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Original article

Evaluation of a handheld device for the measurement of beta-hydroxybutyrate in capillary blood obtained by the puncture of the vulva as well as in venous whole blood in cattle

J.L. Khol[1], K. Freigassner[1], A. Stanitznig[1], A. Tichy[2], T. Wittek[1]

[1] University Clinic for Ruminants, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria
[2] Bioinformatics and Biostatistics Platform, Department for Biomedical Sciences, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

Abstract

A negative energy balance is a common condition in high yielding dairy cows causing the production of ketone bodies (KB), including beta-hydroxybutyrate (BHB), defined as subclinical ketosis (SCK) if clinical signs are missing. The aim of the present study was to evaluate a handheld electronic device for the detection of SCK (BHB-concentration $\geq$ 1.2 mmol/l), in capillary blood and venous whole blood in cows (WellionVet BELUA, MED TRUST Handels GmbH, Marz, Austria) as well as the feasibility of the puncture of the external vulva with a single use lancet. For this purpose, the blood BHB-concentration was tested in 250 venous and capillary blood samples and compared to the results of a certified laboratory.

The majority (76.3%) of the animals displayed no signs of discomfort related to the puncture and in 74.2% the procedure was successful on the first attempt. The BHB-concentrations detected in capillary blood showed good agreement with the reference method, both in capillary (correlation coefficient 0.94 ($p<0.001$), Kappa-value 0.89) and venous whole blood (correlation coefficient of 0.95 ($p<0.001$), Kappa-value 0.89). Altogether, 98% of all the samples were correctly classified as SCK or non-SCK by the handheld device in capillary blood (sensitivity 0.96, specificity 0.98) and 97.4% in venous whole blood (sensitivity 0.889, specificity 0.991), respectively. An increase in the correlation by the adaptation of the cut off level could not be achieved for both sampling sites.

Key words: beta-hydroxybutyrate, subclinical ketosis, capillary blood, handheld device

Correspondence to: J.L. Khol, e-mail: johannes.khol@vetmeduni.ac.at, tel.: +43 1 25077-5205

Introduction

A negative energy balance is a common condition in high yielding dairy cows during early lactation (Andersson 1988). As a consequence, ketone bodies (KB), including beta-hydroxybutyrate (BHB), are excreted by the organism to provide an alternative energy source, leading to the metabolic disease of ketonaemia. This elevation of the concentration of KB is defined as subclinical ketosis (SCK) if clinical signs, such as nervous disorders, are missing (Andersson 1988). Multiple thresholds have been used to define SCK in postpartum cows, ranging from BHB concentrations of 1 mmol/l to 1.8 mmol/l blood, with 1.2 mmol/l and 1.4 mmol/l being the most widely used cut off values (Raboisson et al. 2014). The reported prevalence of SCK in dairy cows varies between 11% and 49% (Suthar et al. 2013, Berge and Vertenten 2014). Increased concentrations of KB not only have been associated with a reduced milk yield, but also with an increased risk for the occurrence of abomasal displacement, metritis, lameness, and clinical ketosis (Duffield et al. 2009, Suthar et al. 2013). Therefore, the adequate treatment and prophylaxis of SCK calls for the early detection of elevated KB in modern dairy farming in high yielding cows during the close up period and early lactation (Marutsova et al. 2015). For this purpose, handheld cow side tests to detect the level of KB in blood, milk, and urine are available. While milk and urine are more easily collected, the analysis of the BHB-concentration in blood has been reported to be the most accurate method for the detection of SCK in cattle (Tatone et al. 2016). These electronic devices, originally intended for use in humans, have been evaluated before and showed satisfying performances in the detection of cows with SCK (Iwersen et al. 2009, 2013). The collection of blood can be challenging and labor intensive, especially if performed at the jugular vein and has to be performed by veterinarians. To overcome these drawbacks, handheld devices have been applied to capillary blood from the external vulva in cows, reporting a comparable performance to venous whole blood samples after the modification of the threshold levels (Kanz et al. 2015, Iwersen et al. 2017,). By lowering the threshold for SCK in capillary blood to 1.0 or 1.1 mmol/l of BHB, a sensitivity between 80% and 100% and a specificity between 76% and 97%, depending on the device used, were reported (Kanz et al. 2015, Iwersen et al. 2017).

