Title: Performance evaluation of a newly designed on-farm blood testing system for determining blood non-esterified fatty acid and β-hydroxybutyrate concentrations in dairy cows

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ABSTRACT

The objective of this study was to evaluate a newly designed on-farm blood testing system (OFBTS) for monitoring blood concentrations of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) in dairy cows. Whole blood samples from 230 Holstein dairy cows between -86 and 343 days in milk were collected into heparinized tubes. A drop of whole blood was used to determine NEFA and BHBA using the OFBTS. Plasma from the remainder of the blood was used to determine both analytes using a commercial kit (gold standard). In the repeatability of the OFBTS, the intra-assay coefficients of variation for NEFA and BHBA were 1.3% and 4.5%, and the inter-assay coefficients of variation were 1.8% and 2.9%, respectively. The slope and coefficient of determination of OFBTS analysis of NEFA compared to the gold standard were 0.92 and 0.94. Those for BHBA were 0.94 and 0.98. The biases (mean of the difference between the gold standard laboratory assays and OFBTS) of NEFA and BHBA were 0.021 and 0.019, respectively. However, the bias became substantial for NEFA in the higher concentration ranges (>1.2 mEq/L). The sensitivity and specificity of NEFA at a cutpoint of 0.4 mEq/L were 93.2% and 99.4%. Increasing the NEFA cutpoint to 0.6 mEq/L changed them to 87.9% and 100%. The sensitivity and specificity of BHBA at a cutpoint of 1.0 mM at a cutpoint of 1.0 were 86.2% and 99.0% and those of 1.2 mM were 94.7% and 99.5%. The reaction time for the NEFA to reach 0.6 mEq/L was 7 minutes. The BHBA reaction reached 1.2 mM within 2 minutes. In conclusion, the OFBTS has excellent performance for detecting cows with high blood NEFA or BHBA concentrations and could be a useful tool for on-farm monitoring of cows with negative energy balance and/or ketosis.

Keywords: dairy cow, non-esterified fatty acids, β-hydroxybutyrate, on-farm blood testing

1. Introduction

Crucial physiological and metabolic adaptations occur in dairy cows during the transition period from late gestation to early lactation. Most dairy cows experience negative energy balance (NEB) after calving. Ketosis, which is practically quantified as hyperketonemia (HYK), occurs when moderate to severe NEB is accompanied by impaired carbohydrate supply (Grummer, 1993). This combination leads to incomplete β-oxidation of mobilized fatty acids and the formation of ketone bodies. Excessive NEB and HYK after calving are both associated with negative health and production outcomes (Kehrli et al., 1989; Hammon et al., 2006; Ospina et al., 2010, Ospina et al., 2013). A smaller proportion of prepartum dairy cows experience NEB; however, when it does occur it is very strongly associated with negative health outcomes postpartum (Ospina et al., 2013). A recent epidemiological study (Macrae et al., 2019) reported that 12.8% of cows had high concentration of serum BHBA (≥ 0.8 mM) in the last 10 days of pregnancy. Blood ketone concentrations determined prior to calving are not highly predictive of postpartum problems (Chapinal et al., 2011, Ospina et al., 2013).

The presence and degree of NEB can be determined by measuring blood concentrations of non-esterified fatty acids (NEFA). Animal handling and diet can affect blood NEFA results (Leroy et al., 2011). Elevated blood NEFA concentrations, either before or after calving, are associated with numerous negative health outcomes. For postpartum cows, blood NEFA was more predictive of negative outcomes than blood ketones (Ospina et al., 2013).

Handheld BHBA meters have recently made it practical to conduct large-scale, on-farm testing for HYK. The performance and usefulness of handheld BHBA meters has been substantiated repeatedly (Iwersen et al., 2013; Kanz et al., 2015; Pineda and Cardoso, 2015; Süss et al., 2016). The use of handheld BHBA meters has allowed for determination

of the prevalence of hyperketonemia in postpartum cows in several different regions of the world (Suthar et al., 2013; Mahrt et al., 2015; McArt et al., 2012). Furthermore, the use of on-farm blood BHBA testing facilitates early detection and early treatment of HYK, which has very favorable impacts on disease risk, milk yield, and culling risk (McArt et al., 2012). The most widely-used cutpoint for defining HYK is blood BHBA ≥ 1.2 mM.

