This study also suggested that blood test is the more reliable method for the SCK detection, while the milk and urine tests show approximately the same performance as the blood testing for the CK detection only, with the advantage of the non-invasive sampling.

### Conflict of interest

The authors declare no conflict of interest.

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Received: June 8, 2019

Accepted: April 7, 2020