Recently, a handheld electronic device for the detection of BHB in capillary as wells as in venous whole blood in different animal species, including a special calibration for cattle, has been developed (WellionVet BELUA, MED TRUST Handels GmbH, Marz, Austria). The aim of the present study was to evaluate this handheld electronic device that was developed for the detection of BHB-concentrations in cows. Furthermore, the feasibility of the puncture of the external vulva with a single use lancet regarding the reaction of the animal toward the procedure as well as the success in producing a sufficient volume of blood for the detection of BHB were assessed.

Materials and Methods

The study was conducted at the University Clinic for Ruminants of the University for Veterinary Medicine Vienna and was approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012-TVG 2012 (BMWFW 68.205/0019 WF/V/3b/2016). A consent to participate in the study was obtained from the animal owners.

Animals enrolled

Altogether, 250 blood samples were included in the study. These samples originated from cattle referred to the University Clinic for Ruminants at the University for Veterinary Medicine in Vienna for various medical reasons. All of the female patients with a minimum age of 6 months admitted to the clinic during the study period were enrolled in the study, without considering their medical status or risk for SCK. If blood had to be drawn several times from these animals during their stay at the clinic, they also were tested for BHB and included in the study multiple times, resulting in the enrollment of 106 different individuals. These animals showed a mean age of 4.8 years (min. 0.6, max. 12.7) and were in different stages of gestation. From the animals tested, 67.9% belonged to the Austrian Simmental breed, 20.7% were Holstein, 7.5% Brown Swiss and 3.9% miscellaneous breeds, representing the typical distribution of cattle breeds in Austria.

Sampling procedures

In the course of the study, capillary blood, gained by the puncture of the external vulva as well as venous whole blood collected from the jugular vein at the same time were tested for the BHB-concentration by a handheld device (WellionVet BELUA, MED TRUST Handels GmbH, Marz, Austria). The blood collected from the jugular vein was further sent to a certified laboratory (Central Laboratory of the University for Veterinary Medicine Vienna) to evaluate the BHB-concentration, serving as the reference method.

Table 1. Reaction toward the puncture of the vulva and the performance of blood collection (n=248).

		Reaction*				
		1	2	3	4	Total
Performance#	1	153 (61.7%)	25 (10.1%)	4 (1.6%)	2 (0.8%)	184 (74.2%)
	2	16 (6.5%)	11 (4.4%)	2 (0.8%)	0 (0.0%)	29 (11.7%)
	3	7 (2.8%)	3 (1.2%)	0 (0.0%)	3 (1.2%)	13 (5.2%)
	4	13 (5.2%)	7 (2.8%)	1 (0.4%)	1 (0.4%)	22 (8.9%)
	Total	189 (76.3%)	46 (18.6%)	7 (2.8%)	6 (2.4%)	248 (100%)

* 1 = no sign of discomfort, 2 = slight signs of discomfort, 3 = distinct signs of discomfort, 4 = severe signs of discomfort including kicking; # 1 = collection easy with one puncture enough blood, 2 = collection easy but more than one attempt necessary, 3 = collection difficult several attempts needed to produce enough blood, 4 = punctures unsuccessful.

The puncture of the vulva was performed using a single use lancet with an 18 gauge needle and a penetration depth of 2.2 mm (Wellion SafetyLancets special, MED TRUST Handels GmbH, Marz, Austria). According to the manufacturer, the puncture with this lancet results in 70-100 µl of blood in humans, with 0.8 µl being necessary to perform one analysis. Before the puncture, the vulva was cleaned with a dry single use paper towel. Cows were tethered at the neck, and the tail was lifted for the procedure. After the puncture, the vulva was squeezed to produce a drop of blood which was directly tested for the concentration of BHB with the handheld device as described in the user's manual.

To evaluate the puncture technique, the success as well as the reaction of the animals to the procedure was recorded on a scale from 1 to 4. The reaction of the animal was rated as a 1 if no sign of discomfort was noticed, 2 if slight signs of discomfort were shown, 3 if the animal displayed distinct signs of discomfort in reaction to the puncture, and 4 if the animal showed severe signs of discomfort, including kicking. The success of the procedure was rated as a 1 if the collection of sufficient blood was easily obtained with one puncture, 2 if it was easy but more than one attempt was necessary, 3 if the collection of enough capillary blood was difficult and needed several attempts, and 4 if several punctures were unsuccessful. If sufficient blood could not be obtained after several attempts at different locations of the vulva, a 16 gauge needle was used instead to produce a sufficient drop of blood.