Blood NEFA testing has been described for use in both pre- and postpartum dairy cows. Unfortunately, on-farm blood NEFA testing is currently impractical because it is limited to laboratory assays of serum or plasma (Ospina et al., 2013). For a blood NEFA test to be suitable for on-farm use, it should use whole blood as the test medium (thus eliminating the need for on-farm blood centrifugation followed by separation of the plasma or serum) and also not require pipetting of liquid reagents. To our knowledge, no test system currently exists that meets these requirements. We therefore designed an on-farm blood testing system (OFBTS) that uses dry chemistry reagents, does not require centrifugation of the blood sample, and simultaneously determines NEFA and BHBA concentrations. The objective of this study was to evaluate the performance of the OFBTS (using whole blood samples) compared to the gold standard laboratory assay using plasma samples for the determination of NEFA and BHBA.

2. Materials and Methods

2.1 Animals and sample collection

Holstein cows on five commercial dairy farms in Hokkaido, Japan, were used in this study. Herd size ranged from approximately 50 to 300 cows. Animals were housed in freestall barns with rubber mats over concrete floors. Animal care on the farms followed the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which

essentially conforms to the guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the United States (1996).

In December 2018, coccygeal blood samples were obtained from 230 cows (-86 to 343 days in milk, average parity 2.4±1.4) and classified into 4 stages (Table 1). The whole blood was immediately placed in a heparinized tube. The samples were refrigerated until assayed. Tests using both the OFBTS and the laboratory biochemical analyzer were conducted within 3 h to avoid hydrolysis of esterified fats to NEFA (Stokol and Nydam, 2005).

2.2 Sample analysis

The OFBTS was developed by modifying a point-of-care testing device designed for human use (BBx; Teramecs, Co., Ltd., Kyoto, Japan). The device was relatively small (205 mm long, 126 mm wide, and 110 mm high) and lightweight (0.8 kg). The assay was performed in a disposable test slide containing the dry chemistry reagents for NEFA and BHBA determination. The test procedure consisted of placing a drop of whole blood (approximately 80 μL) on the cartridge and then inserting the test slide into the device. The cartridge separates plasma from whole blood sample and transports the plasma to enzymatic reaction tanks for NEFA and BHBA determinations.

The principle for NEFA determination was treatment with acyl-CoA synthetase in the presence of ATP and CoA. Thiol esters of CoA then form as acyl-CoA, along with the byproducts adenosine monophosphate and pyrophosphate. In the second portion of the procedure, the acyl-CoA is oxidized by adding acyl-CoA oxidase to produce trans-2,3-dehydroacy-CoA and hydrogen peroxide. In the presence of added peroxidase, hydrogen peroxide converts leuco dye to methylene blue, which is measured at 630 nm (dominant wavelength) and 810 nm (complementary wavelength).

The principle for BHBA determination was oxidation of D-3-hydroxybutyrate in plasma by nicotinamide adenine dinucleotide (NAD) to acetoacetate in the presence of the enzyme 3-hydroxybutyrate dehydrogenase. In the presence of diaphorase, NADH converts tetrazolium salt to a formazan that is measured at 630 nm and 810 nm.

The OFBTS recorded absorbance every 10 seconds, from 10 to 600 seconds. We adopted absorbance at 600 seconds and converted it into concentration using preliminary calibration curves.

In the present study, all measurements by the OFBTS were conducted in our laboratory at room temperature. After a drop of heparinized blood was used for analysis in the OFBTS, the remaining whole blood sample was centrifuged and the plasma was harvested for NEFA and BHBA determinations (the gold standard assays). Commercial kits (NEFA-HRII Wako test kit; Wako Pure Chemical Industries, Osaka, Japan and 3-hydroxy butyrate assay kit; Serotec Co., Ltd., Hokkaido, Japan) were used in an automated biochemistry analyzer (CA-90; Furuno Electric Co., Ltd., Hyogo, Japan). These assays used the same biochemical principles as for the OFBTS.