At the same time as BHB was measured in the capillary blood, a whole blood sample was collected from the jugular vein (Baumgartner et al. 2018), using a vacutainer system and 9 ml tubes with lithium heparin (Greiner Bio one, Kremsmünster, Austria). The BHB concentration in the samples from the jugular vein was measured with the same handheld device as used for capillary blood. In addition, it was sent to the Central Laboratory of the University for Veterinary Medicine in Vienna for the analysis of the BHB concentration, serving as the reference method.

Measuring of beta-hydroxybutyrate

Both the handheld device as well as the laboratory bounded reference method used indirect measuring methods for the detection of the BHB-concentrations.

The handheld device was developed for veterinary use with a special calibration for cattle (MED TRUST Handels GmbH, Marz, Österreich). The concentration of BHB is detected using the enzyme BHB-dehydrogenase in this system with a range of 0.1 to 8.0 mmol/l. As the reference method, the BHB was measured with the Cobas 6000/c501 (Roche Pharma, Freiburg, Germany) and the enzyme 3-hydroxybuyrate-dehydrogenase. While the BHB concentration is determined spectrometrically (optical) by the reference method, the handheld device uses an amperometric (electrochemical) detection system.

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics v. 24 (IBM Corporation, Amonk, USA) and Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). To evaluate the agreement of the BHB results of the handheld device both in capillary and venous whole blood with the reference method, the Kappa-value was calculated. Furthermore, the Pearson Correlation was used to describe the correlation between the different methods with a level of significance of $p<0.01$. Furthermore, the difference between the methods in relation to the mean value was visualized according to Bland and Altman (Bland and Altman 1986).

Based on the literature, the level for SCK was set at a concentration of BHB $\geq$1.2 mmol/l of blood (Mahrt et al. 2015, Pineda and Cardoso 2015). Using this threshold, a receiver operating characteristics (ROC) curve was performed and the Yuden-Index (senstitivity+specificity-1) was calculated to receive the cut-off for the BHB concentration in capillary blood samples.

The results rated as above or below the measuring range of the handheld device were excluded from the analysis.

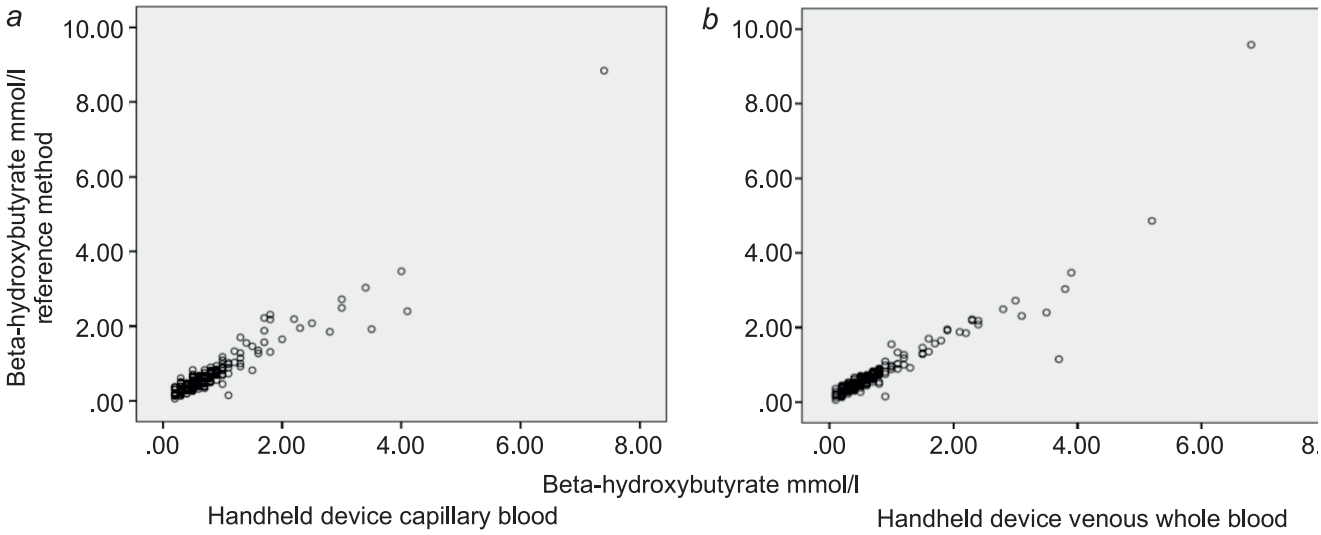

Fig. 1. Beta-hydroxybutyrate in capillary (*a*) and venous whole blood (*b*) compared to the reference method (n=244).

Table 2. Correlation of BHB*-concentrations detected by the handheld device compared to the reference method.

	Reference method[#]	Handheld device capillary[§]	Handheld device venous[†]
Number of samples	248	244	243
Mean (mmol/l)	0.71	0.77	0.73
Min.-Max. (mmol/l)	0.06-9.58	0.20-7.40	0.10-8.00
Standard deviation (mmol/l)	0.94	0.74	0.93
Interquartile range (mmol/l)	0.37	0.40	0.40
Correlation [$]		0.944	0.952
Kappa**		0.894	0.894
AUC[##]		0.998	0.994

* beta-hydroxybutyrate; [#] BHB-concentration detected by reference method (Cobas 6000/c501); [§] BHB-concentration detected by handheld device (WellionVet BELUA) in capillary blood from the external vulva; [†]BHB-concentration detected by handheld device (Wellion-Vet BELUA) in venous whole blood from the jugular vein; [$] Pearson-Correlation coefficient; ** Kappa-value of agreement; [##] Area under the curve (Receiver operating characteristics)

Results

Evaluation of puncture of the vulva

Altogether 248 punctures were available for evaluation, as the performance of the lancet and the reaction of the animals were not recorded in 2 cases. The majority (76.3%) of the animals enrolled in the study showed no signs of discomfort related to the procedure (Table 1). Severe signs of discomfort and kicking were only recorded in 2.4% of the animals (Table 1).

In 74.2% of the cases, the collection of capillary blood by the puncture of the vulva using a single use lancet was successful on the first attempt and was rated as easy in 85.9% (Table 1). Achieving a sufficient amount of capillary blood for the detection of BHB was not possible in 8.9% of the punctures using the lancet (Table 1).

Detection of beta-hydroxybutyrate in capillary blood

For the comparison of the BHB-results gained by the handheld device from capillary blood with the reference method, 244 samplings were available, as 6 samplings had to be excluded because of missing results. The BHB concentrations detected by the two methods showed good agreement (Fig. 1a) with a correlation of 0.94 (p=<0.001). The results of the descriptive statistics are presented in Table 2. The Bland-Altman plot (Fig. 2) showed good agreement between the methods with most of the results within the upper- and lower limits, equally distributed around the mean value.

Altogether, 98% of all the samples were correctly classified as SCK or non-SCK by the handheld device, using the cut off level for BHB of 1.2 mmol /l (Table 3). The sensitivity of the test device applied in capillary blood was calculated to be 0.96 (95% Confidence Inter-