Precision of the OFBTS was evaluated by determining the intra- and inter-assay coefficient of variation (CV). Two samples were used for this evaluation; these samples had 0.47 and 1.03 mEq/L NEFA and 1.21 and 3.66 mM BHBA concentrations. The intra-assay CV was evaluated by repeating the NEFA and BHBA measurements 18 times. Inter-assay CV was evaluated by measuring the same sample 6 times with 3 devices. Accuracy of the OFBTS was evaluated by comparing its results to the gold standard laboratory assays for all 230 samples collected.

2.3 Statistical analysis

Precision of the OFBTS was evaluated by determining the intra-assay CV using JMP (version 13 for Windows, SAS Institute Inc., Cary, NC, USA).

The assumption of a linear relationship between results of OFBTS and gold standard laboratory assays was evaluated by visually inspecting scatterplots of OFBTS versus gold standard laboratory assay results (Figure 1). Simple linear regression with JMP (version 13 for Windows, SAS Institute Inc., Cary, NC, USA) was then used to determine the slope of the regression line and the coefficient of determination (R^2) for the regression.

The sensitivity, specificity, positive predictive value, and negative predictive value of the OFBTS compared to the gold standard laboratory assays were determined using JMP (version 13 for Windows, SAS Institute Inc., Cary, NC, USA). Two cut-points for NEFA (0.4 and 0.6 mEq/L) and for BHBA (1.0 and 1.2 mM) were evaluated. These cut-points were derived previously (Oetzel et al., 2004; Ospina et al., 2010, Ospina et al., 2013, Whitaker et al., 1983).

3. Results and Discussion

The proportions of samples at or above the cut-points for NEFA and BHBA are presented in Table 1. The proportions of cows with high NEFA and high BHBA values were consistent with the results of other published studies. In the present study 13.2% of prepartum cows above the NEFA prepartum cut-point of 0.4 mEq/L. Cook et al. (2006) suggested an alarm level of 10% for prepartum cows with NEFA greater than 0.4 mEq/L. Ospina et al. (2010) suggested a postpartum NEFA cut-point of 0.6 mEq/L but did not propose a cut-point for the proportion of cows above this cut-point. In the present study, 35.4% of the postpartum cows (0 to 30 days in milk) had blood NEFA > 0.6 mEq/L. This was very close to the 32% prevalence of elevated blood NEFA in postpartum cows reported by Ospina et al. (2013).

Table 1

In the current study, the prevalence of HYK (BHBA ≥ 1.0 or 1.2 mM) was 19.8% and 13.5%, respectively, in postpartum cows (0 to 30 days in milk). Reported values for

165 the herd prevalence of HYK in early lactation cows include 4.2 to 9.7% (Iwersen et al.,
166 2009), 11.6 to 36.6% (Suthar et al., 2013), and 10.4% (Süss et al., 2016). The prevalence
167 of blood BHBA ≥ 0.8 mM was 7.9% in prepartum cows. This result was a little lower
168 compared to prevalence of 12.8% reported by Macrae et al. (2019).The intra-assay CV for
169 NEFA and BHBA were 1.3% and 4.5%, and the inter-assay CV were 1.8% and 2.9%,
170 respectively. These results indicate very good precision (repeatability) of the OFBTS.
171 The relationship between OFBTS and gold standard laboratory assays for NEFA did
172 not appear to be linear throughout the range of values (Figure 1A). A plateau in results
173 from the OFBTS was evident at higher concentrations of NEFA (>1.2 mEq/L). Removing
174 the 13 NEFA values over 1.2 mEq/L from the dataset (5.7% of the cows) resulted in a
175 strongly linear appearance of the relationship between the OFBTS and gold standard
176 laboratory assays for NEFA.
177 When all NEFA values were included in the regression analysis, the slope of the
178 regression line was 0.76 and the R^2 was 0.92 ($P < 0.0001$). After removal of the very high
179 NEFA values, the slope of the regression line changed to 0.92 (closer to the desired 1.00)
180 and the R^2 increased to 0.94 ($P < 0.0001$, Figure 1B). These results indicate excellent
181 accuracy of the OFBTS for blood NEFA concentrations below 1.2 mEq/L.
182 The underestimation of very high NEFA values by the OFBTS could be explained
183 by insufficient influx of oxygen into the cartridge to entirely oxidize the NEFA at very
184 high concentrations. Future research is needed to verify the oxidative reaction in the
185 cartridge at high concentrations. Nonetheless, NEFA testing using the OFBTS remains
186 applicable even if very high values are underestimated. Any NEFA concentration over 0.4
187 to 0.6 mEq/L is classified as high; therefore, the few cows with very high NEFA
188 concentrations were still correctly classified by the OFBTS.