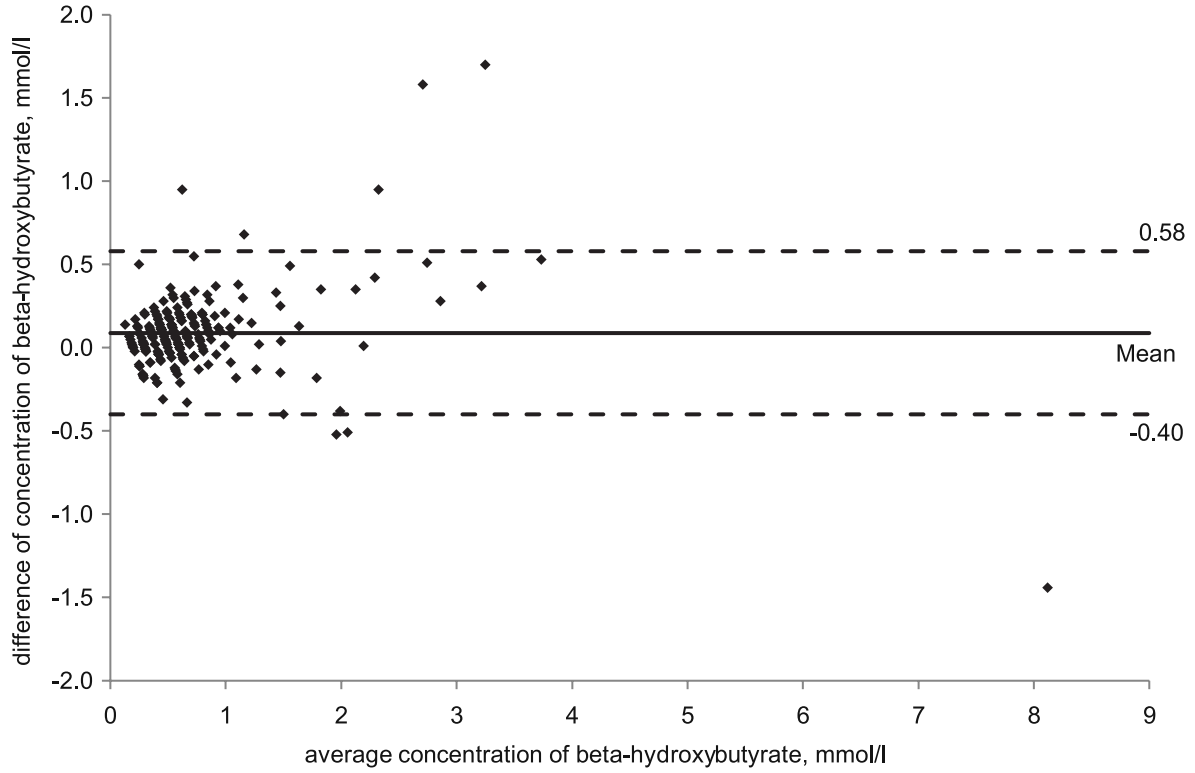

Fig. 2. Bland-Altman plot of beta-hydroxybutyrate detected by the handheld device in capillary blood and reference method.

Table 3. Comparison of BHB[†] – classification in capillary and venous whole blood to the reference method.

Test method[#]		Reference method[*]		
		BHB <1.2[§]	BHB >1.2[§]	Total
Capillary[**] n=244	BHB <1.2	215 (88.1%)	1 (0.4%)	216 (88.5%)
	BHB >1.2	4 (1.6%)	24 (9.8%)	28 (11.5%)
	Total	219 (89.8%)	25 (10.2%)	244 (100%)
Venous[##] n=243	BHB <1.2	214 (88.1%)	3 (1.2%)	217 (89.3%)
	BHB >1.2	2 (0.8%)	24 (9.9%)	26 (10.7%)
	Total	216 (88.9%)	27 (11.1%)	243 (100%)

[†] Beta-hydroxybutyrate; [*]Measuring of BHB by certified laboratory (Cobas 6000/c501); [#] Measuring BHB by handheld device (WellionVet BELUA); [§]BHB concentration above 1.2 mmol/l; [**]results from capillary blood obtained by puncture of the vulva; [##] results from venous whole blood collected from the jugular vein.

val (CI): 0.777; 0.998) and the specificity 0.98 (95% CI: 0.951; 0.994), respectively, compared to the reference method. The Kappa-value between the two methods was 0.89 ($p<0.001$).

The ROC-analysis of the BHB concentrations measured with the handheld device compared to the reference method showed an area under the curve (AUC) of 0.998 (Fig. 3a). An increase in the correlation by the adaptation of the cut off level of 1.2 mmol/l could not be achieved.

Measuring of beta hydroxy butyrate in venous whole blood.

For the comparison of the BHB results gained by the handheld device from venous whole blood with the reference method, 244 samplings were available again, as 6 samplings had to be excluded because of missing results. The BHB concentrations detected in venous whole blood from the jugular vein by the handheld device showed a high level of agreement with the reference method (Fig. 1b) with a correlation coefficient of 0.95 ($p<0.001$). The results of the descriptive statistics can be found in Table 2. The Bland-Altman plot (Fig. 4) confirmed a high agreement between the methods with most of the results within the upper- and lower limit, equally distributed around the mean value.

Of the samples with a BHB-concentration above or below 1.2 mmol/l detected with the reference method, 97.4% were correctly classified by the handheld device (Table 3). The calculated sensitivity

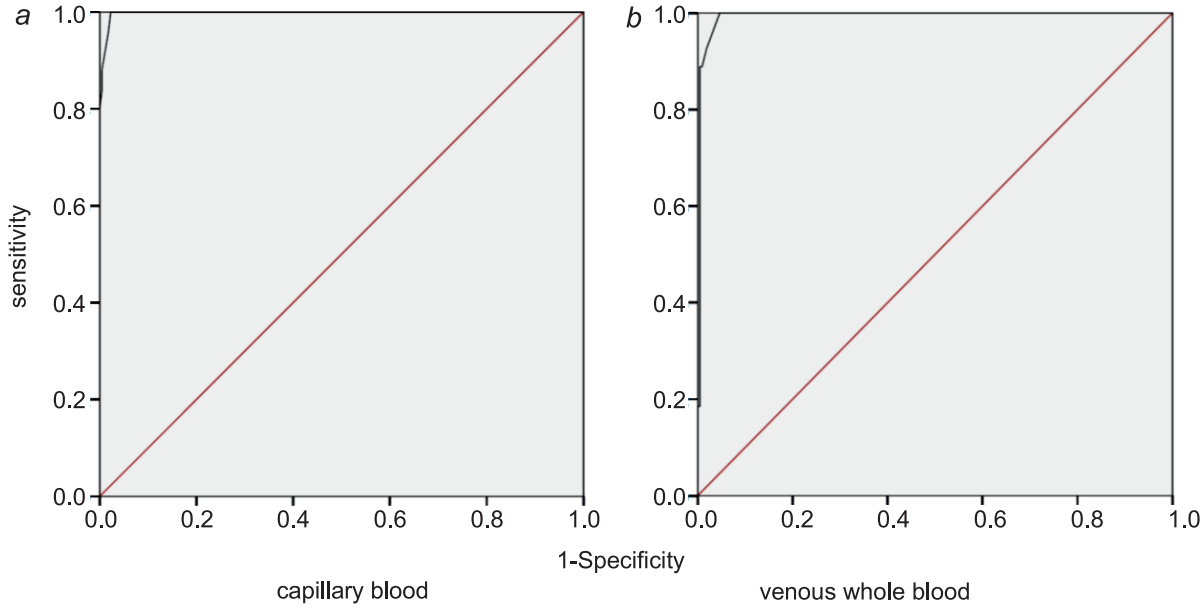

Fig. 3. Receiving operation characteristics of beta-hydroxybutyrate in capillary (*a*) and venous whole blood (*b*).