Figure 1

The relationship between OFBTS and gold standard laboratory assays for BHBA appeared to be strongly linear throughout the range of values (Figure 1C). The slope of the regression line was 0.94 and the R^2 was 0.98 ($P < 0.0001$). These results indicate excellent accuracy of the OFBTS for whole blood BHBA determination.

Bland-Altman plots that visualize the differences between OFBTS and gold standard laboratory assays across the range of results for NEFA and BHBA are presented in Figure 2. The bias, SD, and 95% limits of agreement (±1.96 SD) for the difference between the OFBTS and the gold standard laboratory assays for NEFA were 0.02, 0.12, 0.27, and -0.22 mEq/L (Figure 2A), and for BHBA were 0.02, 0.09, 0.19, and -0.15 mM (Figure 2B). The biases (mean of the difference between the gold standard laboratory assays and OFBTS) of NEFA and BHBA were very close to 0 and their limits of agreement were also very small. These results indicated very good consistency between the OFBTS and gold standard laboratory assays. Kanz et al. (2015) reported less consistency between three different cowside BHBA meters and the gold standard laboratory assay (mean biases of 0.02, -0.10, and -0.06 and SD of the differences of 0.12, 0.21, and 0.17). The OFBTS is not a cowside test and takes considerably longer to run than cowside BHBA tests. As expected, however, the OFBTS delivers more consistent results than cowside BHBA tests. No on-farm NEFA tests are available that can be compared to the OFBTS.

Figure 2

Figure 2

The OFBTS was 93.2% sensitive and 99.4% specific for classifying cows with elevated NEFA at the 0.4 mEq/L cutpoint. At the 0.6 mEq/L cutpoint the OFBTS was 87.9% sensitive and 100% specific (see Table 2). These results demonstrate the excellent value of the OFBTS for identifying cows with high NEFA concentrations. As previously noted, the tendency of the OFBTS to underestimate very high blood NEFA concentrations did not limit its value.

Table 2

213 The OFBTS was 94.7% sensitive and 99.5% specific for classifying cows with
214 elevated BHBA at a cut-point of 1.2 mM. At the 1.0 mM cutpoint the OFBTS was 86.2%
215 and 99.0% (Table 2). These results demonstrate the excellent value of the OFBTS for
216 identifying cows with high BHBA concentrations. Three cowside meters for determining
217 BHBA (Kanz et al., 2015) had similar reported sensitivities (100%, 94%, and 100%) but
218 lower specificities (93%, 85%, and 83%) compared to the OFBTS.
219 The time courses of absorbance for 4 representative levels of NEFA and BHBA
220 (between 10 and 600 seconds) are shown in Figure 3. The reaction for BHBA proceeded
221 rapidly and the absorbance values were clearly separated according to the difference in
222 concentration (Figure 3B). In contrast, the NEFA reaction proceeded slowly and required
223 more time to detect differences in absorbance at each level (Figure 3A). The times
224 required for the reaction to determine the difference around the cutpoint concentrations
225 were 7 min for NEFA and 2 min for BHBA. These results indicate that the OFBTS is
226 capable of conducting simultaneous NEFA and BHBA assays within a reasonable time
227 period for on-farm use.

Figure 3

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229 **4. Conclusions and future work**

230 Evaluating the degree of NEB and HYK can be an important tool for herd-level
231 monitoring and for cow-level early detection and treatment. In particular, diagnosis of
232 NEB using NEFA during late gestation could be more effective than waiting for HYK to
233 develop after calving. To our knowledge, we have developed the first on-farm blood
234 NEFA assay system that has excellent precision and accuracy up to 1.2 mEq/L. This
235 system also measures blood BHBA with similar or greater precision and accuracy than
236 currently available cowside blood BHBA devices. Combining blood NEFA and BHBA

testing within a single device allows for more thorough and complete evaluation of a cow's metabolic condition before and after calving.

Declaration of Competing Interest

The author Takayuki Taguchi works as a director of laboratory of I. B. Co., Ltd.

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Figure legends

Figure 1

Correlation coefficients between blood concentrations measured by the newly developed system (OFBTS) and the plasma concentrations (gold standard laboratory assay) for NEFA (A, B) and BHBA (C). Two hundred thirty samples are plotted with open circles. A and C plot all data. B plots below 1.2 mEq/L. The solid line shows the line of equality (y=x), and the dotted line shows the linear regression line.

Figure 2

Bland-Altman plots with mean bias and 95% limits of agreement for NEFA (A) and BHBA (B) measured with the gold standard laboratory assay and the on-farm blood testing system (OFBTS).

Figure 3

The time course absorbance of whole blood samples from different concentrations of NEFA (A) and BHBA (B) determined using the newly designed on-farm test system (OFBTS).

Table 1. Proportions of blood samples from cows in each stage with NEFA ≧0.4 and 0.6 mEq/L or BHBA ≧1.0 and 1.2 mM

Stage*	n	Number of positive samples/total (%)			
		NEFA		BHBA	
		0.4 mEq/L	0.6 mEq/L	1.0 mM	1.2 mM
Prepartum	38	5/38 (13.2%)	1/38 (2.6%)	1/38 (2.6%)	0/38 (0.0%)
Postpartum 1	96	49/96 (51.0%)	34/96 (35.4%)	19/96 (19.8%)	13/96 (13.5%)
Postpartum 2	46	5/46 (10.9%)	1/46 (2.2%)	5/46 (10.9%)	3/46 (6.5%)
Postpartum 3	50	1/50 (2.0%)	0/50 (0.0%)	4/50 (8.0 %)	3/50 (6.0%)
Total	230	60/230 (26.1%)	36/230 (15.7%)	29/230 (12.6%)	19/230 (8.3%)

*Postpartum 1; 0-30 days in milk, Postpartum 2; = 31-60 days in milk, Postpartum 3; ≧ 61 days in milk

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Table 2. Performance of the newly developed device (NDS) for classification of a cow at blood NEFA concentrations of 0.4 and 0.6 mEq/L, and blood BHBA concentration of 1.0 and 1.2 mM compared with plasma concentrations measured by the gold standard assay (n = 230 samples)

Item	Cut-off	Sensitivity, %	Specificity, %	Positive Predictive Value, %	Negative Predictive Value, %
NEFA	0.4 mEq/L	93.2	99.4	98.2	97.7
	0.6 mEq/L	87.9	100	100	98.0
BHBA	1.0 mM	86.2	99.0	92.6	98.0
	1.2 mM	94.7	99.5	94.7	99.5

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Fig. 1

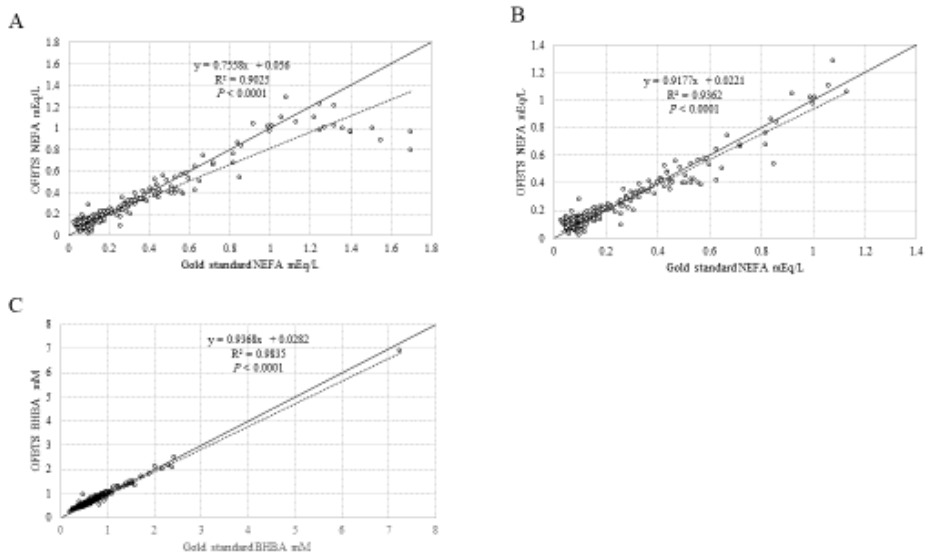
A
y = 0.7558x + 0.056
R² = 0.9025
P < 0.0001
OFBTS NEFA mEq/L
Gold standard NEFA mEq/L
B
y = 0.9177x + 0.0221
R² = 0.9362
P < 0.0001
OFBTS NEFA mEq/L
Gold standard NEFA mEq/L
C
y = 0.9368x + 0.0282
R² = 0.9835
P < 0.0001
OFBTS BHBA mM
Gold standard BHBA mM