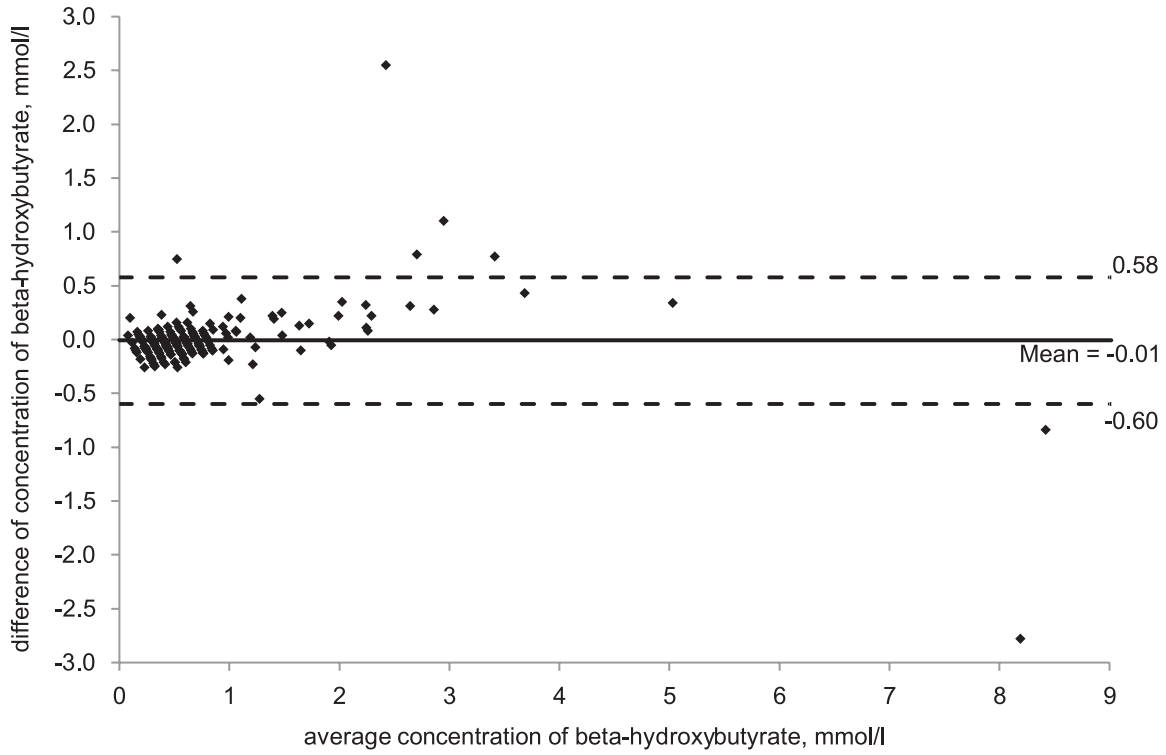

Fig. 4. Bland-Altman plot of beta-hydroxybutyrate detected by the handheld device in venous whole blood and reference method.

of the cow side test was 0.889 (95% CI: 0.697; 0.971) and the specificity 0.991 (95% CI: 0.963; 0.998), respectively, with a Kappa-value of 0.89 ($p<0.001$).

The ROC-analysis of the results obtained with the handheld device from the jugular vein showed an AUC of 0.994 (Fig. 3b), as in capillary blood before, an increase in the correlation with the reference method by the modification of the cut off level was unachievable.

Discussion

The results of the present study confirm that the puncture of the external vulva is a feasible method for the collection of capillary blood in cattle. The majority of the animals enrolled in the present study showed no or slight signs of discomfort as a reaction to the procedure. This indicates a possible advantage of this minimally invasive method over the blood collection by

venipuncture, especially at the jugular vein, concerning animal welfare. Furthermore, the puncture was successfully yielding a blood sample sufficient for the analysis of BHB on the first attempt in 74.2% of punctures, with only 8.9% of unsuccessful samplings. The procedure was rated as easy to perform in most of the punctures in the present study. These results indicate a good performance of the puncture, although the method for recording the result as well as the reaction of the animals used is prone to subjective influences by the sampling person. Therefore, all of the samplings were performed by the same two persons after thoroughly adjusting the rating to minimize their influence. The individual differences concerning the thickness of the skin and the perfusion at the puncture side also might influence the success of the procedure but were not recorded during the study. The single use lancet applied was equipped with an 18 gauge needle with a penetration depth of 2.2 mm, which is intended for use in humans. Meanwhile, a new single use lancet with a higher penetration depth of 3 mm has been developed especially for use in cattle (MED TRUST Handels GmbH, Marz, Austria) and it is believed to further increase the success and amount of capillary blood gained by the puncture of the vulva.

Puncture of the caudal vein to obtain blood samples for BHB-testing is successfully used by many veterinarians worldwide, but was not evaluated in the present study. The possible use of capillary blood by trained farm stuff may further increase its application, although it has to be considered, that in a herd nutrition monitoring programs several other metabolites can be assayed in venous blood sample which complement BHB information such as NEFA and plasma glucose.

The BHB-concentrations obtained with the handheld device showed a strong correlation with the reference method, both in capillary as well as in venous whole blood. The correlation coefficient was 0.94 for capillary blood and 0.95 for venous whole blood, respectively. The correlation of the capillary results with the reference method was stronger in the present study than those reported previously for three different handheld devices with Spearman's rho correlations of 0.83, 0.73, and 0.62, respectively (Kanz et al. 2015). The Bland-Altman plot revealed an equal distribution of the results around the mean value with view outliers only. A trend toward an over- or underestimation of the BHB-concentration in capillary blood in comparison to the reference method, as seen in a previous study (Kanz et al. 2015), was not found in our survey. Using the threshold of 1.2 mmol/l, a sensitivity of 96% (95% CI: 77.7; 99.8) and a specificity of 98% (95% CI: 95.1; 99.4) was calculated for the handheld device in capillary blood in our study. While the specificity is slightly higher than the specificity of 76-89% that was reported by Kanz et al. (2005), the sensitivity was higher (100%) in this study for one of the three devices used. The two remaining tests systems evaluated previously showed a lower sensitivity of 89% and 84% than in the present survey when applied to capillary blood (Kanz et al. 2015).