Fig. 2

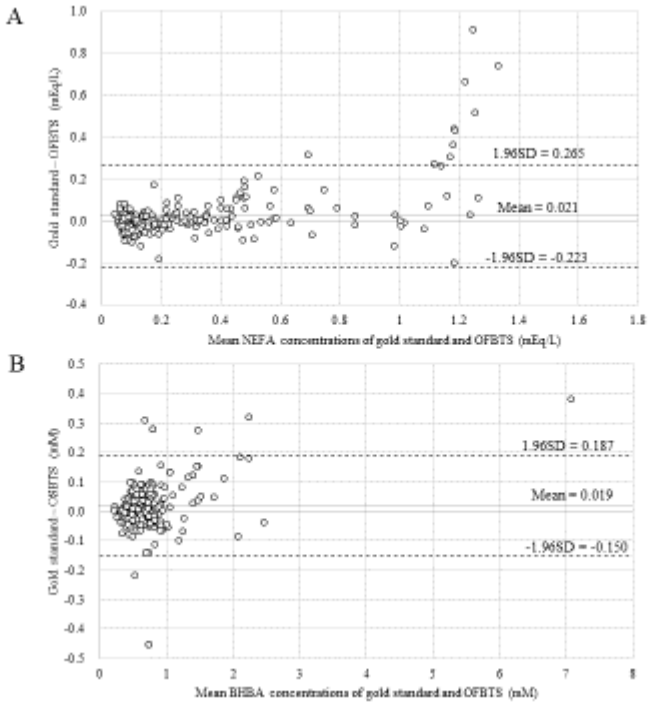

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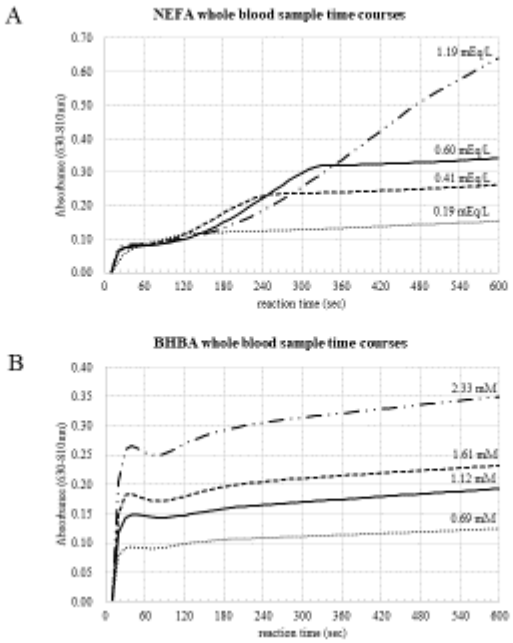
Fig. 3


A
NEFA whole blood sample time courses
1.19 mEq/L
0.60 mEq/L
0.41 mEq/L
0.19 mEq/L
Absorbance (630-810nm)
reaction time (sec)
B
BHBA whole blood sample time courses
2.33 mM
1.61 mM
1.12 mM
0.69 mM
Absorbance (630-810nm)
reaction time (sec)

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