In the present study, the ROC-analysis of the BHB-concentrations in capillary blood resulted in an AUC of 0.998, indicating an excellent (AUC-ROC ≥90%) accuracy of the test system for the diagnosis of SCK. A further increase in the test performance by adjusting the threshold level of 1.2 mmol/l for capillary blood could not be achieved. This is in contradiction to two previous studies, where lowering the threshold to 1.0 or 1.1 mmol/l in capillary blood led to an increased sensitivity and specificity for the detection of SCK (Kanz et al. 2015, Iwersen et al. 2017). The good performance of the handheld device used in our study might point to the advantage of using a test system originally intended for the use in cattle, while the test devices used in previous studies were initially developed for use in humans.

The performance of the handheld device for the detection of the BHB-concentration in venous whole blood used in the present study was comparable to those seen in capillary blood. Handheld devices for the detection of BHB-concentrations in whole blood, serum, and plasma samples have been evaluated before (Iwersen et al. 2009, 2013, Pineda and Cardoso 2015). The sensitivity of 88.9% (95% CI: 69.7; 97.1) for the detection of SCK in venous whole blood of the present study is slightly below the sensitivity of 96% (Iwersen et al. 2009) and 98-100% (Iwersen et al. 2013, Pineda and Cardoso 2015) as seen in other studies but above the reported 80%-86% (Iwersen et al. 2013), depending on the device and threshold used. The specificity of 99.1% (95% CI: 96.3; 99.8) of the handheld device used in our study in venous whole blood was higher than found in previous studies using different test systems with specificities between 51% (Pineda and Cardoso 2015) and 97% (Iwersen et al. 2013), respectively. In an extensive review of a handheld device for the detection of SCK in whole blood samples, different from the one used in our study, a sensitivity of 94.8% (95%CI: 92.6; 97.0) and specificity of 97.5% (95% CI: 96.9; 98.1) were reported (Tatone et al. 2016). The correlation of the BHB-concentrations detected by the handheld device in venous whole blood with the reference method in the present study of 0.95 is in accordance with the correlation coefficient of 0.95 reported in another study for a different handheld device (Iwersen et al. 2009).

The relative low number of the animals with SCK

enrolled in the present study has to be addressed as a possible drawback, warranting further investigations with different study designs to further describe the test performance of the handheld device.

Altogether, it can be concluded that the collection of capillary blood at the vulva is a minimally invasive, safe, and easy to perform sampling procedure. Its adoption in the bovine health management might contribute to an achievable increased monitoring of BHB-concentrations in cattle herds (Kanz et al. 2015, Iwersen et al. 2017). It also has been shown that the site of the puncture at the vulva as well as the frequently required squeezing of the tissue to produce enough blood for the analysis, do not influence the BHB-results (Iwersen et al. 2017). The handheld device used in the present study showed a comparable performance to other handheld devices when used in venous whole blood with a slightly lower sensitivity as reported in other studies but a higher specificity (Iwersen et al. 2009, 2013, Pineda and Cardoso 2015, Tatone et al. 2016). The results for the detection of BHB-concentrations and SCK in capillary blood, obtained by the puncture of the external vulva, indicate a better performance than that reported in other studies using different devices (Kanz et al. 2015, Iwersen et al. 2017). Furthermore, an adaptation of the threshold level for SCK of 1.2 mmol/l of BHB seems to be unnecessary, contributing to a further facilitation of this screening method.

Previous studies have shown the superiority of the BHB-detection in blood samples over test sticks for the use of BHB detection in urine or milk (Iwersen et al. 2009, Tatone et al. 2016). The disadvantage of the more invasive sample collection for blood, hampering a broader acceptance might be overcome by the minimal invasive method of capillary blood testing. Furthermore, this sampling method might also be used for the analysis of further important parameters such as liver specific enzymes or other diagnostic and metabolic parameters in the future, as already performed for decades in human medicine (Lindner et al. 2011).

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